AN INVESTIGATION INTO THE GUT HEALTH PROMOTING MECHANISMS OF A XYLOOLIGOSACCHARIDE BASED PREBIOTIC IN BROILERS

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Copyright statement

Abstract

Poultry meat and eggs are highly significant sources of (nutrient dense) animal proteins in human nutrition. Fast growth, affordability, global acceptability and low environmental impact makes poultry a leading contender in the race for securing food for the future. The EU-wide ban in 2006 on the use of in-feed antibiotics as growth promoters obligated the poultry industry to use plant or microbial derived additives to promote bird health and performance which would ensure sustainable growth of the poultry industry without adding to the threat of antibiotic resistance. Prebiotics are a category of in-feed antibiotic alternatives produced from human inedible agricultural residues and have been shown to improve gut health and thus the overall health of the animals. Amongst prebiotics, xylooligosaccharides (XOS) are gaining significant attention due the vast abundance of its parent molecule, xylan, in the plant kingdom. A limited understanding of the mechanisms of action of XOS has hampered improvements to their efficacy. The aim of this research project was therefore to examine the underlying effects of XOS that may lead to gut health promotion and performance improvement of broiler chickens. Two broiler trials, one under controlled research conditions and another under suboptimal conditions mimicking commercial broiler production units were conducted and an *in vitro* study was undertaken to achieve this aim.

The results demonstrated that in birds raised under controlled research conditions, neither 0.1 g/kg XOS nor xylanase nor their combination had any significant effect on performance or the assessed gut health parameters which included composition and diversity of caecal microbiota, gene expression of biomarkers of gut integrity and caecal short chain fatty acid (SCFA) concentrations. On the other hand, under sub-optimal conditions the same dose of XOS significantly improved performance up to 28- days of age. Although, there was no significant difference in the diversity or overall composition of microbiota in birds raised under sub-optimal conditions, XOS specifically enhanced the numbers of certain members of Lachnospiraceae family (phylum Firmicutes) known to produce SCFAs. In addition, in the same trial, expression of mucin glycoprotein (MUC2) and tight junction protein occludin (OCLN)

in the ileum and caecal acetate and propionate were significantly increased at day 35. In the *in vitro* study, shot-gun proteomics analysis on proteins extracted from the caecal microbiota was used to provide the earliest evidence support the stimbiotic mechanism of XOS which essentially implies that they stimulate the growth of the fibre fibre-degrading microbiota members eventually increasing the nutritive value of feed. The caecal bacteria from chickens fed a XOS supplemented diet, had upregulated the proteins involved in degradation of xylan compared to bacteria from the control group.

Overall, this study supports the use of XOS to improve gut health and performance of broilers raised under challenging environmental conditions or as a "*stimbiotic*" to improve nutritive value of feed by facilitating the breakdown of its fibre fraction.

Dedication

This Ph.D is dedicated to my best half, my husband Amir. Without his moral, financial and IT support and taking on the responsibilities as the main parent for our son Ahmad, this Ph.D. would not have been possible. I also dedicate this Ph.D to my late father, Mohammed Rafi Mulla whose efforts were instrumental in helping me pursue higher education in the UK fifteen years ago and my mother Mrs. Rafat Mulla who did not laden me with household responsibilities (a prevalent practice for a girl child in low-middle income families in India) allowing me to focus on my education. My father passed way two days after the submission of this thesis on 28th June 2021.

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Abbreviations

AX	Arabinoxylan
AXOS	Arabinoxylooligosaccharides
BWG	Body weight gain
BW	Bird weight
Ca	Calcium
CLDN	Claudin
DDA	Data dependent Acquisition
DIA	Data Independent Acquisition
FCR	Feed Conversion Ratio
FFAR	Free fatty acid receptor
FI	Feed intake
GALT	Gut Associated Lymphoid Tissue
GAPDH	Glyceraldehyde phosphate dehyrogenase
GCMS	Gas Chromatography- Mass spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometer
IL	Interleukin
LCMS	Liquid Chromatography- Mass spectrometry
*MUC*2	Mucin-2
NSP	Non-starch polysaccharide
OCLN	Occludin
P	Phosphorous
SBM	Soybean meal
SCFA	short chain fatty acid
sIgA	Secretory Immunoglobulin A
SWATH	Sequential window acquisition of all theoretical mass spectra
TJ	Tight junction
XYL	Xylanase

XOS	Xylo-oligosaccharide
HPSF	High Purity Salt Free

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Chapter 1: Review of Literature

1.1. The importance of poultry in human society

For thousands of years, poultry farming has been a distinctive attribute of human society. This sector has grown from a small-scale backyard activity providing sufficient livelihood for one family to modern intensive production systems, housing approximately 20,000 birds per shed, generating business worth millions of pounds. Poultry production for both meat and eggs continues to grow and industrialise in many parts of the world. As breeding practices have developed, birds have become increasingly productive, and therefore need to be managed in a specialised way. Advances in technology have improved food safety and production efficiency, particularly in areas such as feed and carcass processing. However, these advances tend to be focussed on large scale production and have led to a corresponding scale up of both feed and poultry production. In small scale production, technology has had smaller effects, with family based, traditional systems being crucial to supporting women and families in rural and developing countries (Akinola and Essien, 2019).

Chicken meat and eggs have an important role in human nutrition. They are the best sources of quality protein providing essential amino acids and are rich in B complex vitamins, several minerals and omega-3 fatty acids. In addition, chicken meat is more affordable and faces few if not any religious and cultural barriers compared to other meats. In sub-Saharan Africa and southeast Asia, poultry products can make a significant difference in fighting childhood malnutrition and undernutrition associated with poverty and substantially benefit pregnant women, nursing mothers, and the elderly (FAO, 2013). Another advantage of poultry over other livestock is that its production has a less detrimental impact on the environment and uses less water per tonne of meat produced (Gerbens-Leenes, Mekonnen and Hoekstra, 2013; Röös *et al.*, 2013).

As population size continues to grow, and individual consumption of poultry products increases, the demand for meat and eggs will continue to expand. Projections are for increases in consumption across both developing and developed regions, though the former has a slightly higher projected

growth per capita (FAO, 2018). As the poultry sector develops, maintaining sustainability can contribute towards the Sustainable Development Goals of the UN, specifically, (i) no poverty (ii) zero hunger and (iii) good health and well- being for all (Alders *et al.*, 2019).

1.1.1. Poultry production stats

1.1.1.1. Global meat and egg production

Global egg production is shown in Table 1.1. China is the leading producer of eggs accounting for 40% of the global egg output and thus making Asia the largest egg producing region. The remainder of the world is considerably behind China as the USA and India produce merely 7% and 6% respectively of global egg outputs. Over the last thirty years a significant increase in egg production in Asian countries has led to a threefold increase in global production. Table 1.1 shows that this trend is continuing with a 15% increase between 2010 and 2016 in hen egg production.

Table 1.1: Global egg production (FAOSTAT, 2022)

	Total number of eggs in shell, fresh in millions										
	2010	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
Hen eggs (in shell)	1204	1228	1256	1285	1275	1296	1316	1493	1533	1593	1643
Other bird eggs (in shell)	83.2	84.9	87.2	88.9	91.3	95.3	96.6	92.9	93.6	95.9	100

Other species of poultry currently represent just 8% of global egg production but in this sector too growth is rapid. Non-chicken egg production has increased by 30% from 83 million per annum to 96.6 million between 2010 and 2016. Like chicken eggs, most of this production comes from Asia, with very small proportions in Latin America, the USA, Europe and Oceania. Production of non-chicken eggs in Africa is currently not documented.

Table 1.2 shows the global poultry meat production with broiler chickens being the highest contributors of the total meat produced. In 2010, 56 billion broilers were raised and within a span of six years this increased by over 15% to 65 billion. Turkey production holds a marginal portion of the poultry meat sector with 1% share, similar to geese and guinea fowl, although the production numbers

of the latter varies from year to year. High variability is also seen in duck production but with a generally ascending track record, holding a 4% share of the poultry market. The largest poultry meat producer is the USA with 18% of the output followed by China and Brazil. In 2016, poultry meat represented 36% of the total meat production worldwide.

Table 1.2: Global poultry meat production by species (FAOSTAT, 2022)

	Total production in million tonnes, fresh or chilled										
	2010	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
Chicken	87.2	90.9	94.1	97.6	100	104	107	112	116	118	119
Turkeys	5.5	5.6	5.8	5.6	5.6	5.6	6.0	5.9	6.0	6.0	6.0
Ducks	4.1	4.2	4.3	4.4	4.3	4.3	4.4	4.6	4.9	5.0	5.0
Geese	2.4	2.5	2.7	2.7	2.6	2.5	2.7	2.7	2.8	2.8	2.8

The FAO forecasted the total poultry meat production in the world in 2020 to rise by 2.6% to 137 million tonnes. This rise was driven by the consumers' efforts to substitute pig and bovine meat with alternatives. Consumers appear to have preferred poultry due to its relative affordability, leading to cascading effects through poultry value chains from production to foreign trade However, lower food services sales, intentional production curbs and Avian Influenza (AI) outbreaks dampened production growth rate in 2020 and production figures settled at 133 million tonnes. (FAO, 2020).

1.1.1.2. Meat and egg production in the UK

Table 1.3 shows the production of meat from poultry in the UK. In the UK chicken production is heavily commercialised making up 97% of the total poultry in the country while backyard poultry makes up the remainder. Chicken is a rapidly expanding market in the UK, mirroring the global situation. Between 2010 to 2016 broiler chicken rearing increased by almost 10%. This expansion continued in 2017 and 2018, with annual broiler rearing of 1049 and 1103 million chicks respectively (Burton and Scholey, 2021).

Turkey production is variable each year but holds a fairly constant share of 1.5% of the UK poultry market. Duck rearing is also consistent in holding 1.3% of the UK sector with around 14 million ducklings per annum. Geese make up a very small part of the UK market, but after a period of constant

production up to 2014 of 400 thousand goslings reared annually, there has been a rapid rise in popularity of goose for eating, with annual rearing increasing to 600 thousand birds in 2015 and 2016. As consumers explore new culinary choices this expansion may well continue.

Table 1.3: UK Poultry meat production (Burton and Scholey, 2021)

	Birds placed/yr (millions)						
	2010	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Broilers	904	899	919	945	942	972.4	992.3
Turkeys	15.6	16.9	18.4	17.5	15.4	16.7	15.4
Ducks	13.2	14.7	14.3	13.6	14.3	13.8	14
Geese	0.42	0.42	0.364	0.34	0.407	0.588	0.622

Table 1.4 shows total egg production and market share in the UK by housing system (million cases per year). Although barn and organic production has remained stationery over this period (2013-2017), there has been a 10% switch from colony cage production to free range, leaving free range production with the largest market share in 2017. This switch was driven following a public petition that made the end user, particularly the supermarkets commit to cage free eggs. The push towards cage-free only by 2025 by many retailers suggests that colony production will continue to fall and will be replaced by free range and barn, with many producers choosing to switch production from colony to barn by removal of cage fronts (Karcher and Mench, 2018). However, it is not yet known whether the public will increase consumption of barn eggs from this type of system.

Table 1.4: UK production by housing systems, million cases of egg and market share (Burton and Scholey, 2021)

	2013		**2014**		**2015**		**2016**		**2017**	
	mil cases/yr	**% share**	**mil cases/yr**	**% share**	**mil cases/yr**	**% share**	**mil cases/yr**	**% share**	**mil cases/yr**	**% share**
Colony cage	13.6	50.6	14.1	52	14.2	51	14.4	49.8	13.6	44.4
Free range	11.8	43.9	11.6	42.8	12.3	44.2	13.2	45.7	15.9	51.9
Barn	0.9	3.3	0.8	2.9	0.7	2.4	0.6	2.2	0.4	1.3
Organic	0.6	2.3	0.6	2.3	0.7	2.4	0.7	2.4	0.7	2.4

1.1.2. Contribution of poultry to sustainable future food security

In order to achieve sustainable food and nutrition security in the face of climate change global efforts are required. This is indeed a complex endeavour that involves implementing feasible strategies that suit the local cultural, economic and geographical situations of every country (Alders *et al.*, 2019). Livestock rearing has been an inseparable component of human society since the beginning of civilization meeting the fundamental needs of food and providing livelihood. However, the growing commercialization of animals for meat, milk, eggs and associated products has been heavily criticized for reasons of welfare, impact on the environment, competition of animal feed with food, usage of water among others. Yet, the demand for animal derived food is increasing because of growing population, rising incomes and urbanization (FAO, 2009)

The world has over 23 billion poultry- about three birds per person on the planet (FAOSTAT, 2016). They are also the most numerous livestock in resource-poor areas, where their input to food availability are both direct, through supplying nutrient-rich products for human consumption, and indirect, through enhancing soil fertility for agriculture with the provision of manure and pest control by allowing chickens controlled access to crops and vegetables to feed on insect pests and tick species of cattle in a mixed farming system (Wong *et al.*, 2017). Poultry meat has shown the fastest growth rate in the last decades and is the most sought meat globally due to is acceptance through diverse cultures, traditions and religions. Among terrestrial livestock species, poultry have the shortest production cycles and are the most efficient feed converters. However, poultry feed faces the criticism for having a large majority of its ingredients, mainly cereal grains, in direct competition with human food. Figure 1.1 shows the composition of the global poultry feed ration. When adding cereal grains which represent 58% and other edibles including casava, soybeans, pulses, rapeseed and soya oil which represent 6%, 64% of the total poultry feed intake becomes human edible. If soybean meal were added to this figure, 81% of the global poultry feed ration could be considered as direct

competition with human food (Mottet and Tempio, 2017). Tremendous amount of research has gone into converting the scavenging feed resource base that are less suitable or unavailable for human consumption including plant seeds, earthworms, and insects, into poultry feed in pursuit to mitigate the food vs feed competition (Wong *et al.*, 2017). Poultry can be kept and reared on a wide variety of production systems, intensive, semi-intensive or backyard. In low-middle income countries (LMIC) across Asia and Africa where rural poultry flocks account for 60-90% of the poultry population, chickens contribute to food security directly as a source of food and indirectly via poverty alleviation (Wong *et al.*, 2017; Alemayehu *et al.*, 2018; Akinola and Essien, 2019).

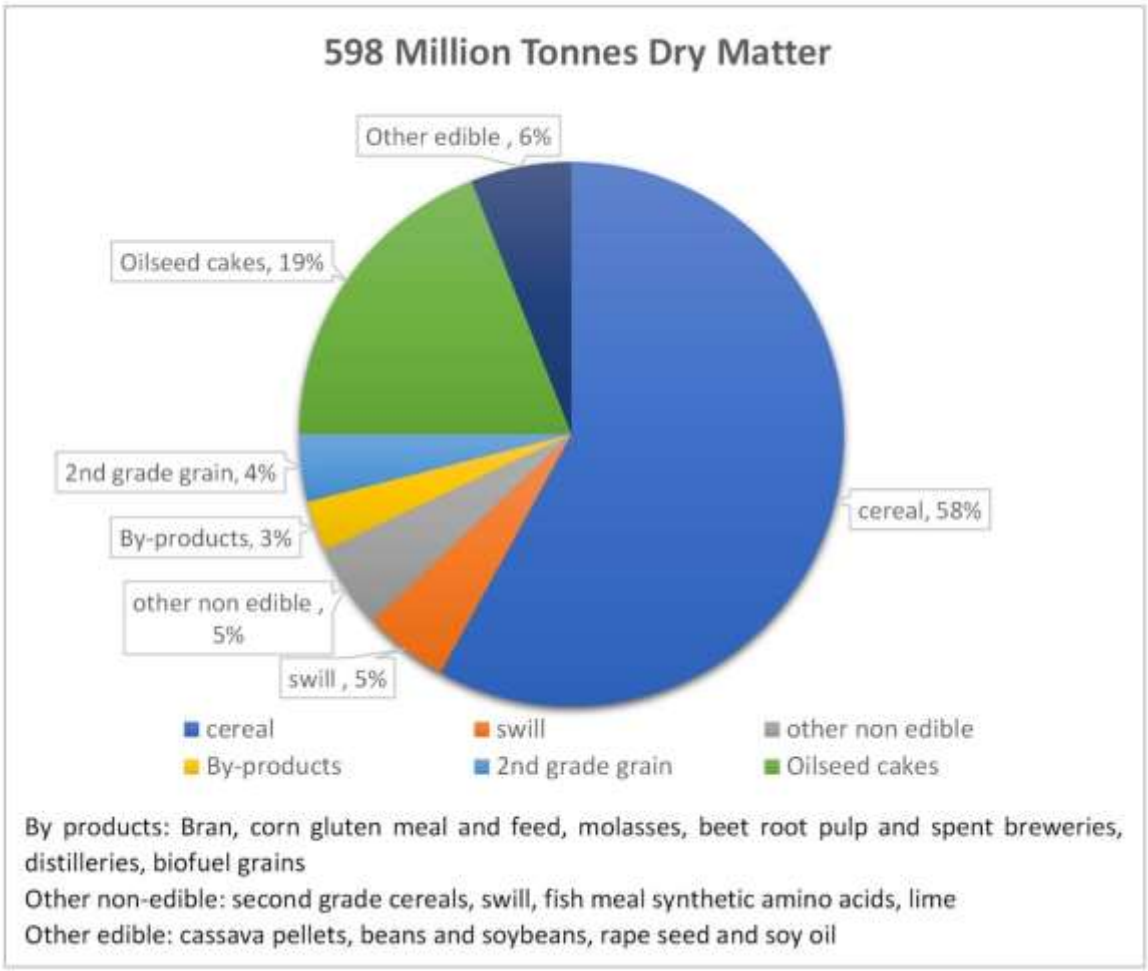


Figure 1.1. Global poultry feed ration (adapted from Mottet and Tempio, 2017). *Reproduced with permission from Taylor and Francis*

Another dimension of food security is resilience in adapting to economic or environmental shocks or changes (FAO, 2006). Village chickens in LMICs are particularly hardy and well adapted to their

environments and serve as a year-round source of food when there are threats to production because of disease or natural disasters (Wong *et al.*, 2017). Livestock production accounts for around 15% of greenhouse gases (GHGs) arising from human activity and amongst intensively raised livestock, poultry meat and egg production has the lowest GHG emissions at 8% compared to 9% of pig meat and 61% of beef and dairy production (Gerber *et al.*, 2013).

The importance of poultry in both developed and developing economies cannot be overstated. It has key role not only in securing food for the present and future generations but also in providing livelihoods and economic opportunities for millions of smallholder farmers and poor people.

1.2. Role of gut health in sustainable poultry production

The performance of poultry (or any farm animal), in the form of weight gain, feed intake, feed conversion ratio (FCR) or egg-related traits are profoundly influenced by the optimal functionality of the gastrointestinal tract (GIT) and its health. Therefore, it would not be incorrect to state that anything that affects gastrointestinal health will unquestionably influence the animal as a whole. Several, complex mechanisms are involved in the regulation of GIT functionality and health; therefore, it is crucial to expand our knowledge of these interactions so that animal performance can be improved through modulation of GIT functionality and health.

Over the last six decades the poultry industry has undergone a remarkable change owing to genetic selection for high growth and weight gain, implementation of advanced husbandry practices and improved understanding of nutritional requirements and digestive physiology. This industry will continue to expand in the coming years to meet the demand for low cost, healthy and convenient products. However, the critical question here is: “has the animal’s performance reached its genetic/physiological limits?” It is within this perspective that the concept of gut health began to attract significant interest within the animal science community (Kogut and Arsenault, 2016). Despite repeated use of the term ‘gut health’, in animal health and nutrition since the 1990s, it was only recently defined. In human health, gut health is often concomitant with absence of clinical diseases,

but this definition could not be applied to farm animals knowing that animal performance can be impaired without any clinical signs of disease. A clear definition of gastrointestinal health and functionality and how it can be measured was needed to monitor animal health and to assess the effects of diet and/or supplements on performance (Celi *et al.*, 2017). A three component gut health description was proposed by Conway in 1994 comprising of diet, mucosa and commensal flora. The mucosa in turn is composed of the digestive epithelium, mucus overlying the epithelium and the Gut Associated Lymphoid tissue (GALT). There exists a complex and dynamic equilibrium between the GALT, microbiota, mucous membrane and host epithelium that ensures the effective functioning of the digestive system. In 2017, Celi *et al*, proposed a comprehensive definition of gut health as, "*a steady state where the microbiome and intestinal tract exist in symbiotic equilibrium and where the welfare and performance of animal is not constrained by intestinal dysfunction*". This definition encompasses the basic components of gut health, namely diet, effective structure and function of the GIT barrier with normal and stable microbiota, for effective digestion and absorption of feed and an effective immune status (Figure 1.2). The cross talk between all of these inter-related components plays a critical role in GIT physiology, animal health, welfare and performance.

Figure 1.2. Schematic representation of the different components of the intestinal ecology important in determining gut health and growth in production animals. Each component interacts with the other in order to maintain a dynamic equilibrium, a state defined as gut health. For each component, the major factors of influence within the component are given (adapted with modifications from https://www.dsm.com/content/dam/dsm/anh/en_US/documents/gut-health-opportunities-and-challenges.pdf, 2021).

1.3. Factors affecting gut health

1.3.1. Diet

The digestive system is an organ with great complexity and dynamism and is the largest interface between host and environment. Its classic function is primarily digestion of feed (by means of enzymes and microbial fermentation) into small molecules which can then be absorbed into the body, and secondly acting as a physical barrier to antigens and pathogens. Finally the GIT is also the largest organ

of the active immune system housing more than 70% of the immune system cells (Vighi *et al.*, 2008). As the ingested nutrients can play an important role in the growth and functionality of the GIT, diet composition (ingredients, nutrients and additives) can influence the development and function of the digestive system, including the immune system and the microbiota (Conway, 1994).

1.3.1.1. Diet composition

There are many dietary factors that negatively impact the health of GIT of non-ruminant farm animals such as certain types of dietary fibre, trypsin inhibitors, phytate, lectins, mycotoxins, undigested proteins in the caeca, pathogenic microorganisms, diets with a poor nutrient balance and many others (Klasing, 1998). The integrity of the gut can be compromised by anti-nutrients, both physiologically and histologically. Feed or raw materials can be processed to reduce temperature labile anti-nutrients, using a combination of, settings of the machinery, pressure and humidity (Celi *et al.*, 2017). The rest of the factors to varying extents can be controlled by feed additives and supplements.

A large number of studies have highlighted the potential of feed supplements, functional foods and nutraceuticals in sustaining animal production performances while maintaining health and welfare (Pluske, 2013; Starkey, 2014; Hoste *et al.*, 2015). For example, antioxidants are routinely supplemented in animal feed to counteract the negative impact of excessive Reactive Oxygen Species (ROS) production, to improve their health and productivity and safeguard the quality of products (Chauhan et al., 2014). In addition, to ROS, heat stress can also lead to oxidative stress which can compromise the intestinal epithelial barrier integrity causing leakiness (Cottrell *et al.*, 2015). Dietary levels of selenium and vitamin E above recommended daily requirements have been used for mitigating the effects of oxidative stress in pigs (Liu *et al.*, 2016). Similarly in heat stressed sheep, supraphysiological levels of dietary antioxidants increased gene expression of heat shock proteins (HSP70 and HSP90) and decreased expression of pro inflammatory genes NF-κβ and TNF-α (Chauhan et al., 2014). Apart from antioxidants several other feed supplements like enzymes, pre and probiotics,

organic acids and essential oils have proven beneficial effects on gastrointestinal functionality. These will be covered in later sections of this review.

Diet can also modulate immune functions by different mechanisms. First, diet can influence the composition and hence metabolic activity of gut microbiota (Yeoman *et al.*, 2012; Yeoman and White, 2014). Gut microbiota in turn interfere with the growth and the adhesion of pathogens to the intestinal mucosa. Second, dietary proteins are an important factor in maintaining gut immune homeostasis. Amino acids from digestion of proteins by digestive enzymes or processing by gut microbiota are absorbed by intestinal epithelial cells and can influence gut immune competence and immune homeostatsis. Specific nutrients of diets, such as amino acids, amines, nucleotides and butyrate, can stimulate gut development (van der Meer *et al.*, 2016). Third, the epithelium of the gut can be directly affected by the diet, by regulation of the intestinal barrier and modulation of cytokine production. Finally, the immune system can be modulated by diet, both locally and systemically, via immune cells migration in blood, or by local activation of immune cells (de Lange *et al.*, 2010).

The production of a "gut friendly" diet requires several characteristics to be considered. The diets should have minimal buffering capacity, low levels of anti-nutrients (such as phytate, arabinoxylans and tannins), a low level of fermentable protein in the hindgut (Rist *et al.*, 2013; Pieper *et al.*, 2016), and ideally functional feed additives which can deliver a beneficial effect (de Lange *et al.*, 2010).

1.3.1.2. Diet form

The form or structure of diet (mash vs crumb vs pellet) and particle size (coarse vs fine) can have a significant impact on the functionality of the GIT. In case of pellets, the processing time and temperature as well as hygiene during pelleting can also influence GIT functionality. In poultry the well-recognized benefits of pelleting are increase in feed efficiency and carcass yield (Saldaña *et al.*, 2015; Jiménez-Moreno *et al.*, 2016; Herrera *et al.*, 2017). Reduction in particle size leads to a greater interaction between the resulting larger surface area of the grains and digestive enzymes and has been associated with increased digestive efficiency in poultry (Preston, McGragken and McAllister, 2000).

On the other hand larger particle size has been shown to improve gizzard function and consequently improve gut motility (Zaefarian, Abdollahi and Ravindran, 2016). I n addition a well-developed gizzard acts as a barrier preventing pathogens from entering the distal GIT thus reducing the risk of enteric diseases such as salmonellosis and coccidiosis (Engberg, Hedemann and Jensen, 2002; Huang *et al.*, 2006).

1.3.2. Effective structure and function of GIT barrier

The mucuslayer and the underlying monolayer layer of intestinal epithelial cells (IEC) together form the physical barriers of the GIT (Figure 1.3). The mucus gel layer provides protection by shielding the epithelium from potentially harmful antigens and molecules while also serving as a lubricant for intestinal motility. Mucus is secreted by specialized epithelial cells called goblet cells which are found along the entire length of the intestinal tract. The main structural protein of mucus, called mucin (coded by the *MUC* gene family) is abundantly glycosylated (up to 80% wt/wt). The glycan groups confer proteolytic resistance and hydrophilicity while the inter and intra molecular disulphide bonds between the cysteine residues of the mucin protein form the backbone of the mucus layer (Lievin-Le Moal and Servin, 2006; Kelsall, 2008). The layer of the mucus closest to the epithelium (about 30 µm), also referred to as the apical glycocalyx or membrane bound mucin, is essentially bacteria free in healthy individuals while the extracellular or secreted mucus above the apical glycocalyx is the site of colonization of commensal bacteria and a guardian to prevent p

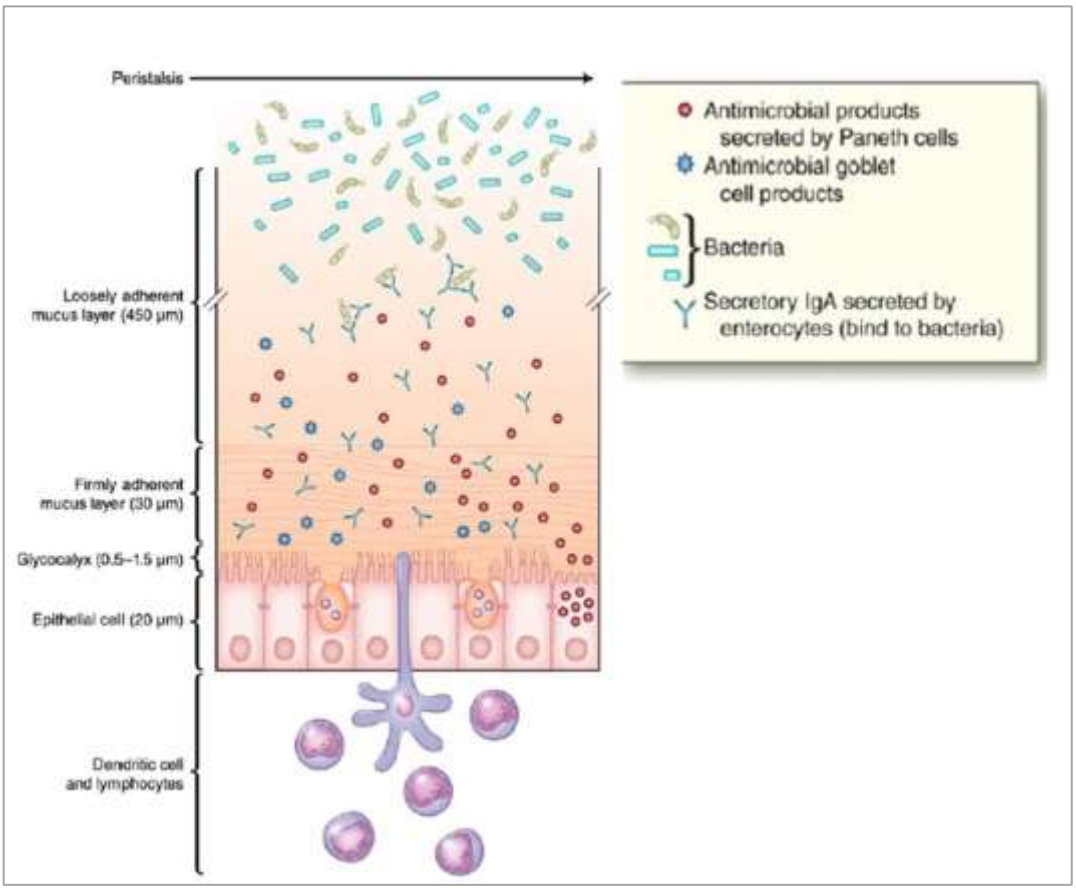


Figure 1.3: A schematic representation of two mucus layers overlying the epithelial cell surface. There are two mucus layers, an outer layer, and an inner, firmly adhered layer which make up the intestinal epithelium. These layers include the MUC2 network which is produced by host defence molecules and by goblet cells. The outer layer of mucin is associated with microbes, and these are absent ion the inner layer. The surface of the epithelium in the small intestine is covered my membrane bound mucins (MUC3 and MUC17), known as glycocalyx, and other glycoproteins. Taken from Kim and Ho (2010). *Reproduced under a CC-BY license.*

The inability of *Campylobacter jejuni* to cause infection in poultry is one of the best studied examples highlighting the role of mucin in gastrointestinal health. *C. jejuni* is the major cause of bacterial gastroenteritis in humans but lives as a commensal in poultry even though it heavily colonizes the poultry GIT. The differences in the mucus composition between humans and chickens underlie the species specific divergence in the outcome post *Campylobacter* exposure (Byrne, Clyne and Bourke, 2007). Furthermore crude chicken mucin attenuated *Campylobacter* binding and internalization into human intestinal cells *in vitro* (Alemka *et al.*, 2010). On the other hand, enteric pathogens have developed diverse methods to degrade mucus and invade the epithelium. In poultry, the ability of

Clostridium perfringens to degrade intestinal mucus is considered the most important criteria in the development of Necrotic Enteritis (NE). *C. perfringens* was shown to preferentially metabolize monosaccharides that are common in mucin glycans by releasing these from the complex mucin oligosaccharides (MacMillan *et al.*, 2019).

The intestinal epithelial cells form the second line of physical barrier in the GIT. The linkage between the adjacent epithelial cells is formed by protein- protein complexes that seal the intercellular space. These transmembrane protein complexes namely desmosomes, adherens junctions and tight junctions interact extracellularly with adjacent cells and intracellularly with adaptor proteins that link to the cytoskeleton. Desmosomes and adherens junctions are thought to be more important in the mechanical bonding of adjacent cells while tight junctions that form the apical most junctional complex are responsible sealing the intercellular space and regulating paracellular transport thus allowing the epithelium to maintain its selective barrier function (Figure 1.4) (Groschwitz and Hogan, 2009). Together these complexes play an important role in absorption of nutrients, electrolytes and water as well as the maintenance intestinal barrier integrity and function and protection of gut from enteric pathogen invasion (Wu *et al.*, 2019). The enterotoxins of *C. perfringens* the causative agent of NE in poultry bind to tight junction proteins, mainly claudin-3 and claudin-4 (Guttman and Finlay, 2009; Saitoh *et al.*, 2015), which eventually leads to pore formation, an increase in paracellular permeability, and cytotoxicity (Mitchell and Koval, 2010; Saitoh *et al.*, 2015). Therefore, during NE, tight junction structure is compromised, thus influencing barrier function and eventually leading to lower performance and higher mortality in birds (Emami *et al.*, 2019).

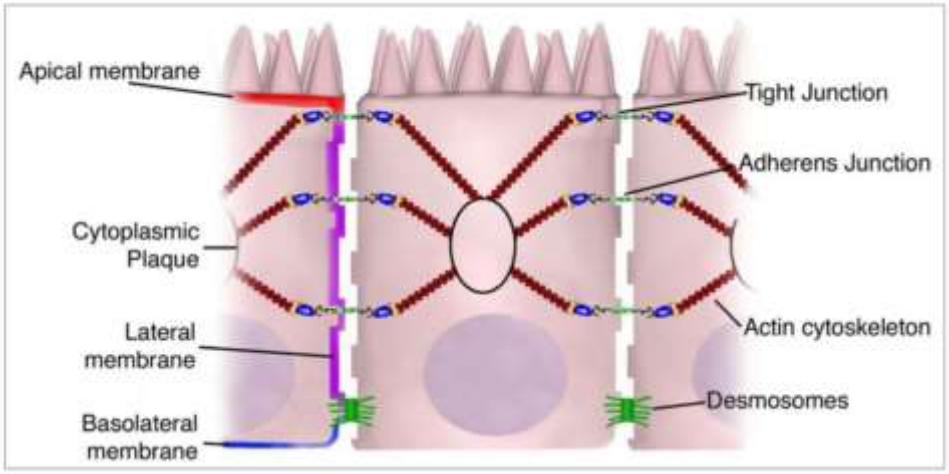


Figure 1.4: 0 A single layer of epithelial cells makes up the intestinal epithelium, with adjacent cells cone cted in three ways; tight junctions, adherens junctions and desmosomes. The latter are attached to keratin filaments and are dense, localised plaques. The former two junctions consist of proteins intracellularly attached via adapter proteins to a cytoskeleton of actin. The collection of proteins in the junctional complexes form "cytoplasmic plaques". Taken from Groschwitz and Hogan (2009). *Reproduced with permission of American Academy of Allergy, Asthma & Immunology*

A number of alternatives to in-feed antibiotics have proven to benefit gut health via their effects on gut barrier integrity. For example, one of the mechanisms by which probiotics have shown to improve gut barrier functions and exclude pathogens is by promoting mucus secretion via increased expression of one or more *MUC* genes as demonstrated in a number of *in vitro* (Mattar *et al.*, 2002; Mack *et al.*, 2003; Otte and Podolsky, 2004; Kim *et al.*, 2008) and *in vivo* studies in rats (Caballero-Franco *et al.*, 2007; Dykstra *et al.*, 2011) and chicken (Smirnov *et al.*, 2005; Shahir *et al.*, 2014). Dietary fibre have also shown to increase mucous secretion in the small intestinal segments of monogastric farm animals (Nyachoti *et al.*, 1997; Montagne, Pluske and Hampson, 2003; Kalantar *et al.*, 2019). In mouse necrotizing enterocolitis model, a *Bifidobacterium* probiotic was shown to stabilize the claudin proteins at tight junctions and prevent intestinal barrier dysfunction (Bergmann *et al.*, 2013). In *Clostridium perfringens* challenge studies in broilers, probiotics have shown to upregulate the expression of tight junction proteins and ameliorating the effects of NE and improving bird performance (Emami *et al.*, 2019; Wu *et al.*, 2019). Another study established that inclusion of

phytogenic feed additives in broiler diets increased expression of certain tight junction proteins indicating an enhancement of intestinal barrier with the phytogenic additive and tendency to improve the feed conversion ratio (Paraskeuas and Mountzouris, 2019). In humans dysregulation of the intestinal barrier has been associated with chronic immune diseases including food allergy, inflammatory bowel disease and celiac disease (Groschwitz and Hogan, 2009). The above evidence supports the role of dietary interventions in enhancing the gut barrier function and prevention of disease pathogenesis.

1.3.3. Effective immune system (Gut Associated Lymphoid Tissue)

The intestinal tract serves as the primary interface between the host and its environment and specifically adapted for assimilation and absorption of nutrients. However, a large and selectively permeable surface implies increased risk of undesirable microorganisms and substances gaining access to the sterile compartments of the body. On the other hand, there are numerous beneficial microbes that colonize the intestines and do not present any threat to the host, in fact, they perform functions necessary for health and well-being. The immune system is therefore posed with the challenging task of maintaining an intricate balance between necessary activation and tolerance through precise mechanisms. The importance and vulnerability of the intestine is reflected in the Gut Associated Lymphoid Tissue (GALT) being the largest organ of the immune system employing more than 70% of the host's immune cells (Kagnoff, 1993).

The intestine responds to pathogens using both innate and adaptive immune responses, as with other immune systems. Initial defences against pathogenic microbes are via the intestinal epithelium which lies between the lumina and the lamina propria beneath. The epithelium is comprised of four types of cells, all progeny from a common stem cell type. These cell types include Paneth cells, which produce peptides with antimicrobial properties; enteroendocrine cells, which produce hormones; goblet cells, which secrete mucus and enterocytes which are absorptive in nature (Yen and Wright, 2006; Abreu, 2010). As described in section 1.3.2 the luminal side of the intestine is covered by mucus whose role

is to prevent direct contact between antigens and the intestinal cell lining. Responses of the innate immune system are triggered when pathogen recognition receptors (PRRs) recognise the pathogen associated molecular patterns (PAMPs) on the pathogens. These PRRs include nucleotide-binding domain leucin-rich repeat-containing receptors, nucleotide-binding oligomerization domain (NOD) proteins and Toll-like receptors (TLRs) (Fukata, Vamadevan and Abreu, 2009). When PRRs on epithelial cells recognize PAMPs on pathogenic microbes, they secrete antimicrobial peptides and pro-inflammatory cytokines which in turn activate lymphocytes, including macrophages and dendritic cells (DCs). These lymphocytes then act in the lamina propria to help defend against infection. B cells then produce secretory IgA (*sIgA*) in response to signals from the PRR in the intestine. This *sIgA* can neutralise pathogens and its production is increased via activation of TLR4, which increases recruitment of B cells to the LP, and causes B cells to switch class to *sIgA*. Other methods of inhibiting microbial pathogens include production of lectins and antimicrobial peptides (AMPs), which are produced mainly in the intestinal crypts, via Paneth cells. AMPs include defensins which inhibit microbes by increasing the permeability of their cell walls.

Although innate immunity is sufficient for protecting the gut on most occasions, adaptive immunity confers more specific and efficient protection against re-encountered pathogens. As the intestine is the largest reservoir of T and B lymphocytes, which are major effector cells of the memory response, the adaptive immune response in the gut plays a central role in protection against infection and ensures that harmless food antigens are well tolerated immunologically (Broom and Kogut, 2018). The adaptive immune response is located the LP and the epithelium cells, with the latter containing intraepithelial lymphocytes (IELs). The LP contains a variety T cells, of which cytotoxic CD8+ are the most common IELs. CD4+ calls are found in both the lymphoid follicles and LP. Gut inflammation is related to high responses of T helper 1 (Th1) and Th17 cells, both of which are part of the CD4+ T cell subset (Casteleyn *et al.*, 2010). There are several mechanisms which maintain intestinal homeostasis by inhibition of antigen presenting cells. These include induction of regulatory T cells to produce

cytokines, such as IL-10 and TGF-β which have an anti-inflammatory effect and therefore supress inflammatory T cell responses (Kim and Lillehoj, 2018).

Chickens have a different reservoir of immune molecules than mammals, including two TLRs which are present in chickens but not in mammals, chTLR15 and chTLR21 (Keestra *et al.*, 2013). They are also suggested to have single isoforms of TLRs 3, 4, 5 and 7, two isoforms of TLR2, and orthologs (genes in different species evolved from a common ancestral gene by a speciation) of TLR1/6/10. In the absence of α-defensin, the only known defensin family in chickens are the β-defensins. Chicken β-defensins are also different to mammalian defensins in their amino acid compositions, the former having arginine as the predominant cationic amino acid while the latter has similar amounts of arginine and lysine (Ganz, 2003; Derache *et al.*, 2009). Although many chemokines and cytokines found in mammals, are also present in chickens, but in limited varieties, particularly in the multigene families (Kaiser *et al.*, 2005). Chickens contain two classes of MHC genes, class I and II, and these are present on the same chromosome, but localised into regions B and Y. There are three classes of immunoglobulins present, including IgY, which is the counterpart of mammalian IgG, there is no evidence they have IgE and IgD (Warr, Magor and Higgins, 1995), and also IgM and IgA. Chickens have a specific primary lymphoid organ, the Bursa of Fabricus which creates a range of B cells, as chickens do not have lymph nodes of the same type and structure as mammals (Kim and Lillehoj, 2018). Chickens also do not have eosinophils or neutrophils but functionally replace the latter with the heterophil and in the gut lining there are also lymphoid aggregates (Kogut, Rothwell and Kaiser, 2005).

In chickens, the GALT includes organized lymphoid structures such as the bursa of Fabricius, caecal tonsils (CT), Payer's patches (PP), Meckel's diverticulum, and lymphocyte aggregates scattered along the intraepithelium and lamina propria (LP) of the gastrointestinal tract. Studies involving oral administration of antigenic materials have shown that helper T cell and IgA precursor B cells in the GALT (and particularly PP) are activated, with subsequent migration to key mucosal effector regions where the antigen-specific responses are mediated via IgA. Following this initial response, the

activated B and T cells migrate to effector tissues (mainly CD4+ memory/effector T cells) where the mucosal immune response developls. The linkage between this T cell activation and the cytokine-driven inflammation response in the intestinal mucosa has been poorly understood until recently when a new lineage of helper T cells, Th17 cells were discovered (Guglani and Khader, 2010) and their contribution to intestinal immune response determined (Min and Lillehoj, 2002; Kim *et al.*, 2012, 2014). Coccidiosis models of intestinal inflammation have also revealed the role of Th17 cytokines in regulating parasite maturation and migration (Zhang *et al.*, 2013; Min *et al.*, 2013; Del Cacho *et al.*, 2014; Kim *et al.*, 2014). In order to avoid inflammation-induced gut pathologies where tolerance is required, the number and activity of pathogenic effector T cells are tightly regulated with Chicken CD4+CD25+ T cells primary suppressor Treg cells abundantly spread across mucosal surfaces (Shanmugasundaram and Selvaraj, 2011). This regulatory system is a pivotal axis in poultry meat production as profit and loss is tightly balanced between excessive immune response and maintaining the gut health required for maximum nutrient absorption. A number of the rapidly emerging phytogenic compounds (PFAs) now registered as feed additives for improving performance in broilers (EU Regulation 1490/2015) are predicated on modulating this axis (Lillehoj *et al.*, 2011). A number of botanical compounds, derived from herbs and spices, and their essential oils have been demonstrate to be highly effective in improvement growth performance (Bravo, Pirgozliev and Rose, 2014). For probiotic additives, registration claims have focussed on improved gut health per se; in particular reduced pathogen load regulation of intestinal epithelial cell function and apoptosis, influence on T-lymphocyte populations, modulation of cytokine profiles, and enhanced antibody secretions (Lee et al., 2010), although many studies report on improved growth and performance in poultry without apparent disease when fed probiotics (Rajput *et al.*, 2013; Palamidi *et al.*, 2016; Yan, Murugesan and Cheng, 2019; Wang *et al.*, 2021)

1.3.4. Gut Microbiota

The GIT of animals is a home to a diverse population of bacteria with the highest cell densities for any ecosystem and in poultry it ranges from 10^7 to 10^{11} bacteria per gram gut content (Apajalahti,

Kettunen and Graham, 2004). The dynamic interactions between a host and its indigenous microbial communities are moulded by a long mutual co-evolution that confers numerous benefits on the host (Ley *et al.*, 2008). The host's gastrointestinal development is significantly influenced by the gut microbiota via biochemical and physiological mechanisms as well as influencing the host gene expression and nonspecific immunity to infections (Lan *et al.*, 2005; Tellez *et al.*, 2006; Yeoman *et al.*, 2012; Rubio, 2019). In chickens, the ileum is dominated by facultative and microaerophilic bacteria (*Lactobacillli* and associated genera) while obligate anaerobes (mainly *Clostridium* related) dominate the caeca (Gong *et al.*, 2007). The current knowledge of the composition of gut microbiome is incomplete, and therefore there is a lack of understanding as to the influence of the microbiome on the health and welfare of the bird. It is estimated from independent analyses via cultures, that the chicken caeca contains many unclassified species, with a total of over 600 species from over 100 genera present (Torok *et al.*, 2011). It would therefore be worthy to identify the gut microbial composition and diversity to improve health and productivity (Pourabedin and Zhao, 2015).

The GIT in chickens contains three phyla in high amounts, with Firmicutes generally being the most abundant, followed by Proteobacteria and Bacteroidetes. There are also some minor phyla present in much lower levels, including Verrucomicrobia and Fusobacteria (Qu *et al.*, 2008) and also Actinobacteria and Tenericutes (Oakley et al., 2014). There are also considerable variations in the bacterial communities in different locations of the GIT. However, there is a similar microbial profile in the early part of the GIT, with crop, gizzard and duodenum all containing mainly Lactobacillus, with some birds having levels up to 99% (Gong *et al.*, 2007; Sekelja *et al.*, 2012). The highest diversity of these Lactobaccili is typically in the crop (Gong *et al.*, 2007; Ranjitkar *et al.*, 2016)., and by the jejunum, there are two main species present, *Lactobacillus salivarius* and *Lactobacillus aviarius* (Gong *et al.*, 2007, Feng *et al.*, 2010). The ileum has a more diverse and variable microbiome compared with the proximal intestine, with *Lactobacillus*, candidatus *Arthromitus, Enterococcus*, *Escherichia_Shigella* and *Clostridium_XI* being the predominant ones (Gong *et al.*, 2007; Mohd Shaufi *et al.*, 2015). At the caecum end of the GIT, the microbial population is the most diverse and the most abundant (Stanley,

Hughes and Moore, 2014), and because of this, there is more information available for caecal microbiota. The most detailed data regarding chicken gut microbiota is available for the caecum (Stanley, Hughes and Moore, 2014). The caecum is a key region for bacterial fermentation of non-digestible carbohydrates and a main site for colonization by pathogens like *Campylobacter, Salmonella* and Enterohemorrhagic *E.coli* and *Clostridium perfringens* (Svihus, Choct and Classen, 2013).

There are several genera present in abundance in the caecum, with *Clostridium* being the most abundant followed by *Lactobacillus* and *Ruminococcus* (Gong et al., 2007). Bacteroidaceae, Enterococcaceae and Enterobacteriaceae are also noted to be present in the caecum (Yin et al., 2010), as are Clostridia, mainly from three families; Lachnospiraceae, Clostridiaceae, and Ruminococcaceae (Danzeisen *et al.*, 2011). Around 40% of the microbiota in the caecum is made up of five species, *Bacteroides fragilis, Lactobacillus crispatus, Lactobacillus johnsonii, Lactobacillus salivarius* and *Lactobacillus reuteri* (Stanley *et al.*, 2015). There are also unclassified residents present in the rich caecal microbiome (Stanley, Geier, Denman, *et al.*, 2013).

In some studies faecal samples have been taken to study the microbiota in a non-invasive way. Faecal microbiota composition is highly variable due to the varying microbiota contributions made by different segments of the GI tract. Videnska et al. (2014) showed that compared to broilers the faecal microbiota of laying hens is normally more complex. However, the core faecal microbiota of both chicken types remains essentially the same represented by members of families, Lactobacillaceae, Peptostreptococcaceae, Enterobacteriaceae, Clostridiaceae, Streptococcaceae, Ruminococcaceae, Lachnospiraceae and Veillonellaceae. Faecal microbiota contains a vast majority of but quantitatively different members from the caecal community indicating that faeces can be used as an effective proxy for caecal sampling where the effect of a treatment or condition on microbiota is to be studied (Stanley *et al.*, 2015).

There also exists a paradox, that even under highly controlled conditions and experiments on the same diet, there remains a strong variation between individual chickens, even when they are the same

breed in the same housing (Nordentoft et al., 2011; Sekelja et al., 2012; Stanley et al., 2013b). There are suggestions that this may be due to the variation in colonisation at hatch, due to eggs hatching without access to maternal bacteria to seed the intestinal microbiota. This allows some hatched chicks access to bacteria from others in the incubator and leads to random colonisation with environmental bacteria which may in part explain individual bird to bird variation (Stanley, Geier, Hughes, *et al.*, 2013).

There are a number of outside factors which affect the microbiome in the chicken gut, including the genotype and sex of the bird (Zhao et al., 2013 and Lumpkins et al., 2008 respectively). In broilers males have been shown to have leaner carcases than females (Lumpkins, Batal and Lee, 2008). It was recently reported that male chicken's caecal microbiota indicated a closer relation with glycan metabolism, while in the female chickens it was more related with lipid metabolism (Cui *et al.*, 2021). This may be the reason for the differences in the male and female carcasses in broilers. Another study, where female and male broilers (age 22 and 42 days) were compared using quantitative PCR (qPCR), showed differences in abundance of *Lactobacillus salivarius, L. crispatus, L. aviarius,* and *E. coli* in their ceca (Torok *et al.*, 2013). Environmental conditions such as housing (Nordentoft et al., 2011), litter (Cressman et al., 2010; Torok et al., 2009) and stocking density (Guardia et al., 2011) can also factor. Diet and feed availability will also effect the gut microbiome (Torok et al., 2009), particularly when birds are feed restricted (Callaway et al., 2009). There is also an age effect on the microbiota, with some bacteria emerging or vanishing during the life of the bird, and complexity increases as the birds age (Yin *et al.*, 2010; Crhanova *et al.*, 2011; Danzeisen *et al.*, 2011; Sekelja *et al.*, 2012). For example, although Firmicutes dominate in younger chicks, once birds are over 7 months, Bacteroidetes are more abundant (Callaway et al., 2009; Videnska et al., 2014), which becomes important when comparing breeders with standard meat birds. In microbiota of laying hens, Videnska et al. (2014) found four diffferent cecal profiles in a 60 week study. From the above examples it is clear that there is variability in microbiota depending on age or time point of sampling and that more frequent sampling is required to provide robust data.

1.4. Significance of microbiota in gut health

1.4.1. Role of microbiota in nutrient provision to the host

Many intestinal bacteria ferment dietary polysaccharides, oligosaccharides and disaccharides to short chain fatty acids (SCFAs) mainly acetate, propionate and butyrate. These short chain fatty acids are utilized by the host as carbon and energy source with butyrate being the preferential energy source for intestinal enterocytes. Rest of the butyrate is absorbed in the blood and transported to the liver by the portal vein where it is metabolised to produce fatty acids, cholesterol and ketone bodies (Guilloteau *et al.*, 2010). Receptors for SCFA have been detected in a variety of tissues but the highest number are found on immune cells. Several studies have indicated that butyrate, besides providing epithelial cells with energy, markedly increases epithelial cell proliferation and differentiation, and improves colonic barrier function (Cook and Sellin, 1998; Mariadason *et al.*, 1999).

Although fermentation of polysaccharides to SCFA can take place in most parts of the GIT (from crop to caecum), it primarily occurs in the caecum which is the most densely populated region (Rehman *et al.*, 2007). Fermentation increases as birds age. Caecal acetate, propionate and butyrate are undetectable in 1-day old chicks but as the microbiome becomes established their concentration in the caeca increases up to 15 days of age and remains sTable thereafter (Van Der Wielen *et al.*, 2000). Some studies have also indicated a gradual increase in SCFA concentrations even up to 42-days of age (Svihus, Choct and Classen, 2013). The absorption of SCFA across the caecal epithelium occurs via passive diffusion (Hooper, Midtvedt and Gordon, 2002). Short Chain Fatty Acids contribute to host nutrition by regulating blood flow to the GIT, stimulating growth and proliferation of enterocytes, increased nutrient digestibility and stimulation of digestive enzymes all of which eventually increase animal performance (Guilloteau *et al.*, 2010).

Gut bacteria also contribute to nitrogen metabolism. In the cloaca, the junction at which the intestinal and ureogenital tracts meet, urine mixes with the faeces. Due to the retrograde peristaltic movement in the rectum some urine may travel to the caeca where the uric acid in the urine gets catabolized to

ammonia by caecal bacteria. Ammonia can be absorbed by the host and used to synthesize some non-essential amino acids such as glutamine (Svihus, Choct and Classen, 2013). Some of the nitrogen is also utilized by the gut bacteria to synthesize their own cellular proteins. Hence gut bacteria can themselves serve as a source of amino acids. However, most of the bacterial proteins are lost with excretion of faeces due to the caecum's inability to digest proteins. Utilization of bacterial proteins is possible where coprophagy (ingestion of faeces) is observed (very common in pigs and poultry) and bacterial proteins can be digested and absorbed in the proximal intestine (Vispo and Karasov, 1997; Koutsos and Arias, 2006).

The gut microbiome of poultry may also provide vitamins especially of the B group to its host. Like bacterial proteins, vitamins synthesized by gut bacteria are defaecated because they cannot be absorbed in the caecum. However, coprophagic birds may benefit from bacterial vitamin synthesis. This was evidenced in a study showing chickens housed in wire cages where coprophagy is hindered, had a greater vitamin requirement compared to chickens raised on hard floors (Vispo and Karasov, 1997).

The gut microbiome has evolved in a symbiotic relationship with its host and, in healthy birds, direct competition for nutrients is negligible as most of the absorption of nutrients takes place in the small intestine where the bacterial density is low and retention time is short. Because of the vital role played by the gut microbiome in feed digestion and absorption several studies have drawn attention to associations between gut microbiome and feed utilization efficiency (Johnson et al., 2018; Singh et al., 2012, Torok et al., 2008). As next generation sequencing technologies continue to advance, the role of the gut microbiota in growth performance of chickens is becoming clear.

1.4.2. Role of microbiota in modulating intestinal morphology and physiology

In the early post hatch period, the digestive organs of chicks undergo rapid anatomical and physiological changes due to the switch from a lipid-rich yolk to carbohydrate- and protein-based diet (Jin, Corless and Sell, 1998). The rapidly developing intestinal tract provides an ideal niche for microbial

colonization. At the same time gut microbiome also plays an important role in intestinal development. Studies using germ free (GF) chickens demonstrated that the small intestine and caecum of GF birds was lighter and had a thinner wall when compared to those of conventional (CV) birds (Furuse and Okumura, 1994; Gabriel *et al.*, 2006). The rate of nutrient passage through the intestinal walls is affected amongst other factors by the thickness of the wall, being faster and slower for the thin and the thick walls respectively (Harrison and Coates, 1972). As a result, lower and higher nutrient digestibility in conventional and GF birds respectively, have been observed (Furuse and Yokota, 1984). It has been shown that SCFAs increase the proliferation of enterocytes *in vitro* which may partially describe the growth stimulating effect on intestines (Blottiere et al., 2003; Fukunaga et al., 2003; Le Blay et al., 2000). This evidence was supported by the study of Muramatsu et al. (1993) who reported that by feeding fermentable carbohydrates, CV chickens had higher gut and organ weight than their GF counterparts which may be attributed to microbial fermentation providing SCFAs for intestinal development.

Intestinal morphology is also affected by the gut microbiome. GF birds or birds colonized with a low bacterial load than CV birds have shorter intestinal villi and shallower crypts (Gabriel *et al.*, 2006; Forder *et al.*, 2007). Dietary supplementation of probiotics has shown to increase villus height and villus height to crypt depth ratio in the small intestinal segments of broilers under physiological conditions (Rajput et al., 2013; Sen et al., 2012) and when challenged with pathogen (Musa *et al.*, 2019). A similar effect on intestinal morphology was observed with supplementation of poultry diets with prebiotics like FOS, MOS and XOS (De Maesschalck et al., 2015; Ding et al., 2018; Pourabedin et al., 2014; Xu et al., 2003) and fermented feed (Chiang et al., 2010; Sun et al., 2013). These studies have indicated that such morphological changes are not a direct effect of supplementation but rather an indirect effect due to modulation of gut microbiome by the supplements.

The gut microbiome can also affect the activity of digestive enzymes. Compared to GF chickens, CV birds, had a higher activity of intestinal alkaline phosphatase (Palmer and Rolls, 1983). Diets that modulate the gut microbiome structure may influence the activity of digestive enzymes. For example,

broilers fed fermented cottonseed meal or FOS had higher activity of amylase and protease when compared to those fed corn-SBM diet (Sun et al, 2013; Xu et al., 2003). Similarly broiler diets with fermented SBM instead of unfermented SBM increased the activities of protease, lipase and trypsin (Feng *et al.*, 2007). The authors concluded that these diets stimulated probiotic bacteria like *Bifidobacterium* and *Lactobacillus* while supressing coliforms like *E.coli* that damage the villus and microvillus and thus impair the secretion of digestive enzymes or secrete their own proteases to degrade host enzymes.

1.4.3. Role of microbiota in immune development & educating the immune system

Various factors such as antimicrobials, environment and stress influence the development of the gut immune system but exposure to microorganisms is considered the most important (Broom and Kogut, 2018). Colonization of the GIT with microorganisms begins soon after hatch in aves or during the passage through the birth canal in case of mammals and microbial succession follows until eventually a complex and dynamic microbiome is established. Extensive interactions take place between the colonizing microbes (the non – self cells) and the immune system which play an important role in maintaining the intestinal homeostasis and preventing excessive inflammation in response to normal gut flora (Brisbin, Gong and Sharif, 2008). Studies in GF animals have demonstrated that intestinal microbes influence the organization of the lymphoid tissue eg Payers Patches, secretion of antimicrobial peptides and the localization of immune cells at mucosal surfaces (Honda and Littman, 2012). Indeed, GF animals have a poorly developed mucosal and systemic immune system and do not generate normal oral tolerance to dietary proteins. On the other hand, there are numerous examples of human and animal disorders that are associated with an altered microbiota status, such as obesity, inflammatory enteropathy and autoimmune diseases (Caesar, Fåk and Bäckhed, 2010; Wlodarska and Finlay, 2010). In poultry, in-feed antimicrobials caused an altered microbiota closely associated with dysbiosis (Li et al., 2010a) and enhanced susceptibility to *Clostridium* pathogens (Li et al., 2010b). The complexity of the enteric microflora in chickens has been shown to significantly influence the profile

of the T-cell receptor β repertoire (Mwangi *et al.*, 2010) and the mucin composition (Forder *et al.*, 2007).

When commensals or pathogenic bacteria breach the intestinal epithelial cell (IEC) barrier via either host mediated or bacterial mediated mechanisms, they are dealt with by cells such as macrophages, dendritic cells (DC), natural killer (NK) cells, heterophils and γδ T cells. These cells are capable of recognizing members of microbiota by binding to large groups of conserved molecules on microorganisms known as microbe associated molecular patterns (MAMP) or pathogen associated molecular patterns (PAMP) via pathogen recognition receptors (PRR). In mammals, it is generally accepted that the recognition of commensal bacteria by PRR is regulated by the expression patterns of these receptors. For example, some studies observed that TLRs are not typically expressed on the apical surfaces of IEC in mammals but are expressed intracellularly or basolaterally indicating a deliberate down regulation in response to commensal bacteria that reside in the lumen (Iwasaki and Medzhitov, 2004; Rakoff-Nahoum *et al.*, 2004). However, this is not absolute as other studies have shown the apical expression of TLR4 on IEC (Lotz *et al.*, 2006; Stokes and Waly, 2006). In chickens TLR expression has been observed throughout the different regions of the intestine (Iqbal, Philbin and Smith, 2005) but their apical or basolateral localization has not yet been examined, it is therefore unknown if similar methods of control exist in chickens. Additionally, intestinal macrophages display phagocytic and bactericidal activity but they have been shown to be hyporesponsive to TLR ligands and do not produce pro inflammatory cytokines thereby ensuring that any bacteria that cross the epithelium are rapidly cleared (by phagocytosing and killing) without causing unnecessary inflammation (Smythies *et al.*, 2005). In chickens, the role of intestinal macrophages in immune homeostasis has not yet been defined (Higgins *et al.*, 2007). Chicken heterophils may play an important role in maintaining intestinal homeostasis by interacting with intestinal microbiota. Heterophils have been shown to express cytokines and chemokines in response to peptidoglycan and CpG from normal mucosal bacteria (He *et al.*, 2005; Kogut *et al.*, 2006). Kogut et al (2005), demonstrated that chicken heterophils recognized peptidoglycan from staphylococci which are

normal residents of the intestine within the first few days of life via TLR2 leading to activation of innate defences. Another anti-inflammatory mechanism to normal flora described in mammals but not in chickens is that intestinal dendritic cells carrying commensal bacteria do not penetrate beyond the mesenteric lymph nodes and are believed to play a vital role in inducing a protective, local IgA response (Macpherson *et al.*, 2000).

It has been shown in chickens that members of the microbiota have the ability to modulate host cytokine gene expression (Oakley and Kogut, 2016) thereby influencing the type of immune response generated within GALT. However, there are very few studies in chickens showing the effect of commensal bacteria on antibody mediated or cell mediated responses. Recently Volf et al. (2017) studied gene expression and protein abundance in the caecum of GF and CV chickens and showed that immunoglobulins, which represent the antibody mediated branch of immune response, were not expressed in the GF birds. They further demonstrated that "expression of immunoglobulins was absolutely dependent on presence of viable microbiota" as GF birds inoculated with heat killed members of normal gut flora did not activate antibody production (Volf *et al.*, 2017). Another study showed that the number of B and T lymphocytes were higher in the caecum, caecal tonsils, and bursa of Fabricius of CV birds compared to GF and antibiotic treated (limited microbiota) birds (Han *et al.*, 2017) which indicates normal development of immunity in response to colonization with gut microflora. The same study also confirmed that the number of CD4+, CD8+ and B cells in caecum of antibiotic treated and GF birds inoculated with *C. jejuni* were higher than in *C.jejuni* inoculated CV birds which shows a more severe infection in the antibiotic treated and GF groups. Gut microbiota of chickens also plays an important role in the initiation of innate responses against viruses. mRNA expression showed that infection of microbiota depleted (via antibiotic treatment) chickens with H9N2 influenza virus resulted in significantly down-regulated type I interferon responses both in the respiratory and gastrointestinal tracts compared to undepleted-H9N2 infected chickens while the antibody mediated response was unaffected (Yitbarek *et al.*, 2018).

Further research is needed to fully understand the relationship between microbiota and immunity in chickens which will aid in the development of in feed antibiotic alternatives as they will have an impact on microbiota, GALT and susceptibility to disease.

1.4.4. Role of microbiota in prevention of colonization by pathogens

The gut microbiota plays a vital role in supporting the gut barrier function by competing with pathogens for space and nutrients. In 1973, Nurmi and Rantala provided clear evidence that orally gavaging newly hatched chicks with the gut contents of health adult chickens profoundly increased their resistance to *Salmonella* infection (Nurmi and Rantala, 1973) which later lead to the concept of 'competitive exclusion' (CE) and contributed to the development of probiotic based products. Their work suggested that early initial colonization by gut microbiota resisted subsequent infections by pathogens. Apart from physical occupation of the gut and resources competition, the commensal bacteria cause direct physical or chemical insult to the invading colonist via production of H_2O_2, bacteriocins and organic acids including SCFA (Oakley et al., 2014; Stern et al., 2006). Microbial SCFA enhance gut barrier integrity via upregulation of mucin and tight junction proteins and affect intestinal immune responses (Guilloteau *et al.*, 2010; Ahsan *et al.*, 2016; Moquet *et al.*, 2016). In addition to their beneficial effects on the host post absorption, the presence of SCFA lowers luminal pH and inhibits pathogenic bacteria such as *Salmonella typhimurium* while favouring the growth of beneficial probiotic bacteria in the gut (Józefiak, Rutkowski and Martin, 2004; Ahsan *et al.*, 2016). Although the gut microbiota from adult birds was initially used to provide protection against *Salmonella*, it has since then been successfully used to protect chicks against pathogenic *E.coli, C. jejuni, C. perfringens* and *L. monocytogenes* (Schneitz, 2005). It is considered as the most effective and harmless method to control intestinal disturbances in poultry.

1.5. Culture independent methods to study gut microbiota

Until recently, the identification of bacteria was almost entirely based on phenotypic characteristics which involved growing the organisms on agar plates also known as microbial culture. Along with the

rise of culture-independent profiling, culture-based techniques have been refined to capture a wider array of organisms than previously possible with the help of more specifically formulated culture media and meticulously controlled conditions. Yet, they lack the necessary resolving power to accurately analyse extremely complex gut microbiota and are therefore unreliable for the purpose identification of gut microbiota (Blaut *et al.*, 2002). Moreover, they are time-consuming, laborious and costly. Identification of intestinal microbiota from culture-based methods could be incomplete and inaccurate because only 10 to 60% of the total intestinal tract bacteria are culturable (Gong *et al.*, 2007).

To overcome these challenges associated with selective growth media and isolation of bacteria from environmental samples, culture-independent methods have become fundamental tools in studying bacterial communities (Rastogi and Sani, 2011). Most of these molecular methods rely on the sequence analysis of the 16S ribosomal RNA (rRNA) gene for identification, quantification, and classification of bacteria. The 16S rRNA is a subunit of the 30S small compartment of prokaryotic ribosomes. Its sequencing was recognized as the new standard for identifying bacteria (Woese *et al.*, 1985; Woese, 1987). The 16S rRNA gene is about 1,550 base pairs (bp) long and is composed of both variable and conserved regions among different species of bacteria (Figure 1.5). 16S rRNA has been used as a phylogenetic marker for identification, classification and quantitation of microbes within complex biological mixtures such as environmental or gut samples because of its ubiquitous distribution across all archaeal and bacterial lineages, its evolutionarily conserved nature, has a number of variable regions and is of sufficient length to give high resolution data for analysis (Woese and Fox, 1977; Schmidt, DeLong and Pace, 1991; Cox, Cookson and Moffatt, 2013). Universal primers are often used to amplify the conserved regions, whereas the sequence of the variable regions in between is used for the comparative taxonomy (Greisen *et al.*, 1994). The 16S rRNA has nine hypervariable regions, V1 to V9, which shows a considerable sequence diversity (Lane *et al.*, 1985).

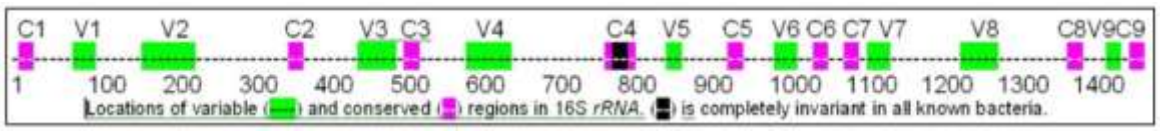


Figure 1.5. Location of conserved and hypervariable regions in the 16S rRNA gene. Numbers below dotted line refer to base pair position in E. coli. Reproduced from Ram *et al*. (2011). *Reproduced with permission from Taylor and Francis*.

16S rRNA analysis has been used for several studies in monogastric farm animals such as to investigate the distribution of microorganisms in different regions of the gastrointestinal tract (Gong *et al.*, 2007; Stanley *et al.*, 2015; Glendinning, Watson and Watson, 2019), effect of environment (Schokker *et al.*, 2014), diet (Ferket *et al*., 2005), age (Lu *et al*., 2003; Ocejo *et al*., 2019), in-feed antibiotics (Schokker *et al.*, 2017; She, Cai and Liu, 2018) and pathogen challenge (Han *et al.*, 2017; Ma *et al.*, 2017; Macdonald *et al.*, 2017) on the gut microbiome. These methodology advances have meant that identification of caecal bacteria has expanded and it has been reported that only 10% of sequenced 16S rRNA relates to bacterial species which were already known, and the remainder are from new species or genera (Apajalahti, Kettunen and Graham, 2004). Cultivation studies were also examined using molecular methods and the same bacterial groups were found, but in different abundance among the cloned sequences (Bjerrum *et al.*, 2006).

Some of the other common culture-independent methods for profiling microbial communities are genetic finger printing techniques such as denaturing- or temperature-gradient gel electrophoresis (DGGE and TGGE), real-time PCR and more recently, whole community analysis approaches such as high throughput sequencing (HTS) platforms (Rastogi & Sani, 2011). In the recent years, the Sanger sequencing method, which was introduced in 1977 (Sanger, Nicklen and Coulson, 1977) has been partially replaced by HTS technologies. HTS platforms such as 454 pyrosequencing (Roche, Basel, Switzerland), SOLiD (Applied Biosystems, MA, USA) and Hi/MiSeq systems (Illumina, CA, USA), have enabled ultra-deep sequencing for studying complex microbial populations (Shendure and Ji, 2008). The 454 pyrosequencing and Illumina systems are the most common platform used for the analysis of microbial communities (Andersson *et al.*, 2008; Diaz-Sanchez *et al.*, 2013) but the discontinuation of

the 454 sequencers by Roche in 2015 and the lack of support for the technology since 2016 has limited its use as a preferred sequencing platform. To date, limited studies have used HTS of 16S rRNA to investigate the poultry GI tract microbiota.

There have been many studies using methods without microbial culture, and these have shown a highly diverse caecal microbiome, containing mainly Gram positive bacteria (Zhu *et al.*, 2002; Gong *et al.*, 2007). It is therefore clear that using sequencing and molecular techniques can provide more complete information about the microbiome (Lan et al., 2002; Zhu et al., 2002).

1.6. Antibiotic use in Poultry diets

1.6.1. History and problem of antibiotic resistance

Antibiotics have been used in poultry feed at subtherapeutic levels for more than 50 years to improve growth performance and feed efficiency and to reduce intestinal pathogens. It has been estimated that the use of antibiotic growth promoters in animal feed improves weight gain by 4 to 8% and feed utilization efficiency by 2 to 5% (Butaye, Devriese and Haesebrouck, 2003). The exact mechanisms by which AGPs promote growth are not clearly understood, but findings that in-feed antibiotics have no growth-promoting effects in germ-free chickens suggest that their mechanism of action must be via the intestinal microbiota (Feighner and Dashkevicz, 1987). Several hypotheses have been proposed to explain how antibiotics improve growth performance. These include: (i) an increase in efficiency of nutrient absorption due to a thinner intestinal epithelium in antibiotics-treated animals, (ii) reduction or elimination of gut pathogen load and subclinical infections, (iii) an increase in nutrient availability due to a reduced microbial destruction of nutrients and (iv) reduction of toxins and growth-depressing metabolites produced by bacteria (Feighner and Dashkevicz, 1987; Butaye, Devriese and Haesebrouck, 2003). Growth promoting antibiotics tend to be less effective in animals when used under hygienic and controlled experimental conditions, suggesting a reduction or inhibition of subclinical infections as the most probable mechanism for their action (Brüssow, 2015). Further to their antimicrobial effect,

it has been proposed that they have anti-inflammatory effects on intestinal phagocytic cells by inhibiting the production and release of catabolic mediators (Niewold, 2007).

Unfortunately, the long term and extensive use of antibiotics resulted in the selection of resistant bacterial strains. In addition, genes coding for resistance have been transferred to previously susceptible bacteria posing a threat to both human and animal health (Montagne, Pluske and Hampson, 2003). The unifying concept of resistance is that bacteria under stress, in this case antibiotics, will sense a deteriorating environment and undergo programmed molecular response by which specific stress inducible proteins are synthesized that act to prevent or repair the molecular damage caused by the stress (Ferket et al., 2005).

Owing to the global campaign on reduction in the use of antibiotics, antibiotic usage by food animal industry has come under intense scrutiny. Sweden became the first country to ban antibiotic growth promotors (AGP) in 1986. In 2000, Denmark restricted the use of antimicrobials to therapeutic use by prescription only (Dibner and Richards, 2005). This was followed withdrawal of AGPs and then a complete ban on use of antibiotics as growth promoters in the EU member nations in 2006. Although such a ban has not yet been initiated in North America, the US FDA asked farmers to voluntarily phase antibiotics from livestock production in April 2012. According to the WHO report (2003), there were no changes in weight gain or mortality in broilers due to termination of antibiotic growth promoters (AGP) in Denmark. The adverse effects of withdrawal of AGPs on weight gain and mortality were more evident in pigs than in poultry due to presence of diseases such as porcine dermatitis, nephritis syndrome and post weaning multisystemic wasting syndrome (Casewell *et al.*, 2003). However, ban on the use of antibiotics in 2006 did see an increase in the incidence of colibacillosis and necrotic enteritis (NE) in the EU. *Clostridium perfringens* induced NE became one of the most noticeable emerging diseases of broilers in Europe (Van Immerseel *et al.*, 2009). Other important consequences were the increase in the susceptibility of foodborne pathogen colonization in the intestine and consequently contamination of poultry products for human consumption. Casewell *et al*. (2003)

argued that removal of antibiotics and subsequent increase in infections, despite efforts to improve animal performance via management practices resulted in substantial increase in use of therapeutic antibiotics for food animals in Europe. On the other hand a study conducted at John Hopkins University, using empirical data collected by US broiler industry, demonstrated that the use of antibiotic growth promotors (AGP) in poultry production does not economically benefit producers because the increased weight gains due to AGPs were not sufficient to balance the cost of antibiotics (Graham, Boland and Silbergeld, 2007). Nonetheless antibiotics were ubiquitously used as they provided a form of insurance against severe infections which were economically very costly when they occurred and possibly due to a habit formed in husbandry practice starting from the time where improvements in performance were seen due to in feed antibiotics resulting in higher financial returns.

Since the removal of AGPs from poultry diets, due to regulation or reasons of consumer preference, there has been tremendous pressure on the poultry industry to look for viable alternatives. However, it is unlikely that a single economically viable replacement to AGPs can be introduced and that a multifactorial approach is needed to address the challenges specific to on-farm situations (Dibner and Richards, 2005). An effective alternative to antibiotics should have significant and sustainable impact on animal performance and health and be safe for both animals and humans, be easy to apply and store and provide substantial returns on investment (Yegani and Korver, 2008)

1.6.2. Alternatives to in-feed antibiotics

The use of dietary supplements has by far been the most common strategy to manage bird performance without the use of antibiotics. The effective use of supplements is dependent upon some degree of understanding of their mechanisms of action. Considering the mode of action of modulation of both microbiome and immunity, any potential supplement should possess these qualities while also improving bird performance or growth (Huyghebaert, Ducatelle and Immerseel, 2011; Seal *et al.*, 2013). Proposed alternatives include enzymes, organic acids, prebiotics, probiotics, synbiotics, and phytogenics and many of these have been tested in poultry research. There are also some novel

alternatives which have become of interest, such as bacteriophages, antimicrobial peptides and hyperimmune egg yolk IgY, but these are often in an early stage of investigation.

1.6.2.1. Probiotics

Probiotics, also referred to as direct fed microbials (DFMs), are being increasingly recognized as potential alternatives to antibiotics to improve production efficiency. They are defined as "*live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance*" (Fuller, 1989). Another definition accepted by FAO/WHO (2001) states that "*Probiotics are mono or mixed cultures of live organisms which when administered in adequate amounts confer a health benefit to the host.*" Probiotics, containing single or multiple strains of microorganisms, alone or in combination with other additives, are usually given in feed or water (Thomke and Elwinger, 1998) but recently novel techniques like spraying on chicks or embryonated eggs and *in-ovo* administration are being explored (Wolfenden *et al.*, 2007; Cox and Dalloul, 2015).

In poultry, the use of probiotics first reported as early as 1973 by Nurmi and Rantala. Since then, several studies have been made and continue to be developed with the use of probiotics. There are several types of probiotics available in the market to be used in poultry, with a variety of bacteria including, *Bacillus, Bifidobacterium, Enterococcus, E. coli, Lactobacillus, Lactococcus, Streptococcus, Pediococcus* species and yeast species (Fuller, 1995; Patterson and Burkholder, 2003; Kabir *et al.*, 2004; Mountzouris *et al.*, 2007). Non-defined mixed cultures, known as competitive exclusion cultures, have also been used to treat one-day chicks with an indefinite microbiota derived from faeces of adult birds. Another characteristic of probiotics is that they may comprise micro-organisms that are the normal residents of the GIT of poultry, like species of *Bifidobacterium*, *Lactobacillus, Enterococcus* and *Streptococcus* or others like *Bacillus* or *Saccharomyces* spp obtained from natural sources. Natural adaptation of lactic acid bacteria to intestinal environment by their production of lactic acid have provided advantages for these organisms over the others used as probiotic (Guerra *et al.*, 2007).

Several mechanisms of action of probiotics have been proposed and have been reviewed in detail by Abd El-Hack et al. (2020); Ahasan et al. (2015); Aziz Mousavi et al. (2018); Jha et al. (2020); Ng et al. (2009); Sherman et al. (2009). Some of the common mechanisms have been depicted in figure 1.6. Briefly they include:

- Creating an unfavourable environment for harmful bacterial species via production of lactic acid and SCFA thereby reducing the pH
- competing for nutrients with harmful bacteria (competitive exclusion)
- production and secretion of antibacterial substances (e.g. bacteriocins by *Lactobacillus, Bacillus* spp.);
- inhibition of bacterial adherence and translocation
- improving barrier function (modulation of cytoskeletal and epithelial tight junctions
- increasing mucin synthesis
- modulating and regulating intestinal immune responses (reducing pro-inflammatory cytokines, increasing secretory IgA production)
- promoting specific and non-specific immune responses against pathogens (activation of macrophages, increase cytokine production by intraepithelial lymphocytes).

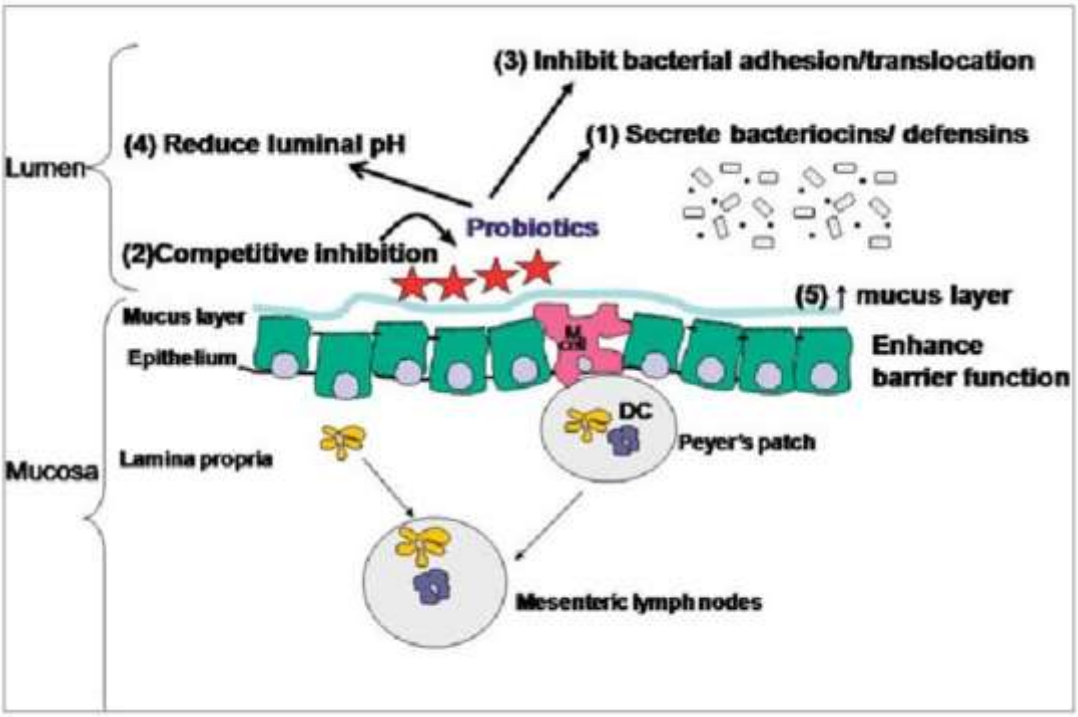


Figure 1.6. Inhibition of enteric bacteria and enhancement of barrier function by probiotic bacteria. Schematic representation of the crosstalk between probiotic bacteria and the intestinal mucosa. Antimicrobial activities of probiotics include the (1) production of bacteriocins/defensins, (2) competitive inhibition with pathogenic bacteria, (3) inhibition of bacterial adherence or translocation, and (4) reduction of luminal pH. Probiotic bacteria can also enhance intestinal barrier function by (5) increasing mucus production. (Adapted from Ng et al., 2009). *'Reproduced with permission Oxford University Press'*

A variety of bacteria and in some *Saccharomyces* spp. have been tested as probiotics in poultry. Although the majority of the conducted research was specifically aimed at investigating the effects of probiotics in reducing the numbers of pathogenic microorganisms in the GIT, a considerable amount of research also examined the effects of probiotics on improving growth and performance in poultry without apparent disease. Several comprehensive and systematic reviews of probiotic use in poultry have been published (Abd El-Hack et al., 2020; Ahasan et al., 2015; Aziz Mousavi et al., 2018; Blajman et al., 2014; De Faria Filho et al., 2006; Jha et al., 2020). Generally, these reviews indicate that probiotics inclusion increased body weight gain and improved feed efficiency, their application via water was more efficacious than through feed, no differences between the use of mono- or multi-strain probiotics and effects were variable with the type of strain used. In addition, probiotics supplementation also enhanced the general immune function of broilers, as evidenced by the

augmented serum/plasma immunoglobulin levels, increased antibody titers to pathogens, and changes in immune cell numbers. These reviews also highlighted that intestines of broilers that were given probiotics showed better development and an increase in villus height and crypt depth ratio, positively modulated the intestinal microbiota and increased numbers of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* spp.

The beneficial effects of probiotics supplementation were also reported in laying hens. Kurtoglu et al. (2004) demonstrated that hens fed diets supplemented with probiotics showed increased egg production compared with controls. Lei et al. (2013) reported that dietary inclusion of *B. licheniformis* improved laying performance and egg mass. Consistent with these findings, various DFM products supplementation was also shown to improve body weight and performance in turkeys (Russell and Grimes, 2009; Wolfenden *et al.*, 2011).

There is a large range of microorganisms used as probiotics, with variations in species and strains of the same species, and therefore, they present variations in their metabolic activity and as a consequence variation in the results of their use. However, other factors such as the origin species, probiotic preparation method, survival of colonizing micro-organisms in the GIT, the environment where the birds are raised, management (application time and route), the immunologic state of the birds, poultry breed evaluated, as well as age and simultaneous use of antibiotics (Otutumi *et al.*, 2012).

1.6.2.2. Prebiotics

A prebiotic is defined as "*a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health*" (Roberfroid *et al.*, 2010). This definition was recently advanced to shift the focus from selective targets to microbial ecological functions within the gut. The new definition of a prebiotic is *"a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on*

the host" (Bindels *et al.*, 2015). Thus in order to be classified as a prebiotic compound, a dietary ingredient has to be:

- Neither digestible nor absorbable through the GI tract
- metabolised by one or limited number of gut commensal bacteria
- able to induce health benefits for the host (Roberfroid *et al.*, 2010).

Prebiotic use in broiler chickens does not have a long history compared to their use in human and pet food (Yang, Iji and Choct, 2009). The most commonly tested oligosaccharides in poultry production include fructo-oligosaccharides (FOS) (Kim et al., 2011; Świątkiewicz et al., 2011), mannan-oligosaccharides (MOS) (Baurhoo, Ferket and Zhao, 2009; Xiao *et al.*, 2012), xylo-oligosaccharides (XOS) (Courtin *et al*., 2008; Ribeiro et al., 2018; Zhenping *et al*., 2012), galacto-oligosaccharides (Slawinska *et al.*, 2019) and soybean meal oligosaccharides (Lan *et al.*, 2007).

Dietary supplementation with prebiotic oligosaccharides have been reported to reduce intestinal *Salmonella* colonization (Eeckhaut *et al.*, 2008), modulate immune cell parameters (Shanmugasundaram and Selvaraj, 2012) and ameliorate inflammation response (Shanmugasundaram, Sifri and Selvaraj, 2013) and severity of lesions during intestinal infection (Lensing *et al.*, 2012). Various potential mechanisms have been proposed for health benefits of prebiotics (Figure 1.7). These include:

- providing a substrate for the gut commensal microbiota, and will affect their growth and metabolic activities
- preventing adhesion of certain bacterial species by occupying carbohydrate-binding sites in bacteria and host cells
- an increase in SCFA production, and will affect immunomodulation and host metabolism (Saulnier *et al.*, 2009; Roberfroid *et al.*, 2010).

In addition, the presence of SCFA in the intestines contributes to a lower pH, a better assimilation of Ca and Mg, and inhibition of potentially harmful bacteria (Teitelbaum and Walker, 2002; Wong *et al.*,

2006). Butyrate, among SCFA, is a favoured source of energy for colonocytes, increases the absorptive capacity of the colonic epithelium, and inhibits the growth of colonic carcinoma cells, both *in vitro* and *in vivo* (Van Craeyveld *et al.*, 2008). The cancer-suppressing properties of dietary fibres seem to correlate with their capability to generate butyrate upon colonic fermentation (Perrin *et al.*, 2001). The selective stimulation by prebiotics of certain colonic bacteria, such as *Bifidobacteria* is in some cases paralleled by suppression of protein fermentation in the colon (De Preter *et al.*, 2004; Geboes *et al.*, 2006). Reduced protein fermentation in the colon is a desired outcome, as the amino acid degradation pathways in bacteria result in the production of potentially toxic catabolites such as ammonia, amines and phenols, some of which have been implicated in bowel cancer and in exacerbation of diseases such as ulcerative colitis (Van Craeyveld et al., 2008).

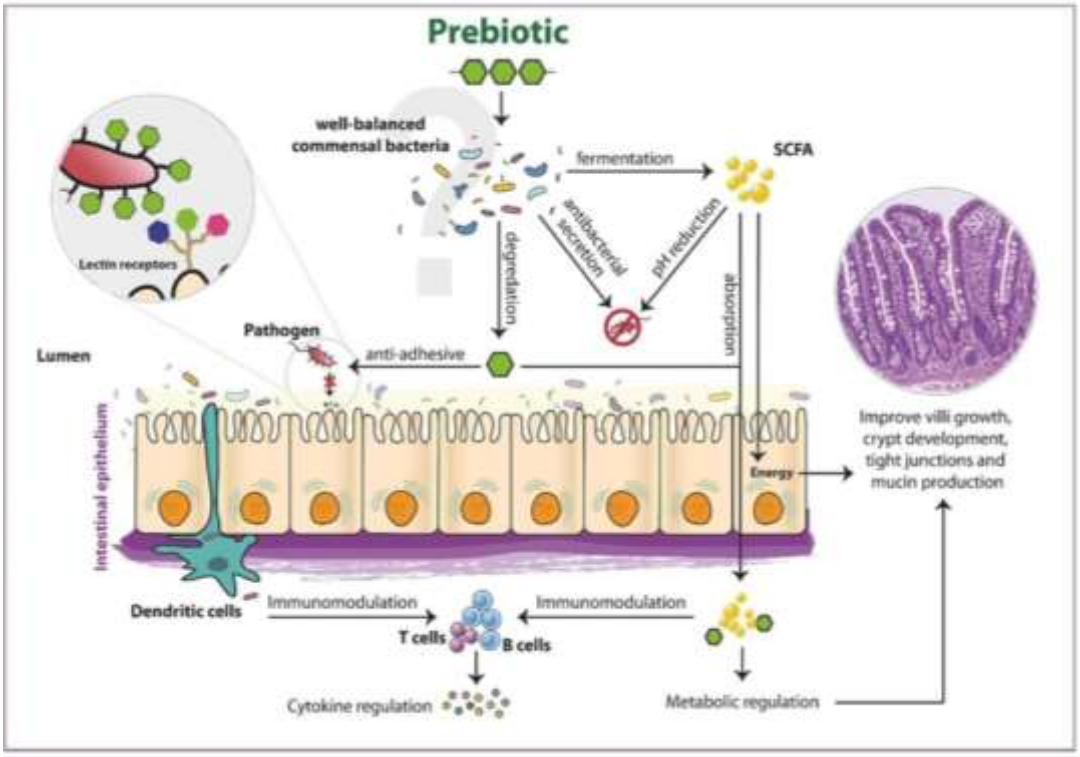


Figure 1.7. Potential mechanisms of action of prebiotics. Prebiotics are metabolized by the gut commensal microbiota. The gut microbiota can ferment prebiotics into SFCA, mainly acetate, propionate and butyrate. SCFA lower the luminal pH, provide energy sources for epithelial cells and have profound effects on inflammation modulators and metabolic regulations. A well-balanced bacterial community can also improve intestinal mucosal structure. Some bacterial strains produce antimicrobial factors or stimulate the immune system by signaling dendritic cells. Oligosaccharides and monosaccharides can reduce pathogen colonization by blocking the receptor sites used by pathogens for attachment to the epithelial cell surface (Adapted from, Pourabedin and Zhao, 2015). '*Reproduced with permission of Oxford University Press'*

The results of the use of prebiotics on performance and gut microbiota of chickens are contradictor but holo- and meta-analysis approaches indicate an overall benefit to growth and performance resulting from their inclusion in broiler feed (Hooge, 2004; Rosen, 2007; Hooge and Connolly, 2011). Performance improvements relating to addition of yeast cell wall products of 1.61% to 5.41%, and 1.99% to 2.54% have been reported for weight gain and FCR respectively (Hooge, 2004; Hooge and Connolly (2011), and more notably, increases in abundance of have been consistently reported since some of the early, culture-based studies beneficial bacteria (i.e., *Bifidobacterium* and *Lactobacillus*), and reduce potential pathogens (i.e., *clostridia* and *E. coli*) (Kim *et al.*, 2011; Peinado et al., 2013; Shanmugasundaram *et al.*, 2013). However, throughout this research era, other studies have shown little or no significant effect (Zhang *et al.*, 2003; Jiang *et al.*, 2006; Biggs, Parsons and Fahey, 2007).

This inconsistency may reflect differences in laboratory techniques used for analyses, experimental conditions, and variation in the gut microbiota of individual animals.

1.6.2.3. Enzymes

The feed rations of monogastric farms are made up of up to 60% plant-based ingredients that are rich in anti-nutritional factors (ANF) such as phytic acid, non-starch polysaccharides (NSP), and cell-wall complex carbohydrates. It is now a common practice to include exogenous enzymes such as phytase and carbohydrases or NSPases (xylanase, cellulase, α-galactosidase, β-mannanase, α-amylase, and pectinase) in feed formulations. These enzymes increase in the overall digestibility and bioavailability of nutrients and thereby improve performance of the animals(Bedford and Schulze, 1998). The effect of various in-feed enzymes in improving the growth and feed efficiency in poultry is well documented and reviewed.

The suggested mechanisms of action of in-feed enzymes include (Choct *et al.*, 2004; Kiarie, Romero and Nyachoti, 2013):

1. increase in the digestibility of nutrients that are otherwise not degraded by host enzymes (e.g. phytic acid)
2. eliminating the nutrient-encapsulating effect of cell-wall polysaccharides and an increase in the availability of starches, amino acids, and minerals
3. inactivation of anti-nutritional factors (e.g., phytic acid or soluble NSP) and reduced intestinal viscosity;
4. an increase in the solubility of non-soluble NSP and promotion of cecal fermentation
5. supplementation of endogenous enzymes that may be insufficient produced, in young animals whose digestive system is not fully developed.

In addition, they are also thought to influence the composition of the gut microbiota. The enzyme-induced microbiota changes are not direct but are facilitated by two main mechanisms:

1) reducing the undigested substrates
2) generating short-chain oligosaccharides from cell-wall NSPs with potential prebiotic effects (Bedford and Cowieson, 2012; Kiarie, Romero and Nyachoti, 2013; Bedford, M.R, 2018)

These mechanisms influence the nutrient supply in the intestinal environment thus exerting a selection pressure favourable for the growth of certain bacterial species (Bedford and Cowieson, 2012; Cheng *et al.*, 2014).

The potential for use of in-feed enzymes, as antibiotic alternatives, to improve performance in poultry is substantial. Hooge et al., (2010) performed a meta-analysis and demonstrated that supplementation of a dietary multi-enzyme blend of phytase and NSPases improved final body weight by 3.73% and lowered FCR by 2.64%. Similar analysis of 7 reported of β-mannanase supplementation studies in broilers concluded an overall improvement in body weight gain and FCR of 4.2% and 4.8 points repectively (Jackson and Hanford, 2014) showed that weight gain and FCR, analyzed across trials, were improved, respectively. At a similar time, Swann and Romero (2014) investigated the effects of a particular xylanase, amylase, and protease cocktail across ten independent trials and concluded that particular combination substantially increased the protein, fat and starch digestibility. Unfortunately, generalisations from this meta-analysis are difficult due to differences in the enzyme strain, source and volume, alongside changes in diet and genetic variations between bird strains by gepgrpahical region and timeframe (Son and Ravindran, 2012; Cheng *et al.*, 2014).

1.6.2.4. Organic acids

Dietary organic acids have been considered as potential alternatives to AGPs, owing to their antibacterial nature. Chemically, the ones used in animal feed are either simple monocarboxylic acids (e.g., formic, acetic, propionic, and butyric acids) or carboxylic acids with a hydroxyl group (e.g., lactic, malic, tartaric, and citric acids) (Dibner and Buttin, 2002). They can be given in the feed or drinking

water and either individually as organic acids or their salts (sodium, potassium, or calcium) or as combinations of multiple acids or their salts (Huyghebaert *et al*., 2011).

The mechanism of action of organic acids is largely due to their antibacterial effects, although some benefits are also derived through their modulation of the host gastro-testinal physiology. Organic acids reduced pH in the crop, proventriculus, and gizzard), leading to physiological changes in the intestinal mucosa (Samanta, Haldar and Ghosh, 2008; Panda *et al.*, 2016). There is also a direct bactericidal effect on pathogenic bacteria and a contrasting benefit to the acid-tolerant species such as *Lactobacilllus* spp through reduced competition for nutrients (Biggs and Parsons, 2008; Nava *et al.*, 2009; Czerwiński *et al.*, 2010; Boroojeni *et al.*, 2014). This reduced competition for nutrients results directly contributes to increased protein and mineral digestion in the host (Rafacz-Livingston, Parsons and Jungk, 2005; Nezhad *et al.*, 2011) and also indirectly improves nutrient use efficiency by improved absorptive surface area by providing SCFA as a direct energy source for epithelial cells.

Similarly to enzymes, the potentially beneficial effects of organic acids are inconsistent, possibly due to variation in inclusion rates, organic acid source and the buffering capacity of other dietary ingredients (Dibner and Buttin, 2002; Kim et al., 2015). The immense variation in diet design across the world and the range of available raw materials makes it challenging to quantify the impact of these factors and to understand their mechanisms of action.

1.6.2.5. Phytobiotics

Phytobiotics or phytogenic feed additives (PFAs) are naturally occurring bioactive compounds derived from plants that are commonly claimed to benefit growth performance (Franz et al., 2010; Windisch et al., 2008). Depending on how the active ingredients are derived, PFA made be described essential oils (EOs; volatile lipophilic substances obtained by cold extraction or by steam or alcohol distillation) and oleoresins (extracts derived by non-aqueous solvents) (Van Der Klis and Vinyeta-Punti, 2014; Windisch et al., 2008). In the majority of PFAs the key active components are polyphenols. One of the major challenges associated with PFAs is the number of factors influencing their composition and

concentration. Plant variety within species, region of pant used, geographical origin, point of harvesting, environmental factors greatly influence polyphenol concentration before the added impact of storage conditions, and processing techniques are considered (Applegate et al., 2010; Windisch et al., 2008).

The mechanism of action of PFAs is not clearly understood and depends greatly upon the composition of the active ingredients in the product being used. As with organic acids, much of their beneficial effects are attributed to their antimicrobial activity (Tiihonen et al., 2010; Viveros et al., 2011; Zhang et al., 2013) and immunomodulatory properties (Kim et al., 2010; Lee et al., 2010; Park et al., 2011; Pourhossein et al., 2015) but they also exhibit antioxidant properties that enhance intestinal health (Liu et al., 2014; Settle et al., 2014; Zhang et al., 2013). On a more systemic level, PFAs have also been reported to increase intestinal and pancreatic enzyme production and activity and increase bile flow, leading to improved apparent nutrient digestibility and performance (Hashemipour et al., 2014, 2013; Jang et al., 2007; Lee et al., 2003; Malayoğlu et al., 2010). Beyond these physiological change sin the host, PFAs also benefit the epithelial surface through absorptive surface (via increased villus height to crypt depth ratio; Ghazanfari et al., 2015; Murugesan et al., 2015) and may improve intestinal barrier function (Placha et al., 2014).

1.7. Xylo-oligosaccharides (XOS) as an emerging prebiotic

1.7.1. Chemistry and production of XOS

Xylo-oligosaccharides are chains of β- 1,4- linked D- xylopyranoside units, produced by partial hydrolytic degradation of arabinoxylans, one of the main components of cereal fibre fractions (Linares-Pasten, Aronsson and Karlsson, 2016). Depending upon various xylan sources used for XOS production, the structures of XOS vary in degree of polymerization (DP), monomeric units, and types of linkages (Figure 1.8). The number of xylose residues involved in their formation can vary from 2 to 10. Production of XOS from xylan containing lignocellulosic material can be achieved by autohydrolysis using steam (Garrote, Domínguez and Parajó, 1999, 2001; Kabel *et al.*, 2002), chemical methods or

direct enzymatic hydrolysis of a suiTable substrate (Katapodis *et al.*, 2002; Christakopoulos *et al.*, 2003) or a combination of chemical and enzymatic methods (Yuan *et al.*, 2004; Yang *et al.*, 2005). XOS have been manufactured from a variety of agricultural residues like, hardwoods, corncobs, corn fibre, barley hulls and spent grans, brewery spent grains, rice hulls, coconut husks and sugarcane bagasse (Aachary and Prapulla, 2011a; Samanta *et al.*, 2015). Autohydrolysis or steam treatment for the manufacture of XOS involves deacetylation of xylans at high temperatures. Although this method eliminates the use of corrosive chemicals, it requires special equipment that can be operated at those temperatures. To produce XOS with chemical or enzymatic techniques, xylan is by and large extracted from appropriate lignocellulosic material with an alkali, like KOH or NaOH, and then converted to XOS by xylanases. Enzymatic production of XOS has a distinct advantage over production using steam or chemicals in that it does not require downstream purification from undesired fractions of monosaccharides, acetic acid or insoluble lignin fractions (Aachary and Prapulla, 2011a).

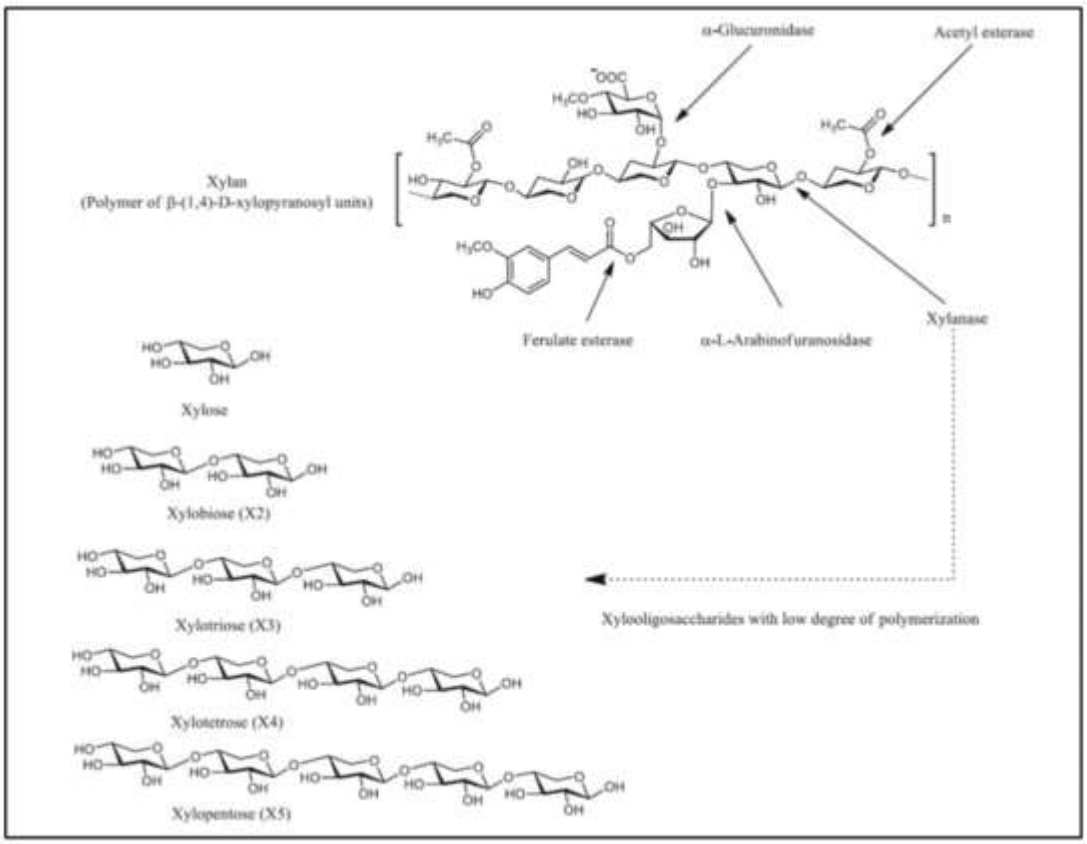


Figure 1.8. Chemical structure of xylan and xylooligosaccharides (linkage of 2-10 xylose units) produced by enzymatic hydrolysis (Adapted from Mano *et al.*, 2018) *This image has been removed by the author for copyright reasons. 'Reproduced with permission from Springer-Verlag GmbH Germany'*

1.7.2. Degradation and utilization of XOS by probiotic/intestinal microorganisms

In vitro fermentation of xylose, xylobiose, xylotriose, and other saccharides as a carbon source by *Bifidobacterium adolescentis*, *Bifidobacterium longum*, and *Bifidobacterium infantis* was employed by Okazaki et al. (1990a, 1990b). The authors highlighted the remarkable ability of *B. adolescentis* to use both xylobiose and xylotriose. On the other hand Hopkins et al. (1998) using commercial XOS showed that the ability of *Bifidobacterium* for growth on XOS was influenced by the strain under consideration. The bifidogenic potential of XOS was also demonstrated by Moniz et al., (2016). In this study two substrates of XOS obtained from corn fibre with DP 2-4 and 9-21 were inoculated with human faecal slurry. All the substrates were utilized by the microbiota resulting in increased Bifidobacterial populations and SCFA production. Several other studies have also confirmed the bifidogenic property of XOS obtained from a variety of plant sources and increased production of SCFA on fermentation of XOS by *Bifidobacterium* spp (Crittenden et al., 2002; Monteagudo-Mera et al., 2018; Moura et al., 2007a, 2007b; Palframan et al., 2003; Broekaert et al., 2011; Fehlbaum et al., 2018). Additionally, some studies have shown that branched chain structures of arabino-XOS are more bifidogenic compared to the linear structures due to the preference of Bifidobacteria for the branched chains (van Laere *et al.*, 2000; Moniz *et al.*, 2016)

Carbohydrate utilization capacity of *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus lactis* was tested by Kontula et al., (1998) using oat bran oligosaccharides. All three organisms utilized oat β-glucooligosaccharides, while only *L. plantarum* degraded XOS. The main products of fermentation of carbohydrates by lactic acid bacteria were acetic acid, formic acid, lactic acid and ethanol. The results indicated that oat β-glucooligosaccharides and XOS induce lactic acid bacteria to form the end-products characteristic of anaerobic respiration of cells. Some strains used XOS with DP 2, 3, and 4 preferentially, whereas other strains preferred xylose.

XOS have been shown to be selective not only for Bifidobacteria but also other intestinal organisms like *Bacteroides* spp., *Clostridium* cluster IV and XIVa, *Lactobacillus acidophilus*, and *Klebsiella pneumoniae* with these organisms showing moderate growth on oat XOS (Jaskari *et al.*, 1998; van Laere *et al.*, 2000). Another study found that XOS was efficiently fermented by Bacteroides but not by pathogens like *E.coli, Clostridium difficle* and *Clostridium perfringens* (Crittenden *et al.*, 2002). Kabel et al. (2002) studied *in vitro* fermentability of differently substituted XOS and concluded that the neutral-XOS, arabino-XOS, acetylated-XOS, and acidic-XOS obtained from hydrothermally treated xylan-rich byproducts were fermented by human fecal inoculum.

A few other probiotic organisms capable of hydrolysing XOS or xylan include *Leuconostoc lactis* and *Saccharomyces cerevisiae* (Moura *et al*., 2007; Ohara *et al*., 2006; Smiricky-Tjardes *et al*., 2003)

1.7.3. Benefits of XOS in poultry diets

XOS as poultry feed additive has recently received significant attention. Table 1.5 summarizes the studies on the effect of XOS on bird performance in broiler trials. There are limited but contradictory results available in literature on the effects of XOS on performance of broilers.

Table 1.5: Effect of XOS on growth performance of broiler chickens from published studies

Study	Source of XOS	Dietary cereal/s	Doze of XOS (%)	Bird Breed	Trial Duration	Performance Results
Courtin et al. (2008) Trial 1	Wheat bran	Wheat & maize	0.03, 0.06, 0.12, 0.25 & 0.5	Cobb 500 males	21 days	NSD in FCR at 0.03, 0.06. 0.12 and 0.25% inclusion at 11-21 and 1 -21 days Significantly lower FCR in 0.5% XOS group due to low FI at d11 & 21
Courtin et al. (2008) Trial 2	Wheat bran	Maize only	0.1 & 0.25	Cobb 500 male	21 days	NSD in FCR at 0.1% inclusion at 11-21 and 1 -21 days Significantly lower FCR at 0.25 due to low FI at d11 &21
Zhenping et al. (2012)	Straw	Maize	0.5, 1 & 2	Arbor Acres male & female	59 days	Significantly higher BWG and significantly lower FCR at 1%, numerically higher BWG and lower FCR at 0.5% & 2% XOS, significantly lower FI at 2%
De Maesschalck et al. (2015)	Corn Cob (XOS 35P)	Wheat & Rye	Starter 0.2, Grower & Finisher 0.5	Ross 308 Male & female	39 days	Significantly lower FCR & numerically higher BWG at d26 & 39
Pourabedin et al. (2015)	Not stated	Corn & soya-bean meal	0.1 & 0.2	Ross x Ross	35 days	NSD in body weight at d21 or d35 NSD in body weight or FCR with 0.1% inclusion Significantly lower FCR at d21 but not d35 with 0.2% inclusion level

Samanta et al. (2015)	Corn husk	Maize	0.5	Vencob	3 weeks	NSD in FCR or BWG at 1, 2 or 3 weeks of age
Yuan et al. (2018)	Not stated	Corn	0.0002 (2mg/kg)	Arbor Acres or Ross 308	42 days	NSD in BWG or FCR at d21 Significantly higher BWG but no difference in FCR at d42
Ribeiro et al. (2018) Trial 1	Corn Cob XOS 95P	Wheat & Corn	0.006% (0.06g/kg)	Ross 308 male	28 days	Significantly higher BWG & sig low FCR at d0-28
Ribeiro *et al* 2018 Trial 2	Corn Cob XOS 95P	Wheat & Corn	0.01 & 0.1	Ross 308	42 days	Significantly higher BWG but no diff in FCR at d0-42 FCR sig low at d28-35 for both inclusion rates
Ribeiro *et al* 2018 Trial 3	Corn Cob XOS 95P	Corn	0.01, 0.1 & 1	Ross 308	42 days	Significantly higher BWG for 0.01% & 0.1% but no difference for 1% at d0-42 NSD in FCR for any inclusion rate at d0-42
Craig et al. (2018)	Corn Cob XOS	Wheat - corn	Not stated	Ross 308	22 days	NSD in FCR at d1 -21
Craig et al. (2020)	Corn Cob XOS	Wheat-soya	0.025% and 0.1%	Ross 308	29 days	NSD in BWG or FCR at 0.025 or 0.1% inclusion at d14 or d28

XOS= xylooligosaccahride, BWG = Body Weight Gain, FI= Feed Intake, FCR = Feed Conversion Ratio, NSD = no significant difference, d= day

These differences in performance of birds reported in the above studies may be due to the differences in the source of XOS, composition of the basal diets, levels of inclusion, duration of supplementation or housing management practices.

Nevertheless, many studies are in agreement regarding the positive influence of XOS on gut health in chickens. Pourabedin *et al.* (2015) showed increased production of acetate in caecum of broilers fed a XOS supplemented diet. In addition, some members of *Clostridium* cluster XIVa are able to convert acetate and lactate into butyrate (De Maesschalck *et al.*, 2015). The beneficial effects of butyrate on performance and gut health of poultry have been reviewed in many publications (Onrust *et al.*, 2015; Ahsan *et al.*, 2016; Moquet *et al.*, 2016). In a layer trial XOS supplementation significantly increased villus height to crypt depth ratio in jejunum, the relative length of jejunum and the number of bifidobacteria in the caecum. However lactobacilli and *E. coli* in the caecum were not affected (Ding et al., 2018). XOS has also been reported to ameliorate the effects of pathogens or inhibit their colonization in poultry. For example, wheat bran derived arabinoxylooligisaccaharides were shown to have a protective effective in intestinal colonization and systemic translocation of *Salmonella* (Eeckhaut *et al.*, 2008). Similarly another study showed that five days post challenge the mean counts of *S. enteritidis* in caecum in XOS group was significantly lower than positive control (Pourabedin *et al.*, 2017). A synbiotic supplementation of XOS and probiotic *Bifidobacterium longum* was successful in reducing *C. jejuni* and *Campylobacter* spp when administered to chicks from the first day of life (Baffoni *et al.*, 2017).

A more recently proposed mechanism of action of XOS is its role as a stimbiotic (Bedford, 2019; González-Ortiz *et al.*, 2019). This essentially means that the small quantities of XOS supplemented in the diet are picked up by bacteria that can themselves produce xylanase. Effectively these XOS are being recognized as a stimulant by bacteria to produce their own xylanses in large quantities which can then degrade the xylan fragment of the cell walls of cereals such wheat, maize, barley and soybeans which are commonly used in poultry diets.

1.7.4. Other biological effects of XOS from *in vitro* and *in vivo* studies

Xylooligosaccharide, alone or as active constituent of pharmaceutical preparations has shown to exhibit a variety of biological activities other than prebiotic effects related to gut modulation. These include antioxidant activity (conferred by phenolic substituents) (Yuan et al., 2004), anti-inflammatory properties, immunomodulatory action and anti-hyperlipidemic effects (Izumi et al., 2004). These properties are mainly attributed to acidic oligosaccharides containing uronic substituents, which can be produced from hardwoods by a combination of enzymatic and/or chemical treatments.

Immunostimulating effects have been reported for arabino-(glucurono) xylans isolated from *Echinacea purpurea*, *Eupatorium perfoliatum*, and *Sabal serrulata* (Proksch and Wagner, 1987; Wagner and Jurcic, 1991). Anti-inflammatory activity has been reported for the 4-*O*-methylglucuronoxylan from *Chamommilla recutita* (Whistler *et al.*, 1976) and the acidic, highly branched heteroxylan from *Plantago species* (Yamada *et al.*, 1985; Samuelsen *et al.*, 1995). Partially *O*-acetylated XOS and de-acetylated forms of the almond shell XOS showed direct mitogenic activity and enhancement of the T-mitogen-induced proliferation of rat thymocytes, indicating the immunostimulatory potential (Nabarlatz *et al.*, 2007). Ebringerová et al. (1998) showed that water-soluble arabinoglucuronoxylan from corncobs showed dose-dependent mitogenic (promotes mitosis or cell division) as well as comitogenic (does not induce cell growth alone but promotes the effect of the mitogen) activities.

Ando et al. (2004) examined the effect of hot compressed water extracted and fractionated bamboo products, (fraction A containing xylose, XOS and water-soluble lignin and fraction B composed of glucose and cellooligosaccharides) on the viability of human cultured cell lines derived from leukemia patients and human peripheral blood lymphocytes obtained from normal adults. It was found that fraction B expressed a negligible cytotoxic effect against leukemia cells, while fraction A markedly reduced the viability of leukemia cell lines derived from acute lymphoblastic leukemia in a dose-dependent manner. Furthermore, microscopic inspection of acute lymphoblastic leukemia derived

cells treated with fraction A showed distinctive apoptotic morphological changes. These results indicated that the cytotoxic effect of fraction A may be attributed to apoptosis, induced by XOS, and that it is specific for acute lymphoblastic leukemia cells. Another study examined the inhibitory effects of XOS on colon cancer in Sprague-Dawley rats (Hsu *et al.*, 2004) and showed that XOS markedly reduced the number of aberrant crypt foci, a biomarker of colon carcinogenesis, in 1,2-dimethylhydrazine (DMH) treated rats. Similar effects of corn cob derived XOS on DMH induced colon cancer in rats were also reported by Aachary (2009). However, these studies did not elucidate mechanisms underlying the anti-cancer properties of XOS.

XOS have great potential as agents to maintain and improve a balanced intestinal microflora for enhanced health and well-being. The published studies mentioned above on nutritional, physiological, and microbial benefits of XOS either *in vitro* or from animal or clinical trials give a distinct direction to future research on its health benefits. Challenge remains to further exploit XOS to authentic health benefits for human and animals. Available experimental evidence supports the hypothesis that XOS and other prebiotics can offer an opportunity to prevent or mitigate gastrointestinal disorders. Even though encouraging results have been obtained for other prebiotics in preliminary trials, the data on XOS are limited. More investigations are needed to further elucidate the mechanisms involved in the reduction of cancer risk and in the cancer chemo- and/or radiotherapy-potentiating effects of XOS. XOS offers a new dimension for the development of functional feed and foods.

1.8. Aims and Objectives

The overarching aim of this project was to explore ways of supporting gut health and production parameters of broilers via XOS supplementation of diets. The hypothesis was that XOS or xylanase, alone or together improve the performance and gut health of broilers by modulating the microbial composition and diversity subsequently improving SCFA supply to the host. The following specific objectives were set to test these hypotheses:

1) To determine the effect of XOS alone and in combination with xylanase on the production performance of broilers raised under controlled conditions.
2) To determine the effect of XOS on composition and diversity of caecal microbiota
3) To elucidate the mechanisms via which XOS improves performance and gut integrity

Chapter 2: Materials and Methods

2.1. Introduction

This chapter describes the general materials and methods used in this thesis and how they correspond to subsequent thesis chapters. A total of 3 studies involving 2 bird trials were conducted as summarised below in Table 2.1. Bird Trial 1, at the Poultry Research Unit (Nottingham Trent University, Brackenhurst) and bird Trial 2, on a farm (Holme Farm, Southwell Road, Gonalston Nottingham) were conducted to investigate the effects of corncob-derived XOS35 (Longlive Bio-technology, Shandong, China) on performance and gut health parameters of broiler chickens. XOS35 is a mixture of 35% XOS and 65% maltodextrin with the degree of polymerization (DP) of XOS being between 2 and 7.

Table 2.1: Description of individual studies conducted

Study	Areas investigated	Chapter
Trial 1 (Coded bird trial Oligo26)	Effect of XOS alone and in combination with xylanase on performance, cecal microbiota, cecal short chain fatty acids and gene expression of biomarkers of gut integrity broilers raised under controlled research conditions	3
Trial 2 (Coded bird trial Oligo13)	Effect of XOS on weight gain, cecal microbiota, caecal short chain fatty acids and gene expression of biomarkers of gut integrity of raised on farm barn	4
Trail 3	Effect of XOS on proteins expressed by gut microbiota. Is XOS a *stimbiotic? (Analysis of samples from Oligo13 trial)*	5

2.2 Materials and Methods

2.2.1. Bird trials

Institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986) were followed and all experimental procedures involving animals were approved by the School of Animal, Rural and Environmental Sciences Ethical Review Group and logged as ARE627 (Trial 1) and ARE716 (Trail 2). All bird trials used Ross 308, male broiler chicks, supplied within 24 hours of hatching by PD Hook, Cote Hatchery, Oxfordshire and transported to the trial locations by NTU poultry research staff.

2.2.1.1. Bird Trial 1

2.2.1.1. Birds and Husbandry

A total of 384 birds were used in this trial. Birds were within the weight range of 40 -50 g and were from breeder flocks aged between 40-45 weeks. Birds were weighed using dynamic weighing which measured the average weight over a period of 3 sec (Mettler Toledo International). The chicks were randomised by weight and housed in preheated 0.64 m^2 pens in a purpose built, insulated poultry house. The birds were bedded on clean wood shavings (approximately 3 cm thick) and fresh shavings were replenished as required to keep them dry and friable during the trial. Birds were always allowed *ad libitum* access to the treatment diets and water for the duration of the trial. Commercial guidelines for the care and husbandry of Ross 308 broilers were followed in all studies (Aviagen, 2007). The room was thermostatically controlled to produce an initial temperature of 32°C reduced to 21°C by day 21 using heating fans. The lighting regimen used was 24 hours light on day 1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached and this was maintained throughout the remainder of the study. Birds were checked twice daily to monitor the environmental conditions; heating and ventilation were adjusted accordingly. Any mortalities were recorded

along with the date and weight of the bird and reason if culled. All birds sampled were euthanised by cervical dislocation as determined by DEFRA (DEFRA, 2015).

2.2.1.2. Diet Formulation

Diet was manufactured on site as mash. The particle size of each diet was uniform, consistent and typical for broiler diets. The composition and analysis of the trial diet are detailed in the corresponding chapter. When making the diet, each ingredient was individually weighed out and mixed dry for 5 min in a ribbon mixer (Rigal Bennett, Goole, UK) before addition of oil (Appendix A). The diet was then mixed for a further five minutes. The mixer was brushed down at various stages throughout the mixing process to ensure oil clumps were removed. Diet was randomly allocated to pens within the room, to eliminate any effect of room position. A grab sample was taken during the feed weighing prior to the trial to allow for proximate analysis at a later date. Diet was made in 50 kg batches for each treatment and feeding phase and then were weighed into bags (new individual bags for each feeding phase; starter, grower and finisher) for each pen to allow feed intake to be measured. Bags were topped up with feed as required and added feed weights recorded.

2.2.1.3. Feeding procedure and feed intake

Each pen of chicks was fed exclusively from an individual experimental bag of diet that was pre-weighed prior to the trial. Feed troughs were positioned horizontally to minimise spillage. Pens were monitored daily for spillage of feed. A pen was recorded as having unusable feed intake data when spillage that could not be weighed back was estimated to be more than 20 grams on two or more days within a weigh period. Data from these pens were excluded from calculated feed intake values for that weigh period. On sampling days remaining feed in the trough and bag, and any spilt feed if able to be collected, were weighed. Feed intake was

measured on day 7, 14, 21, 28 and 35. Feed intake was measured as total intake per pen then the average amount consumed per bird calculated.

2.2.1.4. Bird Weights

Chicks were weighed on arrival, and any outside the range of 40 - 50 g were not included in the trial. Birds were distributed into pens based on average weight per pen, ensuring there were no significant differences in starting pen weight between dietary treatments. Birds were weighed on day 7, 14, 21, 28 and 35. Bird weights were measured by weighing the whole pen, and then calculating the average bird weight, unless stated in the specific trial methodology. The increase in average bird weight was used, alongside the average feed intake value, to calculate the average feed conversion ratio (FCR) per pen.

$$Feed\ conversion\ ratio\ =\ feed\ intake\ \div\ average\ daily\ gain$$

2.2.1.2. Trial 2

2.2.2.1. Birds and Husbandry

Prior to commencement of trial the farm shed was divided in 4 pens approximately 18 m^2 and covered in wood shavings (approximately 3 cm thick). The shed was heated with overhead electric lamps. Pens were labelled with treatment, diet and pen number. A diary was placed outside the pens in the bird shed to record any observations considered appropriate such as birds with suspected health conditions, any unusual environmental conditions, water leaks or wasted feed in the pen. A schematic representation of the set up in the shed is shown in Figure 2.1. A total of 830 birds were randomised by weight and distributed across the 4 pens on the day of hatch with pen 1 and 2 housing 225 birds each and pen 3 and 4, 190 birds each. The birds were within the weight range of 35 - 50 g and from breeder flocks aged approximately 40 weeks. Sixty birds per pen were wing tagged by licensed personnel using six

different colour tags (10 birds per colour) and the weight of each tagged bird was recorded. Feed was provided in feed hoppers, four per pen for pens 1 and 2 and three per pen for pens 3 and 4. Water was provided continuously via bell drinkers, three each in pens 1 and 2 and two each in pens 3 and 4.

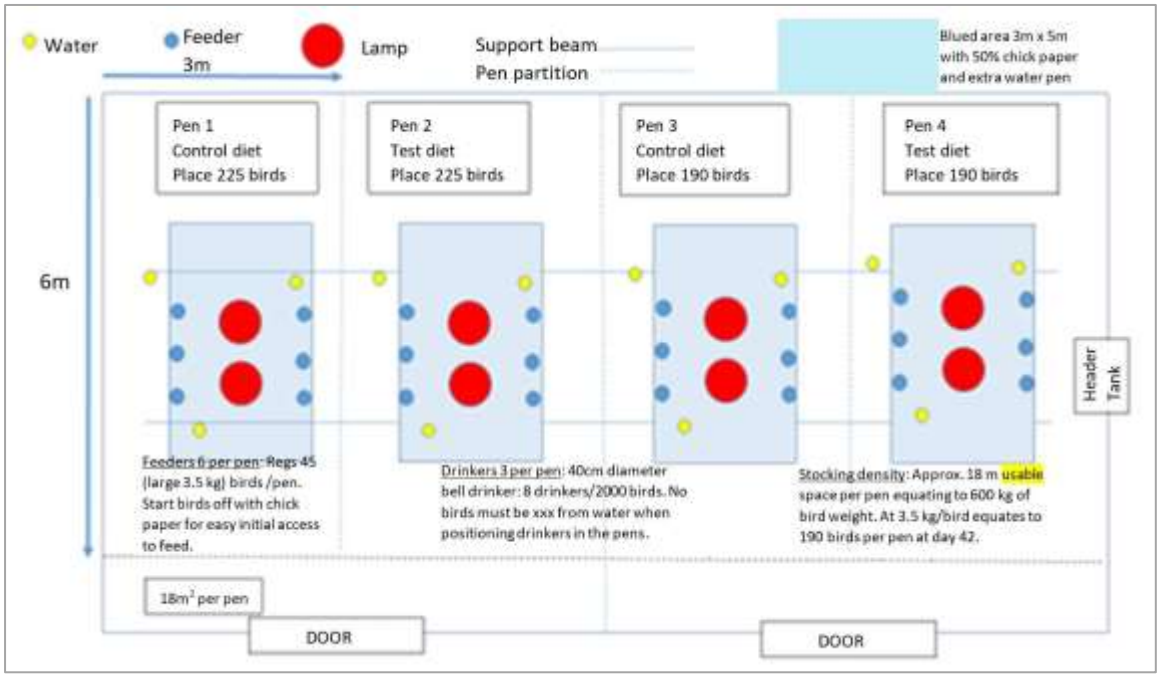


Figure 2.1: A schematic representation of the arrangement of shed

Additionally feed was also provided on chick paper for easy initial access to feed for up to 7 days from start of trial. Birds had *ad libitum* access to feed and water throughout the entire trial. Temperature and ventilation within the shed were manually controlled based upon the age of the bird housed as stipulated in the breed guide (Aviagen, 2007). Additional heating when required was provided by gas heaters placed in each pen. The ammonia level within the room was monitored through husbandry observations. Lighting was also manually controlled with approximately 6 to 8 hours of darkness per day throughout the entire trial period. Holme farm staff checked the birds and turned off the lights at the end of the day and turned on first thing, the following morning (approx 11 pm to 6 am). Birds were inspected twice daily by NTU poultry research staff primarily to confirm that the environmental conditions are adequate

and that bird welfare is not compromised and to feed the birds. Any dead birds were removed immediately from the pen and sick or malformed birds were culled via cervical dislocation as in trial 1 and dead weights were recorded on the health records.

2.2.2.2. Diet Formulation

The diets for this trial were manufactured by Research Diet Services, Utrecht, The Netherlands, as a crumb starter and pelleted grower and finisher and supplied in 50 kg bags. A 1 kg grab sample of each experimental diet was taken by collecting and pooling samples of feed from different points of the bags and stored at -20°C for proximate analysis.

2.2.2.3. Feeding Procedure and Feed Intake

All pens had bags of appropriate treatment diet placed outside the pens. Pens were fed from their designated bag. Each additional bag was labelled with pen number and bag number consecutively. Pen labels were checked against bag labels to ensure the correct diet is fed. At the end of the trial the number of bags consumed and hence an estimated feed intake for each pen was calculated. Due to the trial design, it was not possible to measure the entire pen weight and hence an accurate weekly feed intake measurement to calculate FCR was deemed unnecessary.

2.2.2.4. Bird Weights

Chicks were weighed on arrival, and any outside the range of 35 – 50g were not included in the trial. Performance was assessed by measuring the weight gain of sixty representative birds per pen that were wing tagged birds at the start of the trial. These representative birds were individually weighed on day 0, 7, 14, 21, 28, 35 and 42 and weekly weight gain was calculated.

2.2.2. Sampling

In Trial 1, three birds per pen were sampled on day 8 and 1 bird per pen was sampled on days 22 and 35. Birds were euthanized in a separate room via cervical dislocation by trained NTU staff. In case of Trial 2, twenty untagged birds from each treatment were randomly selected on each sampling day (days 7, 21 and 35) and transported from the farm to the NTU Poultry Research Unit where they were euthanized in the same manner as birds from trial 1. On each sampling occasion, caeca, blood and ileal tissue were collected as described in sections below.

2.2.2.1. Caeca Collection

Both ceaca from each bird were excised post-mortem and collected separately in clean labelled bags. The ceca were weighed before being snap frozen on dry ice. Caeca were then stored at -20^{o}C until anlaysis.

2.2.2.2. Blood Plasma Collection

Postmortem blood samples were collected immediately post euthanasia into EDTA coated tubes. Samples were centrifuged at 3000 rpm for 5 min to separate the plasma which was collected in sterile Eppendorf tubes and stored at -20^{o}C.

2.2.2.3. Ileal Tissue Collection

Prior to collection of the tissue, bench surface, forceps, scalpel blades and gloves to be used in the procedure were thoroughly cleaned with 70% ethanol followed by cleaning with RNaseZap^{TM} to remove any contaminating RNA from the environment. Approximately 2 cm piece of the ileum was cut using dissection scissors from the Meckel's diverticulum and placed on sterile petri plates. The piece of ileum was cleaned with ultra pure water to remove digesta and then washed in RNAlater. The tissue was cut in 0.5 cm pieces with scalpel blades and placed in Eppendorf tubes containing 1.5 ml of RNAlater and incubated at 4^{o}C overnight to stabilize cellular RNA. The samples were then stored at -80^{o}C.

2.2.3. Analytical Procedures for Feed Analysis

2.2.3.1. Dry Matter Determination

Dry matter content of the diet was analysed by accurately weighing approximately 5-10 g of finely ground sample into pre-weighed crucibles. The crucibles were then placed in a drying oven set at 105°C for approximately 4 days (according to the standard operating procedure used at the poultry research unit) until as constant weight was reached (samples were removed from the oven after 2 days and weighed, then they returned to the oven for additional 2 days). The dried samples were cooled in a desiccator for and reweighed.

2.2.3.2. Ash Determination

Ash content of diet was analysed by accurately weighing approximately 2-5 g of sample into a pre-weighed ceramic crucible. The crucibles were then placed in a muffle furnace (Nabertherm, B180) on a program that brought them from room temperature up to 650°C over a two-hour period, then maintained them for 14 hours at 650°C, before automatically shutting off and allowing them to cool back to room temperature. The ashed samples were then cooled in a desiccator and reweighed.

2.2.3.3. Calcium and Phosphorous Determination Using Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES)

Diet was analysed for Ca and P by Inductively Coupled Plasma mass spectroscopy with Optical Emission Spectrometry (ICP-OES) (ICP-MS model PQ Excell, VG Elemental, USA). Prior to the assay, all glassware was acid washed for a minimum of 12 hours, rinsed with ultra-pure water and dried, to ensure there was no cross contamination. Approximately 0.5 g of sample was weighed in duplicate into 50 ml conical flasks. The samples were then incubated for a minimum of 16 hours with 10 ml of aqua regia (1 part nitric acid and 3 parts hydrochloric acid) before heating until dissolved (approximately 90 min) in a fume cupboard. If necessary, an extra 5ml of aqua regia was added and an additional 30 min of heating was carried out to

ensure complete dissolution. One blank flask containing just aqua regia was prepared for each 5 samples. The samples were then cooled before the flask contents were diluted with ultra-pure water and filtered into 50 ml volumetric flasks through Whatman 541 hardened, ashless filter papers. The volumetric flasks were then brought to volume with ultra-pure water, and the contents were mixed and transferred into 15 ml, duplicate tubes per sample. ICP-OES standards were prepared with differing levels of Ca and P (dependent on the predicted levels of the sample being analysed) using 1000 ppm ICP-OES grade standards (Fisher Scientific, Loughborough, UK) diluted in ultra-pure water. The samples were analysed on the ICP-OES, set to analyse Ca at wavelength 317.933 nm and P at wavelength 213.617 nm. The readings on the ICP-OES are presented as concentration in mg/L; and the following equation used to convert to g/kg:

$$Ca\ or\ P\ in\ sample\ (g/kg) = Ca\ or\ P\ in\ sample\ (mg/L) * volume\ of\ sample\ (ml) \div weight\ of\ sample\ (g)/1000$$

2.2.3.4. Crude Protein Determination

Protein content of each diet was analyzed using the Dumatherm Nitrogen Anlayzer (Gerhardt, UK). The instrument works on the principle of Dumas method which is a quick combustion of liquid or solid samples in a pure oxygen atmosphere, followed by analysing the resulting gases. The measurement of the thermal conductivity with a TCD-detector gives a signal which corresponds to the amount of nitrogen in the combusted sample. The simplified flow diagram is shown in Figure 2.2.

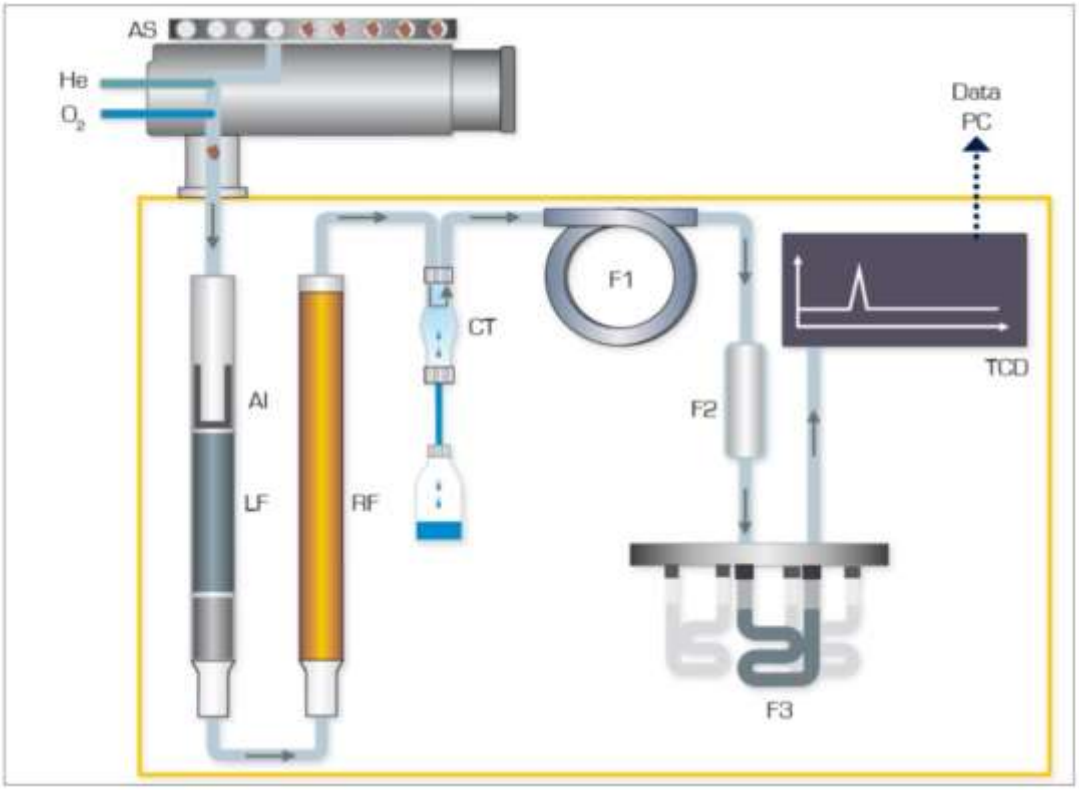


Figure 2.2: Simplified flow chart of the Dumatherm (AS = Autosampler, AI = Ash insert, LF = Combustion furnace, RF = Reduction furnace, CT = Condensation trap, F1 = Membrane system (Nafion®), F2 = Absorption trap, F3 = Self regenerating adsorption trap, TCD = Thermal conductivity detector).
(https://www.dijkstra.net/media/catalog/category/Dijkstra_Vereenigde_Gerhardt_Dumatherm.pdf)

Helium circulates as a carrier gas through the entire analytical circuit, which consists of autosampler (AS), combustion reactor (LF), reduction reactor (RF), traps for water (F1, F2) and carbon dioxide (F3) and a thermal conductivity detector (TCD). Before the sample drops from the autosampler into the combustion reactor, the carrier flow is switched from helium/Argon to oxygen. The exothermic reaction when the oxygen gets in touch with the tin capsule including the sample at a temperature of about 1030 °C raises the temperature in the reactor from 1000°C up to 1700 - 1800°C within seconds. At this temperature - and in the presence of specially designed heterogeneous oxidation catalysts - the sample is completely converted into its elemental oxides. After combustion, the oxygen flow is switched back to Helium which

serves as the transport gas for the combustion products through the rest of the analytical circuit.

The by-product water is separated from this gas mixture by two filters - F1 and F2. F1 is the Perma Pure gas dryer, which separates the majority of water from the gas mixture. The second filter F2 is packed with magnesium perchlorate to remove the residual amount of water from the gas stream. Carbon dioxide (CO_2) is separated from the gas mixture in the CO_2 absorber/desorber system (F3 in Figure 2.2). The CO_2 absorber tubes are regenerated in the degassing furnace. The remaining gas mixture passes the TC detector leading to an output signal which is proportional to the nitrogen concentration in the combusted sample. Standard samples with a known concentration of nitrogen (e.g. EDTA or Glycin) are used for calibration of the TCD.

Protein content was calculated by nitrogen content x 6.25 (standard multiplier)

$$6.25\ x\ \%\ Nitrogen\ =\ \%\ crude\ protein$$

To run the analysis 0.5 g of the sample was weighed in a tin foil. The tin foil was then placed in the shaping tool provided with the instrument. The tin foil along with the sample was compressed in the form of an airtight Tablet by pressing and turning the closing cap clockwise as shown in figure 2.3. The Tablets thus made were placed in the sample tray (Figure xx) which is then inserted in the sample loader in the Dumatherm.

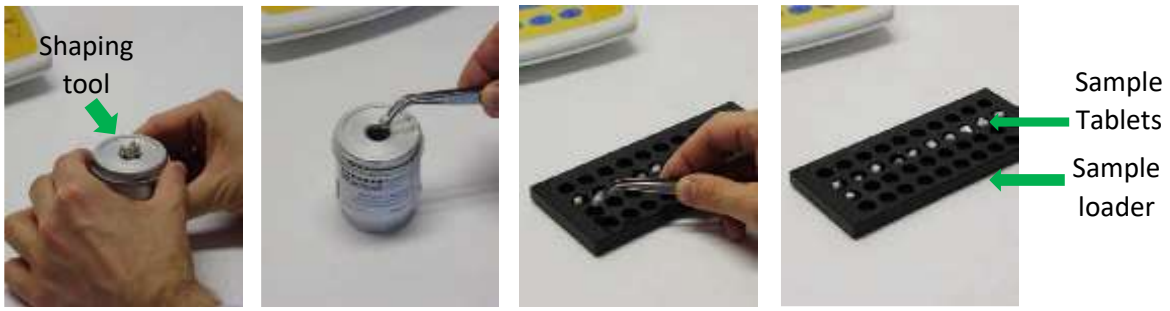


Figure 2.3: Preparation of sample for analysis showing the shaping tool and placement of tablets in the sample tray (Gerhradt Dummatherm instruction manual**,** DT 20. March 2009_GB)

2.2.3.5. Extractable Fat Determination

The Soxtherm fat extraction system (Gerhardt, UK; Figure 2.4) is based on the conventional principles of the conventional Soxhlet fat extraction. Clean dry extraction flasks with boiling stones were accurately weighed at the start of the fat extraction process. 5 g of dried diet was accurately weighed and inserted into extraction thimbles which were then placed in fat extraction beakers. The fat extraction process took a total of 2 hours and constituted of the following programmable steps:

a. Hot extraction phase: 170ml petroleum ether (CAS 64742-49-0; Fisher Scientific, UK) was poured into the extraction flask containing dried samples and brought to boil at 150^{o}C for 30 minutes. Fat was liberated from the sample during this process.
b. Evaporating phase A: the level of the solvent was lowered below the extraction thimble. Excess solvent was collected in the rear solvent recovery tank.
c. Extraction phase: petroleum ether was refluxed to further extract fat from sample for 1h.
d. Evaporating phase B: the remaining solvent was distilled and collected in the rear solvent recovery tank.
e. Evaporating phase C: a further recovery of the remaining solvent which was distilled and collected in the rear solvent recovery tank.

The extraction flasks with remaining petroleum ether and boiling stones were placed on a hot plate to evaporate off the solvent. Flasks were then placed in an oven for 2h set at 105^{o}C until constant weight was reached. Flasks including contents (fat and boiling stones) were weighed after cooling down in a desiccator. Fat was determined using the following formula:

$$\%\, extractable\ fat\ =\ [(M2 - M1)\ \div\ MO]\, x\, 100$$

$Where$:

$MO = weight\ of\ sample\ (g)$

$M1 = weight\ of\ flask + anti-bumping\ granules\ (g)$

$M2 = weight\ of\ flask + fat + anti-bumping\ granules\ (g)$

Figure 2.4: Soxtherm fat extractor (Gerhardt, UK)

2.2.3.6. Gross Energy Analysis

Gross energy analysis was done by an external laboratory, Pemberton analytical services, UK. The procedure followed by the laboratory has been detailed in this section. Gross energy of the feed was measured using a bomb calorimeter (Instrument 1261, Parr Instruments, Illinois, USA) (Rutherfurd, Chung and Moughan 2007, Woyengo, Kiarie and Nyachoti 2010). Pellets of feed sample, weighing approximately 1 g, were made by adding a small amount of water to the sample before pelleting it with a pellet press (Parr Instruments, USA). The pellets were dried overnight in a drying oven at 105°C, before being weighed into tin crucibles (Sartorius CP1245) and placed in the bomb. The bucket in the bomb jacket was filled with 2 l of water. 10 cm of fuse wire was threaded through the hole, ensuring the wire touched the pellet. The bomb was then assembled, ensuring the top was tightly screwed on, and then filled with

oxygen. Once filled, the bomb was put into the bucket of water, the electrodes were pushed into the bomb, and the lid of the bomb jacket was shut. Sample weight was entered and the process was started; the calorimeter measures the energy produced (in MJ/kg) when the pellet is exploded.

2.2.4. Procedures For Analysis Of Samples From Birds

2.2.4.1. Preparation Of Samples For 16S rRNA Sequencing

2.2.4.1.1 DNA Extraction

One of the two caeca from the bird sampled from each pen on days 22 and 35 was removed from -20^{o}C and defrosted overnight in the fridge. On day 7, three birds per pen were sampled. Hence one caecum from each of the three birds was used for DNA extraction. Prior to commencement of DNA extraction all surfaces, pipettes, weighing balance were disinfected with freshly prepared virkon. In addition, the pipettes, pipette tips, racks etc were treated with UV in the UV hood. DNA was extracted at the Poultry Research Unit lab using the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol with some modifications described below. Briefly, 0.25 – 0.26 g of cecal contents were weighed into the power bead tubes and the weight of each sample was recorded. After addition of 750 µl of power bead solution and 60 µl of solution C1, the tubes were vortexed till the sample, beads and solutions were uniformly mixed. The samples were homogenized using Precellys 24 homogenizer (Bertin Technologies, France) with bead beating at 6500 rpm for 40s (x3) with 30 s interval between two cycles. Then steps 5 to 18 of the manufacturer's Quick-Start Protocol (August, 2016) was followed except that in step 17, 60µl of solution C6 was added to the centre of the white filter membrane instead of 100 µl.

The DNA concentration and purity of extracted DNA was determined using a NanoPhotometer NP80 (Implen, Germany). The spectrophotometer lens was cleaned with ethanol and lens tissue prior to use. 1-2 µl of DNA solution was pipetted at the centre of the

lens and the concentration values displayed were recorded. This was done twice for each sample. Samples with DNA concentration above 30 ng/µl, 260:280 ratio (ratio absorbance at 260 nm and 280 nm) of approx. 1.8 and 260:230 ratio (ratio of absorbance at 260 and 230) between 2.0 – 2.2 were accepted for 16S rRNA analysis. A 260:280 ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. Extraction was repeated with samples where they were found to be contaminated with RNA or protein based on the 260:280 and 260:230 ratio values. The extracted DNA was then stored at -80^{o}C.

2.2.4.1.2. DNA Normalization

Prior to sequencing the extracted DNA was defrosted at 4-6^{o}C and was diluted to 5 ng/µl using 10 mM Tris-Cl, pH 8.5, quantified using Quant-iT^{TM} 1x dsDNA HS assay kit (Life technologies Corp., Oregon, USA) according to manufacturer's protocol and fluorescence was read using Qubit^{TM}4 fluorometer (Life Technologies Holdings Pte Ltd, Singapore).

2.2.4.1.3. 16S rRNA Sequencing

The purified genomic DNA of each sample normalized to a concentration of 5 ng/µl was used for preparation of the sequencing library following the Illumina 16S Metagenomic Sequencing library preparation protocol (Part No 15044223 Rev. B). It involves amplification of the V3 - V4 region of the gene encoding 16S rRNA using a two-step PCR.

The following 16S, V3 - V4 specific primers were used (containing both 16S specific primers as well as adapter tails for adding indices and Illumina flow cell adapters) and were selected from Klindworth *et al* (2014)

Forward Primer = 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer=

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The first step was PCR amplifications (Amplicon PCR), which were carried out using 25 µl reaction mixtures of 2.5 µl microbial DNA (5 ng/µl); 5 µl Amplicon PCR Forward Primer (1 µM); 5 µl Amplicon PCR Reverse Primer (1 µM) and 12.5 µl 2x KAPA HiFi HotStart ReadyMix (KAPA-Germany) in a 96 well plate. The plate was sealed using microseal before PCR was performed in a thermal cycler (Techne,TC-512, UK) using the following program: 95°C for 3 minutes; 25 cycles of: 95°C for 30 seconds; 55°C for 30 seconds; 72°C for 30 seconds; 72°C for 5 minutes. Then PCR amplicons were cleaned up as follows: 20 µl of AMPure XP beads was added to each well of the PCR plate, and mixed by gently pipetted up and down and incubated at room temperature for 5 min. Then the plate was placed on a magnetic stand for 2 min until the supernatant cleared. The clear supernatant was discarded without disturbing the beads. The beads were washed with 200 µl freshly prepared 80% ethanol twice. Then excess ethanol was carefully removed, the beads were air dried and then the plate was taken off the magnetic stand. Next, 53 µl of 10 mM Tris-Cl, pH 8.5 was added to each well of the PCR plate and the beads were fully resuspended by pipetting up and down and then incubated at room temperature for 2 minutes. The plate was again placed on the magnetic stand till a clear supernatant was obtained. Fifty µl of the supernatant was transferred to a new 96 well plate and the plate with magnetic beads was discarded. The purified amplicons of 15 random samples were diluted 1:5 in molecular grade water before mixing with 2 µl sample buffer and run on Agilent High Sensitivity D1000 screen tape (Agilent Technologies, INC, CA, USA) according to manufacturer's instructions to verify amplicon size.

The next step was the Index PCR to attach the dual indices and the Illumina sequencing adapters which was performed as follows: A fresh 96 well PCR plate placed in the TruSeq Index Plate Fixture (Illumina, USA) and the Index 1 and Index 2 primers were arranged on the same fixture (Figure 2.5). The following reactions were set up: 5 µl amplicon DNA, 5 µl Nextera XT Index Primer 1 (N71-12) horizontally, 5 µl Nextera XT Index Primer 2 (S51-8) vertically, 25 µl of 2x KAPA HiFi HotStart ReadyMix, 10 µl molecular grade water.

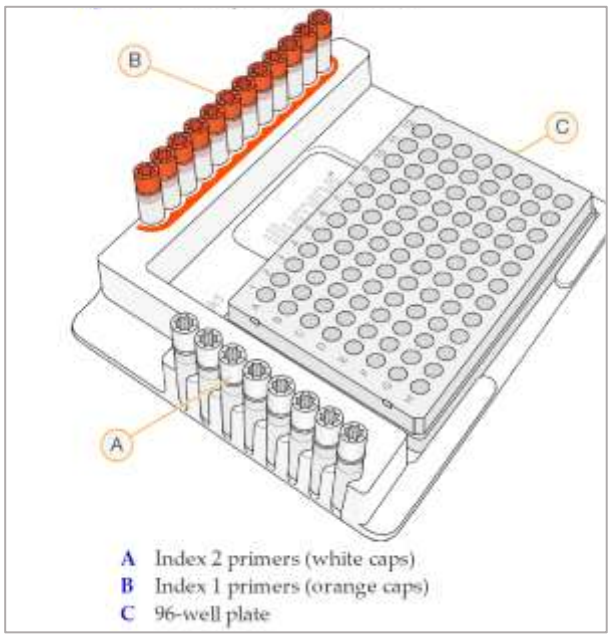


Figure 2.5. Illumina TrueSeq plate fixture showing arrangement of indices and 96-well plate (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf)

This mixture was gently mixed and the plate was sealed with microseal. PCR was performed on a thermal cycler using this programme: 95°C for 3 min, 8 cycles of: 95°C for 30 sec 55°C for 30 sec 72°C for 30 sec, 72°C for 5 min, then hold at 4°C. The Index PCR products were cleaned up using AMPure XP beads in the same manner as described for Amplicon PCR above except that after the ethanol washes the beads were resuspended in 27.5 µl of 10 mM Tris-Cl, pH 8.5 and 25 µl of the supernatant free of beads was transferred to a new plate. Size of

the indexed amplicon was verified using Agilent High Sensitivity D1000 screen tape as described above.

The last step was library quantification, normalization and pooling. The libraries were quantified using Qubit™ dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and fluorescence read using Qubit™ 4 fluorometer (Life Technologies Holdings Pte Ltd, Singapore). DNA concentration was calculated in nM, based on the size of DNA amplicons based on the average size of DNA amplicons as determined by on Agilent High Sensitivity D1000 screen tape using the formula,

$$Concentration\ in\ nM\ =$$

$$\left\{Concentration\ in\ \left(\frac{ng}{\mu l}\right) \div 660\ \left(\frac{g}{mol}\right) \times Average\ library\ size\right\} \times 10^6$$

$where,$

$$Average\ library\ size\ =\ Average\ base\ pair\ from\ Agilent\ Screen\ Tape$$

$$660\ (g/mol)\ =\ molecular\ weight\ of\ double\ stranded\ DNA$$

The DNA was diluted to a 4 nM concentration and 5 µl aliquot of the diluted DNA from each library was pooled as follow; first the MiSeq reagent cartridge was removed from -15°C storage and thawed at room temperature. Then DNA was denatured by combining the following volumes in a microcentrifuge tube: 4 nM pooled library (5 µl) and 0.2 N NaOH (5 µl), vortexed briefly then centrifuged at 280 × g at 20°C for 1 minute before incubating for 5 min at room temperature. Then 990 µl of pre - chilled Hybridization Buffer HT1 was added to the tube containing denatured DNA (10 µl) Library resulting in a 20 pM denatured library in 1 mM NaOH and placed on ice until use. PhiX as a sequence control was denatured and diluted to 4 nM by combining 10 nM PhiX library (2 µl) and 10 mM Tris pH 8.5 (3 µl). Then 4 nM PhiX

library (5 µl) and 0.2 N NaOH (5 µl) was combined in a microcentrifuge tube and vortexed briefly before incubating for 5 minutes at room temperature to denature the PhiX library into single strands. Then 990 µl pre - chilled HT1 was added to the tube containing 10 µl denatured PhiX library to result in a 20 pM PhiX library. This was then diluted to the same loading concentration as the Amplicon library to get 8pM by mixing 240 µl of 20 pM denatured library and pre - chilled HT1 (360 µl). The Amplicon Library and PhiX Control were combined in volume of 570 µl and 30 µl respectively. This was then set aside on ice until it was time to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge at which point the mixture was incubated at 96°C for 2 minutes by using a heat block. Afterward the tube was mixed and placed in the ice-water bath. Finally the template allocations of samples was set up in the Illumina sheet, then the combined sample library and PhiX was loaded into the well in the Miseq cartridge then loaded in the machine. A paired-end sequencing (2 × 300 bp for Trial 1 and 2 x 150 for Trial 2) approach was used on two runs of the Illumina MiSeq (Illumina Inc., USA) platform, one for Trial 1 and another for Trial 2.

2.2.4.1.4. Bioinformatic Analyses

Raw reads were trimmed for Illumina Nextera XT adapters and read through using Trimmomatic version 0.38 (Bolger, Lohse and Usadel, 2014). Reads trimmed with Trimmomatic were checked for quality through FastQC version 0.11.7 (ref: https://github.com/s-andrews/FastQC) for adapter sequences and average sequencing quality drop off below Phred 20. Reads trimmed with Trimmomatic were also hard trimmed at the 3' ends for each forward and reverse read by 16 and 60 bp (Trial 1) or 16 and 10 bp (Trial 2) respectively, removing poor quality read ends. Reads were further curated through Sickle version 1.33 (see manual citation here: https://github.com/ucdavis-bioinformatics/sickle) trimming reads with base quality below Phred 20 and removal of reads

less than 245 bp in length in case of Trial 1 or 225 bp in length in case of Trial 2. The difference in the number of bp trimmed for sequences from Trial 1 and Trial 2 was due the different versions of the Illumina kit used (technician error while placing orders). Trial 1 was sequenced using the 2 x 300 bp kit and 2 x 150 bp kit. Hence less trimming was done at the 3' end for sequences from Trial 2 as these sequences were already shorter.

Dada2 v1.14.0 was used to estimate error rates within the fastq files, and further trim the sequences (the forward read to a maximum length of 255bp, the reverse read to a maximum length of 235 bp; the 5' of both forward and reverse reads were trimmed by 20bp). PhiX reads were filtered, and reads were discarded if they contained any Ns. Reads were merged and denoised, and chimeric reads removed. All unique reads from the positive control (mock reference) were extracted and compared against a FASTA of 16S sequences from the strains in the ATCC mock. Taxonomy was assigned to sequences by comparison to the Silva database v132.

At this point, data was transferred to Phyloseq v1.30.0. Phyloseq requires a count for each OTU (or unique read), a taxa Table (obtained through the comparison with the Silva database) and metadata. OTU names were changed from nucleotide sequence and given an amplicon sequence variant (ASV) number. Samples were pruned, to remove negative and positive control as well as samples that failed PCR or sequencing reaction. Any ASV that was present in less than two samples was also removed from the dataset.

2.2.4.1.5. Ecological and Statistical analyses of sequencing data

Further downstream analyses and graphical outputs were generated using Marker Data Profiling module of MicrobiomeAnalyst a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data (Dhariwal *et al.*, 2017).

Marker Data Profiling module was used as according to Chong et al. (2020). Under data filtering, the low count and low variance (based on inter-quantile range) were both selected to be 10%. Data was neither rarified nor transformed and total sum scaling (TSS) was selected for normalization. Both alpha and beta diversity metrics were used to estimate microbial community diversity. Species richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson index) were selected for alpha diversity estimations. To compare alpha diversity metrics among groups, non-parametric Kruskal-Wallis test was selected. For beta diversity analysis, dissimilarity matrix between samples Bray Curtis method was selected, and was further visualized with a Non-Metric Multidimensional Scaling (NMDS) ordination technique. Bray Curtis dissimilarity was also used to perform hierarchical clustering (dendrogram) with average as the clustering algorithm.

Linear Discriminant Analysis Effect Size (LEfSe) algorithm with LDA effect size threshold of 2 (on a log10 scale) was selected at genus level for evidencing potential biomarkers linked to age and diet. Core microbiome was identified for age at family and genus level and was defined as taxa detected in all age-groups with at least 80% prevalence within the group.

To identify the shared and unique ASVs among the age-groups, Venn diagrams were constructed with the online tool accessed through

http://bioinformatics.psb.ugent.be/webtools/Venn/

2.2.4.2. Preparation of samples for gene expression studies

2.2.4.2.1. Total RNA extraction from ileal tissue

Ileal tissue that had been prepared according to method described in section 2.3.3 had its total RNA extracted using the RNeasy Mini Kit (Qiagen, Manchester, UK) and following the manufacturer's instruction. Stabilised illeal tissue samples in RNAlater previously frozen at -80°C were thawed at room temperature and 25 – 40 mg of tissue was weighed (Sartorius,UK)

into a 2ml eppendorf tube containing 600 µl RLT buffer then lysed and homogenised using cordless motor Pellet Pestle, (Z359955, Sigma, UK) to release cellular RNA. Buffer RLT contains β-mercaptoethanol and guanidine thiocyanate which lyses the cells and protects the RNA by inactivating RNases. Ethanol was then added to lysates to provide suiTable binding conditions before samples were bound to RNeasy silica membranes (spin columns) by centrifugation at 16000 x g for 15 seconds. After washing the spin column with buffer RW1, on-column DNase digestion was performed by adding 80 µl of DNase I – RDD buffer incubation mixture to the centre of the membrane in the spin column and incubated at room temperature for 15 min. Then buffer RW1 and buffer RPE washes were carried out respectively to remove contaminants from the spin column. Finally, RNA was eluted into clean 1.5 ml eppendorf tubes, using 55 µl RNase-free water, by centrifugation at 16000 x g for 15 seconds.

2.2.4.2.2. Quantification and purity of RNA

The concentration and quality of RNA was determined using spectral analysis by NanoDrop spectrophotometer ND-1000 (Fisher Scientific, UK). RNA concentration is automatically calculated in ng/µl which was at least 100 ng/µl to be used in the cDNA synthesis. RNA purity is also calculated as the ratio of absorbance at 260 nm and 280 nm (A260/A280) and ratio of ~2.0 is considered pure for RNA. Then the extracted RNA kept at -80°C until cDNA synthesis.

2.2.4.2.3. Assessment Of RNA Integrity

Integrity of the extracted RNA was assessed using agarose gel electrophoresis. A 5 µl aliquot of RNA normalized to 100 ng/µl was heated to 70^{o}C for 1 min in a thermocycler and then mixed with 1 µl of loading buffer. A final volume of 2 µl (200 ng of RNA) was then loaded in a 1.2% (w/v) agarose gel with SYBR™ Safe Stain (5 µl of stain in 100 ml gel) (Invitrogen, USA). The gel was then submerged in 1X TAE buffer in an electrophoresis tank and run at 100 V for

35 min. The gel was then visualized under UV transilluminator. The extracted RNA was kept at -80°C until cDNA synthesis

2.2.4.2.4. cDNA Synthesis

The DNase treated RNA was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA). The reaction mix was made by mixing 4 µl of 5x iScript, 1 µl iScript reverse transcriptase and 15 µl diluted RNA with nuclease–free water to make a total volume of 20 µl. The conditions for cDNA synthesis using Thermal Cycler (AB Applied Biosystems, UK) were: 25°C for 5 min, 46°C for 20 min and 95°C for 1 min. The cDNA samples were then stored at -80°C until use for qRT-PCR.

2.2.4.2.5. Selection Of Primers For The Target Genes And Primer Efficiency Testing

The primers and house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were chosen from previously published papers listed in Table 2.2 and double checked for target identity using GenBank in the National Centre for Biotechnology Information (NCBI). All primers are listed in Table 2.2. Primer Blast of the NCBI database was used to check that the selected primers

- had a G-C content in the range of 45 – 70%
- avoided runs of an identical nucleotide, especially for guanine, where runs of four or more Gs should be avoided
- had Tm in the range of 58 – 63°C
- the five nucleotides at the 3´end of each primer had no more than two G and/or C bases.
- had amplicon sizes in the range of 50 – 250 bp

Primers were purchased from Eurofins (Germany) with the synthesis scale 0.2 µmol and purification: High Purity Salt Free (HPSF®).

Table 2.2: Oligonucleotide primers used for the study of gene expression of selected targets by quantitative real time PCR.

RNA Target	Genbank accession no.	Primer Sequence (5' – 3')	Tm	Product size	Reference
MUC2	NM_001318434.1	F: TCACCCTGCATGGATACTTGCTCA R: TGTCCATCTGCCTGAATCACAGGT	62	228	Palamidi and Mountzouris, 2018
CLDN 1	424910	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	62	92	Slawinska et al 2019
CLDN 5	NM_204201.1	F: CATCACTTCTCCTTCGTCAGC R: GCACAAAGATCTCCCAGGTC	59	111	Palamidi and Mountzouris, 2018
OCLN	NM_205128.1	F: TCATCGCCTCCATCGTCTAC R: TCTTACTGCGCGTCTTCTGG	62	240	Palamidi and Mountzouris, 2018
sIgA	S40610	F: GTCACCGTCACCTGGACTACA R: ACCGATGGTCTCCTTCACATC	59	192	Paraskeuas and Mountzouris, 2019
IL-1β	NM_204524.1	F: CAGCCCGTGGGCATCA R: CTTAGCTTGTAGGTGGCGATGTT	58	59	Chen et al 2015
FFAR-2	100859369	F: GCTCGACCCCTTCATCTTCT R: ACACATTGTGCCCCGAATTG	59	88	Slawinska et al 2019
GAPDH	NM_204305.1	F: GCTGAATGGGAAGCTTACTG R: AAGGTGGAGGAATGGCTG	60	216	Paraskeuas and Mountzouris, 2019

For primer efficiency testing, 5 µl cDNA from several samples was pooled together in one tube to generate a single sample that was representative of the entire study. This was used as the stock. Appropriate dilutions of the cDNA stock were made, and a qRT-PCR reaction was performed for each target gene and its primer pair in duplicates as described in section 2.5.3.6. A 5-point standard curve was generated by plotting the average Ct value of each duplicate

dilution against the log of the cDNA quantity (Figure 2.6). The amplification factor (E) was calculated using the formula

$$E = 10^{-1/slope}$$

and primer efficiency (E) *was calculated as a percentage using the equation*

$$Efficiency, (\%) = (10^{-1/slope} - 1)\ x\ 100$$

Only primer pairs with efficiency values between 90 – 110% selected for the study.

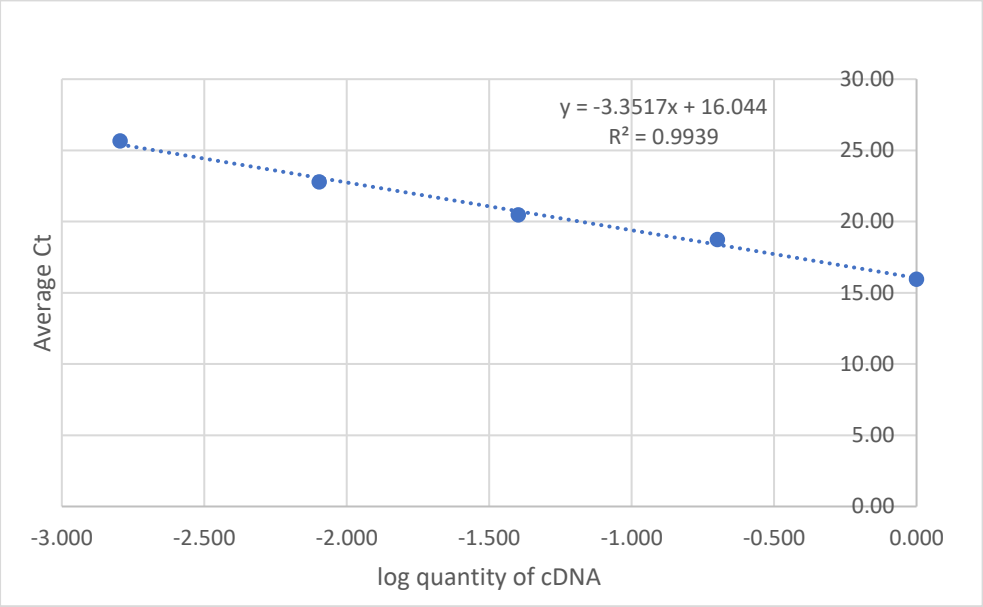


Figure 2.6: Example of a typical standard curve from efficiency testing of GAPDH primers

2.2.4.2.6. qPCR assay

All qPCR experiments were set up in a PCR hood after decontaminating material under UV light for at least 15 min. The "sample maximisation strategy" (Bustin et al., 2009) was used so that all samples were run on the same plate for each target gene to minimise inter-run variability (Hellemans et al., 2007). The full protocol for Real-Time Quantitative PCR reactions was carried out in duplicate using iQ™ SYBR Green Supermix kit (BioRad, Hercules, CA) on CFXConnect™ Real time System (Bio-Rad, USA) according to the manufacturer's instructions. Amplification was carried out in a total volume of 20 µl in Hard-Shell® 96-Well PCR Plates (BioRad, Hercules, CA). Reaction mixture was composed of 10 µl iQ SYBR Green supermix

(BioRad, Hercules, CA), 1 µl each of 10 µM forward and reverse primers, 1 µl of 1:2 dilution of sample cDNA and 7 µl H_2O. RT-PCR conditions were 95°C for 3 min, followed by 40 cycles of 95° C for 10 sec, Tm°C (according to Table 2.2) for 25 sec and melt curve analysis from 55 to 90° C with 0.5° C increment. Each run and each target gene was set up with a no template control and GAPDH primers with undiluted stock as positive control.

2.2.4.2.7. Analysis of qPCR results by Pfaffl method

The relative gene expression ratio (R) is the relative quantity of mRNA transcripts in experimental sample to that of the control sample. The relative gene expression ratio (R) of the target genes was calculated using the equation given by Pfaffl (2001)

$$\boldsymbol{R} = \boldsymbol{E}_{\mathbf{GOI}}{}^{\Delta \mathbf{Ct(GOI)}} / \boldsymbol{E}_{\mathbf{REF}}{}^{\Delta \mathbf{Ct(REF)}}$$

$where\ E = amplification\ factor$

$GOI = gene\ of\ interest$

$REF = reference\ gene$

$\Delta Ct = Average\ Ct\ (control\ birds) - Average\ Ct\ (treatment\ birds)$

2.2.4.3. Quantification Of Short Chain Fatty Acid Using Gas Chromatography Mass Spectrometry (GCMS)

The samples and standards were prepared and derivatized according to the Agilent Technologies (Japan) protocol for GCMS detection of short chain fatty acids in mammalian faeces in aqueous solution by, Japan with a minor modification in the derivatization process described in 2.5.3.3. The procedure involves preparation of samples and standards and followed by derivatization.

2.2.4.3.1 Sample preparation

The chicken caecum sample collected according to method described in 2.3.1 were thawed at room temperature. Immediately after thaw approximately 200 – 300 mg of caecal contents were weighed into 2-mL screw cap tubes with ceramic beads (KT03961 1, Bertin Technologies,

France). After the addition of 1 ml 10% isobutanol, the samples were homogenized mechanically (Precellys Evolution; Bertin Technologies, France) over two cycles, 6,000 rpm for 20 seconds with a 30 sec interval between the two cycle. Each sample was then centrifuged 16,000 x g for 5 min and 675 µl supernatant transferred to a new tube.

In order to monitor recoveries, 10 µl of 3-methylpentanoic acid (3-methyl valeric acid) was added to the supernatant. Then, 125 µL of 20 mM NaOH solution and 400µL of chloroform were added, and the sample vortexed and centrifuged at 21,000 x g for two minutes. A 400 µl aliquot of the upper aqueous phase was transferred into a new tube, and 80 µl isobutanol and 100µl of pyridine were added along with ultrahigh quality water to adjust to volume to 650 µl. In order to minimize foaming during the following derivatisation stage, one boiling chip was also placed into the tube. The sample were stored at -20^{o}C until GCMS analysis could be completed.

2.2.4.3.2. Calibration standards preparation

The desired concentration of the SCFAs standard were mixed with 125 µl of 20 mM NaOH, 100 µL pyridine, and 80 µl isobutanol were combined in a tube. The final volume was adjusted to 650 µL with ultrahigh purity water. To avoid foaming, one boiling chip was added into the tube.

2.2.4.3.3. Derivatization process

Calibration standards and samples were subjected to the same derivatization procedure. An aliquot of 50µl isobutyl chloroformate was carefully added to the entire volume of sample or standard solution. In order to release the gases generated by the reaction, the tube lid was kept open for 1 min, then closed and the sample vortexed. The samples were then incubated at room temperature for 10 min. One hundred and fifty µl of hexane, was then added, and the tubes centrifuged at 16,000 x g for 2 min. Fifty µl of the upper hexane-isobutanol phase

was then transferred into an autosampler vial prior to GC-MS analysis. Figure 2.7 illustrates the reaction mechanism for the derivatisation process. All reagents for sample preparation, fatty acid extraction, derivatisation, and analysis, were obtained from Sigma Aldrich (Merck KGaA, St Louis, USA).

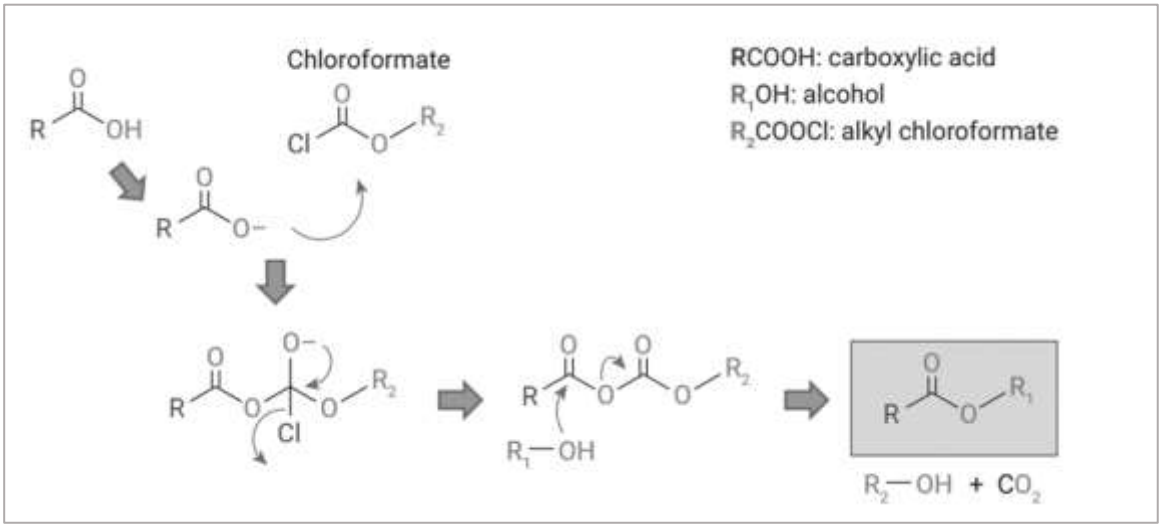


Figure 2.7. Derivatization reaction mechanism

2.2.4.3.4. Instrumentation

For SCFA quantification, GC-MS measurements were carried out using a Shimadzu 2010ultra GC-MS single quadrupole mass spectrometer (Figure 2.8) equipped with a Shimadzu GC2010 and AOC20i+s autosampler (Shimadzu Corp., Kyoto, Japan). Table 2.3 details the GC-MS analytical conditions.

Table 2.3. Parameter settings used in the GC-MS Analytical method

Parameter Value	
GC-MS System	Shimadzu 2010
Column	HP-5ms 30 m × 0.25 mm, 0.5 µm
Column flow	1.0 ml min^{-1}
Liner	Ultra Inert liner, Universal, Low PSI drop, Wool
Injection mode	Split (50:1)
Injection temperature	260 °C
Oven temperature	40 °C for 5 min, 10 °C min^{-1} to 310 °C
Transfer line temperature	280 °C
MS mode	Scan; 70eV
Scan range	m/z 30–350
Ion source temperature	250 °C
Quadrupole temperature	150 °C

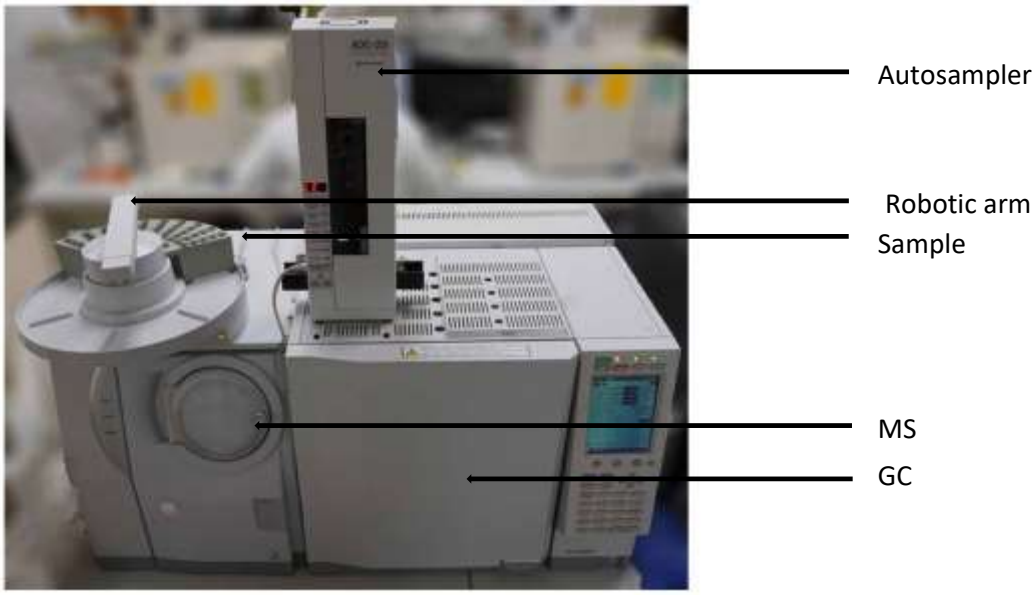


Figure 2.8. Image of Shimadzu QP2010Ultra GC-MS. The gas chromatograph (GC) is situated on the right and the mass spectrometer (MS) to the left. The sample injector is the box on top of the GC. The autosampler, into which sample vials are placed prior to analysis. A robotic arm picks the appropriate sample vial from the autosampler and places it into the injector. A syringe (located within the sample injector box) then takes a known volume of the analyte from the sample vial and injects it into the GC. The injection port of the GC is situated beneath the sample injector and is not visible. The entire instrument is controlled by a computerised data system (not pictured).

The derivatized samples were placed in the sample carousel from where they were picked by the robotic arm and injected into the GC. The GC comprises of a heated inlet port, an oven, and a fused silica column containing the ***stationary phase.*** Samples entering the inlet port in the GC were heated to >200^{o}C, where the molecules were vaporised and became gaseous.

He gas (***mobile phase)*** carried the gaseous sample through the differentially heated column, at differing speeds according to their individual chemical structure, so that separated SCFA molecules left the GC after their unique retention time to enter the MS where each separated compound was broken down into charged molecular fragments by electron ionisation so the mass to charge ratio (m/z) could be calculated. ***Electron Ionisation*** (EI) yielded singly charged ions, such that m/z = m. Each fragment then underwent a process of acceleration and was exposed to an alternating electric field. By constantly changing the electric field a 'scan' of fragment ions over a range of mass to charge ratios was taken. The ions eventually hit the detector, where the mass to charge ratio (m/z) and relative abundances (how many of those fragment ions were present in the sample) were calculated.

This data was used to construct a graph called a mass spectrum, showing the signal intensity or abundance of each detected fragment's mass to charge ratio (Figure 2.9 and 2.10).

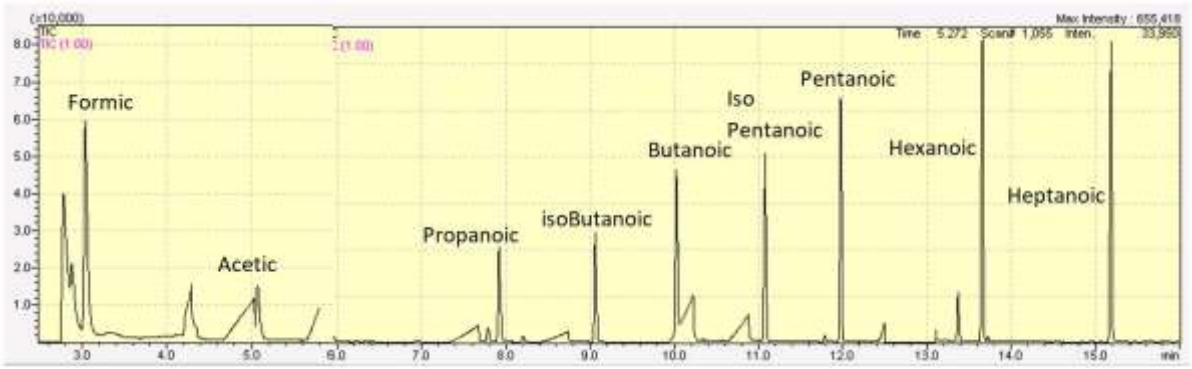


Figure 2.9. Typical GC-MS Total Ion Chromatogram (TIC) trace from the analysis of reference SCFA isobutyl derivatives.

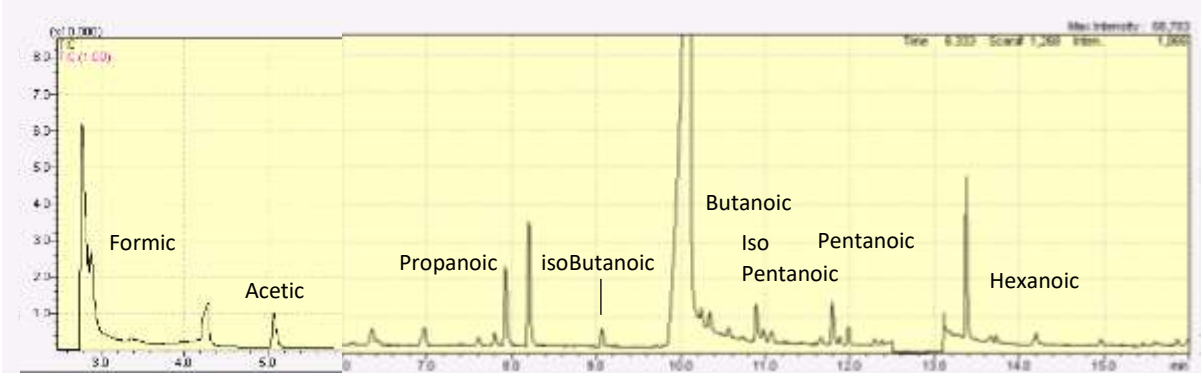


Figure 2.10. Typical GC-MS Total Ion Chromatogram (TIC) trace from the SCFA analysis of chicken caecum. Labels indicate isobutyl ester derivatives of the relevant acids.

The mass spectral intensities were also used to produce a graph called a ***chromatogram***, where each separated substance was represented by a ***peak***. The number of peaks showed the number of separated compounds in the sample. The position of each peak showed the retention time for each compound. The area under each peak indicated how much of that component was present.

For trace quantification only signals from ions specific to the analytes under study were acquired which allowed for the ***sensitivity*** and ***specificity*** to be increased. This technique is called ***Single Ion Monitoring (SIM)*** or ***Multiple Ion Detection (MID)*** which was employed in this study (Figure 2.11).

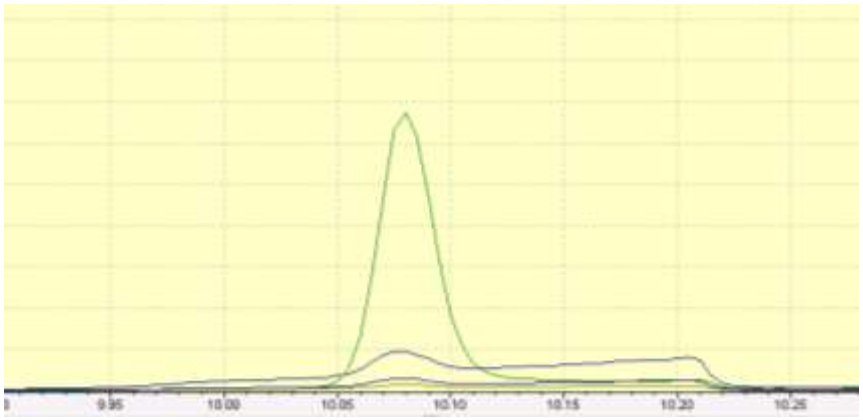


Figure 2.11. Illustration of the advantage of acquiring GC-MS data in SIM mode, rather than scan mode. The green trace shows the signal of the quantification mass (m/z 71.05) for isobutanoic acid in SIM mode, while the black trace is for the same compound, on the same intensity scale, acquired in scan mode, where all masses are measured. The selectivity reduced baseline and increased signal to noise ratio of the green trace is apparent.

2.2.4.4 Determination of mucin layer thickness

Mucin layer thickness was measured as described by Smirnov et al. (2004). A 10mm section of jejunum was incised from the Meckel's end of the tract and weighed (4D.P). The tissue was gently flushed with distilled water and then placed in 10g/l Alcian blue (160mmol/l sucrose with 50mmol sodium acetate) for 2 hours. The tissue was then washed in 250mmol/l sucrose to remove excess dye. The bound dye was then extracted using 10g/l docusate sodium salt overnight at room temperature. The supernatant was centrifuged at 700 x g for 10 minutes to remove particulate matter. The absorbance was then read at 620nm on a spectrophotometer (Jenway 7315, Bibby Scientific Ltd, UK) against a standard curve of diluted Alcian blue. Results were expressed as µg Alcian blue released per gram of tissue.

2.2.5. Statistical analysis of data

In case of Trial 1, each pen was the experimental unit while in case of Trial 2, the individual tagged birds were the experimental units. Outliers were removed from performance data set of if they fell above or below 2x the standard deviation of the mean. Normality was checked using Kolmogorov-Smirnov (ks.test function). All data was analysed using R software, version 3.6.1. The effect of XOS or xylanase supplementation under controlled conditions alone or in combination (Trial 1 – Oligo26) on the performance was analysed using ANOVA according to the following model

$$Yij = Mean + XOS(i) + XYL(j) + XOS*XYL(ij) + E(ij)$$

And relative abundance of microbial taxa, gene expression in the ileum and SCFA concentrations in caeca was analysed using ANOVA according to the following model

$$Y(ijk) = Mean + Age(i) + XOS(j) + XYL(k) + Age*XOS(ij) + Age*XYL(ik) + XOS*XYL(jk) + Age*XOS*XYL(ijk) + E(ijk).$$

Where Y(ijk) is the response variable, Mean is the overall mean, Age(i) is the effect of bird age, XOS(j) is the effect of the XOS level, XYL(k) is the effect of the XYL level, Age*XOS(ij) is the effect of the interaction between bird age and XOS, + Age*XYL(ik) is the effect of the interaction between bird age and XYL, XOS*XYL(jk) is the effect between XOS and XYL, Age*XOS*XYL(ijk) is the effect of the interaction among bird age, XOS and XYL and E(ijk) is the residual.

The effect of XOS on performance of birds raised under sub-optimal conditions (Trial 2 – Oligo13) was analysed using ANOVA according to the following model

$$Y(ij) = Mean + B(i) + TRT(j) + E(ij)$$

Y(ij): response variable, B(i): effect of block, TRT (j): effect of treatment (XOS vs. control) and E(ij) is the residual

Relative abundance of microbial taxa, SCFA, gene expression data were analysed using ANOVA according to the following model

$$Y(ij) = Mean + Age(i) + TRT(j) + Age*trt(ij) + E(ij)$$

Y(ij): response variable, Age (ij): effect of bird age, TRT (ij): effect of treatment (XOS vs. control) and age*trt (ij): age-treatment interaction and E(ij) is the residual

AOV function of R was used to perform ANOVA. Means were compared using LSD method and p values were adjusted using Tukey method

Chapter 3: Effect of XOS and xylanase alone and in combination on performance and gut health parameters of broilers raised under controlled research conditions

3.1 Introduction

This trial seeks to investigate the effects of a corn cobb derived xylo- oligosaccharide (XOS) and xylanase alone and in combination, on the whole bird response as well as alternations in microbiome and gene expression. XOS have a great prebiotic potential and have shown to exert their nutritional benefits in various animal species. XOS for use in animal feed are generally extracted from xylan rich inexpensive agricultural residues such as cereal straw, sugarcane bagasse, coconut husk, corn husk and corn cobb (Samanta *et al.*, 2015). Xylan, the precursor of XOS, is polysaccharide accounting for 25–35% of the dry biomass of woody tissues of dicots and lignified tissues of monocots and comprises up to 50% in some grasses and tissues of cereal grains (Samanta *et al.*, 2015). Depending on the source or raw material used, the number of xylose residues involved in the formation (known as degree of polymerization or DP) of XOS can vary from 2 to 10 linked by β(1→4) glycosidic bonds. They may be present with side chains with different types of linkages and may also differ in the degree of substitution. This results in the production of XOS with diverse biological properties (Aachary and Prapulla, 2011).

The application of xylanase is ubiquitous in poultry diets containing wheat and barley due to its widely accepted role in reduction of viscosity (Bedford, 2018). But the viscosity effect alone could not explain the improvement in performance of birds fed different xylanases in wheat based diets, some of which reduced viscosity and some did not (Choct *et al.*, 2004). The authors therefore suggested an alternative mechanism that had not been sufficiently explored (Choct *et al.*, 2004). The other proposed mode of action of xylanases from *in vitro* studies is their ability to degrade endosperm cell walls thereby enhancing digestibility by enabling endogenous proteases and amylases more rapid access to the

encapsulated protein and starch (Aftab and Bedford, 2018). However this mechanism too was challenged by the fact that in *in vitro* studies where commercial doses of enzymes were used there was negligible destruction of cell wall (Morgan *et al.*, 1995). Secondly the pH profile of the enzymes employed coupled with the transit time of digesta in the broiler suggest that there is not enough time for exogenously applied enzymes to appreciably degrade cell walls directly by the mid jejunum the point at which microscopic work has shown cell wall degradation (Singh et al., 2012). There has been heightened interest concerning the role of xylanase in generation of XOS from the xylan fractions in the cell wall of cereals in the gastrointestinal tract of poultry which would subsequently serve as prebiotic modulating the microbiota and improving bird health and performance (Craig *et al.*, 2020; Morgan *et al.*, 2020).

Several studies investigating the effect of XOS on performance of chickens have been published with inconsistent results. A summary of chicken trials evaluating the effect of XOS on performance is presented in Table 1.5 (Craig *et al*., 2018; De Maesschalck *et al*., 2015; Pourabedin *et al*., 2015; Ribeiro *et al*., 2018; Samanta *et al*., 2016; Zhenping *et al*., 2012; Suo *et al.,* 2015; Yuan *et al*., 2018). However, these studies differ in the composition of the basal diet, source of XOS and levels of inclusion, duration of supplementation or housing management practices which may have contributed to the differences in performances. Nevertheless, most studies are in agreement regarding the positive influence of XOS on gut health via modulation of gut microbiota, production of short chain fatty acids and ameliorating the effects of pathogens in challenge studies.

In the present study corn-cob derived XOS35 (Longlive Biotechnology, Shandong, China) was used as the feed additive. XOS35 is a mixture of 35% XOS with degree of

polymerization between 2 and 7 and 65% maltodextrin. The xylanase (Econase XT, AB Vista, Marlborough, UK) used contained 160000 units of endo-1,4-β-xylanse activity. One unit of xylanase activity is defined as the amount of enzyme required to liberate 1 nmol of reducing sugars from xylan using a standardised test.

The hypothesis of this study was that XOS or xylanase, either alone or in combination would improve performance of broilers by improving their gut health.

3.2. Aims

The aims of this trial were to evaluate the effect of XOS and xylanase alone and in combination on

1) performance
2) mucin layer thickness
3) diversity and composition of caecal microbiota
4) gene expression of biomarkers of gut integrity and SCFA receptors in the ileum
5) caecal SCFA concentrations

3.3. Materials and Methods

3.3.1. Bird husbandry

A total of 384 one day old male Ross 308 birds from a flock of 40 – 45 weeks of age were obtained from P.D.Hook Hatcheries Ltd (Cote, Bampton, Oxfordshire, UK). They were placed in 48 pens, containing 8 birds and fed one of four dietary treatments. The diets were allocated using a stratified random allocation. There were four dietary treatments with 12 replicates for each treatment, made up of 12 small floor pens (area = 0.43m^2). Birds were kept with a stocking density aiming for a commercial stocking density of 30 kg per m^2 at the end of the trial. The litter provided was as wood shavings at a depth of 3 cm and was topped up if

necessary, to maintain adequate environmental welfare. Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was obtained and is recorded as project ARE643.

3.3.2. Experimental diets

The rations were based on the nutritional requirements for the strain of bird and contained approximately 35% wheat. The basal diet was free of antibiotics and manufactured using the formulation in Table 3.1. The trial lasted 35 days with a three-phase feeding programme; starter diets were fed from 1 to 15 days; grower diets were fed from 15 to 29 days and finisher diets were fed from 29 to 35 days. The four dietary treatments were

1) Control (CON)

2) Control + XOS at 100 g per tonne (XOS)

3) Control + xylanase at 100 g per tonne (XYL)

4) Control + XOS at 100 g per tonne + xylanase at 100 g per tonne (XOS + XYL)

Table 3.1: Formulation of control diet presented as rates of inclusion (%)

Ingredient (%)	Starter	Grower	Finisher
Wheat (Feed grade)	35	35	35
Corn	24	27	29
Soybean meal	34.3	31.1	27.6
Soya oil	2.76	3.85	4.96
Salt	0.3	0.31	0.31
Limestone	0.1	0.06	0.03
Dicalcium Phos. 18%	2.26	2	1.84
Sodium bicarbonate	0.1	0.1	0.1
Lysine HCL	0.23	0.18	0.18
DL-Methionine	0.31	0.27	0.26
Threonine	0.13	0.09	0.08
Vitamin & Mineral premix*	0.5	0.5	0.5
Quantum Blue 5G (Phytase)	0.01	0.01	0.01
Titanium (IV) dioxide	0.5	0.5	0.5

*Premix content (volume/kg diet): Mn 100 mg, Zn 80 mg, Fe 20 mg, Cu 10 mg, I 1 mg, Mb 0.48 mg, Se 0.2 mg, Retinol 13.5 mg, Cholecalciferol 3 mg, Tocopherol 25 mg, Menadione 5.0 mg, Thiamine 3 mg, Riboflavin 10.0 mg, Pantothenic acid 15 mg, Pyroxidine 3.0 mg, Niacin 60 mg, Cobalamin 30 µg, Folic acid 1.5 mg, Biotin 125 mg

3.3.3 Treatment schedule / randomisation plan

A replicate consisted of a pen containing 8 birds, with only birds weighing between 40 g and 50 g placed. The weight of each pen was recorded on day 0 and treatments randomly allotted to pens around the trial room using an online randomiser allocated (by an individual not involved in the study to prevent bias), to reduce any possible effects of ventilation and room placement. Pen layout and diet allocation is shown in figure 3.1.

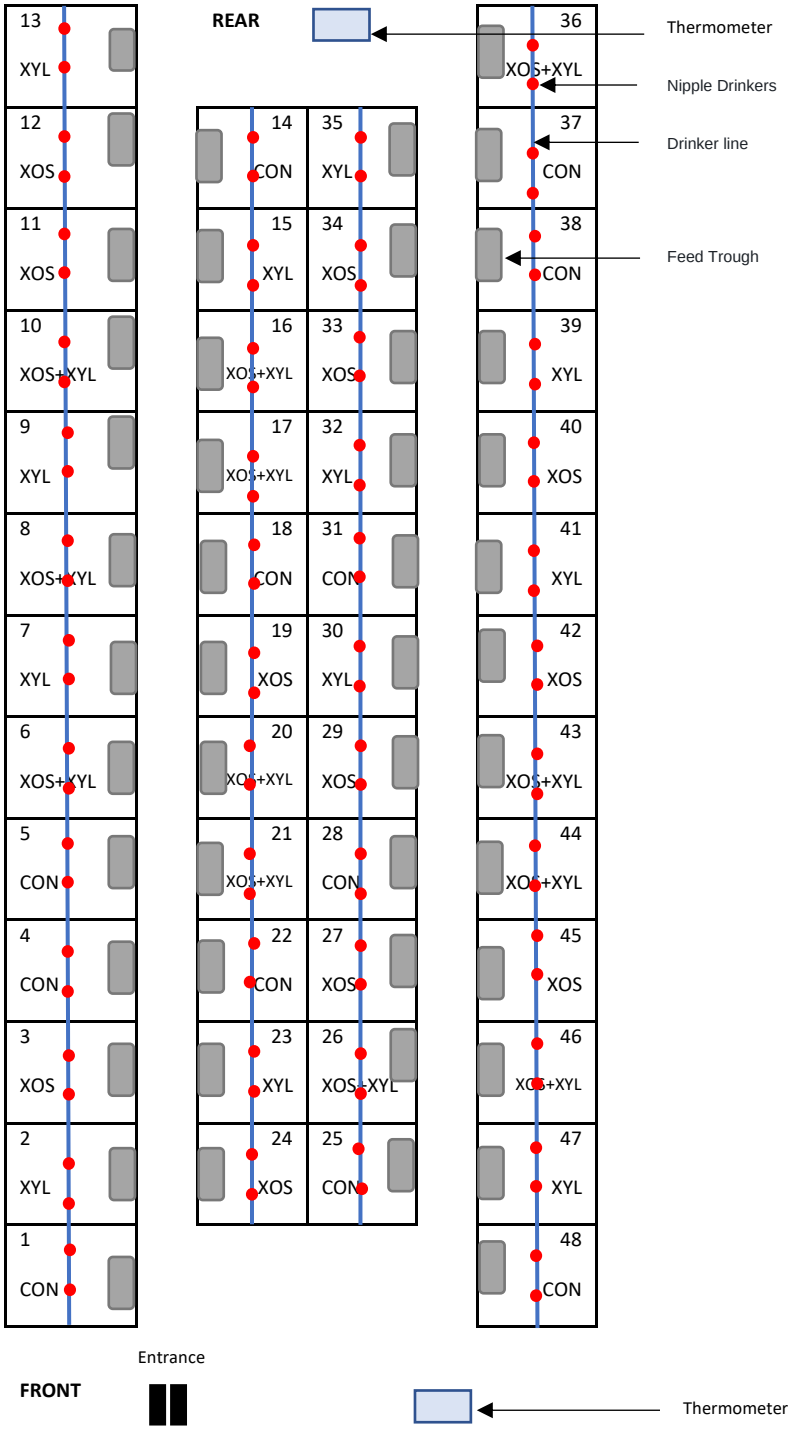


Figure 3.1.: Pen/Room layout with diet allocation

3.3.4. Determined parameters

Birds weight and feed intake per pen were recorded weekly on days 0, 8, 15, 22, 29 and 35.

The weekly FCR was calculated using this information for each phase. Three birds per pen on

day 8 and 1 bird per pen on days 22 and 35 with weights closest to the mean weight for the pen were selected and humanely killed via cervical dislocation. Caeca (one each for microbiome sequencing and determination of short chain fatty acids concentration) and a section of the ileum (for determination of mucin layer thickness and gene expression levels) were collected as described in sections 2.3.1 – 2.3.3 of chapter 2. Proximate analysis (protein, fat, dry matter and ash) of feed was done and Ca and P content was determined in house as described in sections 2.4.1 – 2.4.5 of chapter 2 while 500 g of feed was despatched to an external lab for gross energy, phytase and xylanase analysis.

3.3.5. Analysis of samples collected from birds

16S rRNA sequencing of caecal microbiota, determination of SCFA in caecal chyme and gene expression in ileal tissue was done according to sections 2.4.1 – 2.4.3 of Chapter 2.

3.3.6 Statistical analysis of data

Statistical analysis was performed in R version 3.6.1. Data was analysed using the 2 x 2 factorial arrangement. The additive or interactive effect of XOS or xylanase supplementation on the performance was analysed using the model

$$Yij = Mean + XOS(i) + XYL(j) + XOS*XYL(ij) + E(ij)$$

And the relative abundance of microbial taxa, gene expression in the ileum and SCFA concentrations in caeca was analysed using ANOVA according to the following model

$$Y(ijk) = Mean + Age(i) + XOS(j) + XYL(k) + Age*XOS(ij) + Age*XYL(ik) + XOS*XYL(jk) + Age*XOS*XYL(ijk) + E(ijk).$$

Where Y(ijk) is the response variable, Mean is the overall mean, Age(i) is the effect of bird age, XOS(j) is the effect of the XOS level, XYL(k) is the effect of the XYL level, Age*XOS(ij) is

the effect of the interaction between bird age and XOS, + Age*XYL(ik) is the effect of the interaction between bird age and XYL, XOS*XYL(jk) is the effect between XOS and XYL, Age*XOS*XYL(ijk) is the effect of the interaction among bird age, XOS and XYL and E(ijk) is the residual.

For microbial community dynamics (microbiota composition variability) between samples the relative abundance values at each taxonomic level were used in the above model and histograms were generated in excel.

Proportion of mortality was compared according to Altman *et al.,* (2000) at 0.05 level of significance. The z statistic for the difference between proportion was calculated using the flowing equation:

$$z = \frac{(\rho_1 - \rho_2) - 0}{\sqrt{\rho(1-\rho)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

Where ρ_1 is the proportion of mortality is one treatment group and ρ_2 is the proportion of the mortality is the other treatment group being compared and ρ proportion in the combined group (all the individuals in the first and second samples together). P value of z statistics was identified using z table.

Marker Data Profiling module of MicrobiomeAnalyst was used as described below for statistical, visual and meta-analysis of microbiome data (Chong *et al.*, 2020). Under data filtering, the low count and low variance (based on inter-quantile range) were both selected to be 10%. Data was neither rarefied nor transformed and total sum scaling (TSS) was selected for normalization. Both alpha and beta diversity metrics were used to estimate microbial community diversity. Species richness (Observed ASVs and Chao1) and evenness (Shannon

and Simpson index) were selected for alpha diversity estimations. To compare alpha diversity metrics among groups, non-parametric Kruskal-Wallis test was selected. For beta diversity analysis, dissimilarity matrix between samples Bray Curtis method was selected, and was further visualized with a Non-Metric Multidimensional Scaling (NMDS) ordination technique. Bray Curtis dissimilarity was also used to perform hierarchical clustering (dendrogram) with average as the clustering algorithm.

Linear Discriminant Analysis Effect Size (LEfSe) algorithm with LDA effect size threshold of 2 (on a log10 scale) was selected at genus level for evidencing potential biomarkers linked to age and diet. Core microbiome was identified for age at family and genus level and was defined as taxa detected in all age-groups with at least 80% prevalence within the group.

To identify the shared and unique ASVs among the age-groups, Venn diagrams were constructed with the online tool accessed through http://bioinformatics.psb.ugent.be/webtools/Venn/

3.4 Results

3.4.1 Diet Analysis

The measured values of macromolecules (fat, crude protein, dry matter, ash), minerals, energy and enzyme activities in the diets are represented in Table 3.2

Table 3.2.: Proximate analysis, energy and enzyme activity of starter, grower and finisher diets

Phase	Treatment	Gross energy (MJ/kg)	Ca (g/kg of feed as fed)	P (g/kg of feed as fed)	Crude protein (g/kg of feed as fed)	Dry Matter (g/kg of feed)	Ash (g/kg of feed as fed)	Fat (g/kg of feed as fed)	Phytase Activity (FTU/kg)	Xylanase Activity (BXU/kg)
Starter	Con	16.6	9.23	7.64	216	874	6.94	40.9	485	<2000
	XOS	16.6	7.92	6.83	215	871	6.34	38.7	587	<2000
	XYL	16.7	8.34	7.25	219	866	6.04	35.1	603	16100
	XOS + XYL	16.6	7.61	6.55	214	876	6.40	28.5	631	18600
Grower	Con	16.9	7.75	6.61	197	871	5.68	41.5	662	<2000
	XOS	16.9	7.84	6.64	203	872	5.52	45.6	783	<2000
	XYL	16.9	7.81	6.65	200	871	5.65	44.4	820	17000
	XOS + XYL	17.0	7.83	6.92	200	872	5.86	43.5	701	18100
Finisher	Con	17.0	10.2	7.36	213	872	4.80	56.7	715	<2000
	XOS	17.1	10.1	7.49	207	872	5.04	57.8	606	<2000
	XYL	17.0	7.73	6.55	189	873	4.68	55.8	502	17200
	XOS + XYL	17.3	8.63	7.10	175	872	4.20	53.5	568	19100

3.4.2 Environment

No environmental abnormalities occurred during this trial

3.4.3 Health and Condition

Mortality data shown in Table 3.3 demonstrates that there were no concerns regarding the health of the birds. There was no statistical difference in mortality between treatments over the entire trial period. Total flock mortality for the study was 3.4%, which is considered standard for trials conducted at the NTU poultry research unit and in line with the mortality rate on commercial farms which is expected to be no more than 5% (Red Tractor Standards: Broilers and Poussin, 2019).

Table 3.3. Bird mortality over the entire trial period

Treatment	No. of deaths
Control	1
XOS	6
XYL	4
XOS + XYL	3
Total Mortality	13
p	>0.05*
% Mortality	3.38

* proportion of mortality was compared according to Altman *et al* 2000 at 0.05 level of significance. p value of all comparisons were >0.05

3.4.4. Bird weight uniformity

The mean start weights are shown in Table 3.4. There was no statistical difference in the start weight of the chicks between treatments

Table 3.4.: The average weight of chicks on day 0

Treatment	D0 Body weight (g)
Control	43.9
XOS	43.7
XYL	43.9
XOS + XYL	43.9
SEM	
P value	0.991

SEM – stand error mean

3.4.5 Cumulative performance

To account for impact of sickness or mortality on mean pen feed intake and mean pen bird growth, pens with dead birds or birds culled due to sickness or lameness or runt birds were removed from the analysis of performance data for the entire week during which the

impacting event occurred. However, such pens were reintroduced in the statistical analysis of data if no deaths were recorded in subsequent weeks at the next weigh point. This approach of excluding the affected week was adopted rather than the "bird days approach" (where mean daily performance is calculated to allow adjustments for the number of birds in the pen) because the bird days approach does not account for the impact of illness in the days prior to death. Additionally, feed spillage from troughs affected data collection for this trial and outliers were removed following the process described in Chapter 2 section 2.2.1.3. Details on the outliers removed from the performance data are shown in Appendix C

Table 3.5 shows that when comparing the interaction means for the entire trial period birds fed diet containing XOS only had a significantly lower ($p < 0.05$) body weight gain compared to the other groups but there was no difference in feed intake or FCR. The diet supplemented with both XOS and XYL together produced numerically heavier birds and improved the FCR compared to the control. The a main effect of xylanase ($p < 0.05$) for body weight gain was significantly higher but not for feed intake or FCR

Table 3.5.: Performance of birds over the entire trial period (D0 – 35)

	BWG (g/bird)	FI (g/bird)	FCR*
Main effect			
XOS			
0 g XOS /tonne	2057	3222	1.57
100 g XOS/ tonne	2036	3258	1.61
XYL			
0 g XYL/tonne	1994[b]	3232	1.62
100 g XYL /tonne	2099[a]	3247	1.55
Interaction[1]			
CON	2033[a]	3244	1.61
XOS	1955[b]	3221	1.65
XYL	2081[a]	3199	1.54
XOS + XYL	2117[a]	3296	1.56
SEM[2]	49.75	83.90	0.037
P values[3]			
XOS	0.616	0.688	0.306
XYL	0.045	0.868	0.056
XOS*XYL	0.026	0.502	0.759

[a,b] different superscripts with the same column indicate means that are significantly ($P < 0.05$) different.

[1] Interaction means from replicate pens of treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] standard error mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

*mortality corrected

3.4.6 Weekly Performance

On d22 there was XOS*XYL interaction for body weights with birds fed a XOS only supplemented diet having a significant lower ($p < 0.05$) body weight compared to the birds fed a non-supplemented diet or diet supplemented with xylanase or their combination. There was no XOS*XYL interaction at any other time point. However there was main effect of XYL on body weight at d35, the xylanase fed birds being significantly heavier ($p < 0.05$) than birds fed diets without xylanase.

Table 3.6.: Weekly average body weight (g) of birds fed XOS and xylanase alone and in combination

	D8	D14	D22	D28	D35
Main effect					
XOS					
0 g XOS /tonne	152	332	808	1420	2085
100 g XOS/ tonne	151	325	778	1388	2074
XYL					
0 g XYL/tonne	154	327	774	1367	2022[b]
100 g XYL /tonne	150	330	812	1441	2137[a]
Interaction[1]					
CON	157	336	825[a]	1420	2046
XOS	150	318	724[b]	1314	1999
XYL	148	328	791[a]	1421	2125
XOS + XYL	152	332	832[a]	1462	2148
SEM[2]	5.04	14.0	27.5	45.0	51.3
P values[3]					
XOS	0.831	0.627	0.280	0.471	0.821
XYL	0.447	0.814	0.182	0.103	0.031
XOS * XYL	0.273	0.450	0.012	0.110	0.501

[a,b] different superscripts with the same column indicate means that are significantly ($P < 0.05$) different

[1] Interaction means from replicate pens of treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] pooled standard error of mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

Similar to body weights, weight gain results also show that there was significant XOS*XYL interaction for weight gain at d22. The average weekly body weight gain was non significantly higher in the XOS+XYL group from the third week onwards (Table 3.7). Like main effect of xylanase for body weight at d35, the weight gain too was significantly higher.

Table 3.7: Weekly body weight gain (g/bird) of birds fed XOS and xylanase alone and in combination

	D0-8	D8-14	D14-22	D22-28	D28-35
Main effect					
XOS					
0 g XOS /tonne	111	178	476	629	674
100 g XOS/ tonne	107	174	464	621	655
XYL					
0 g XYL/tonne	110	174	458	610	646[b]
100 g XYL /tonne	108	178	481	641	683[a]
Interaction[1]					
CON	113	177	489[a]	614	635
XOS	107	171	427[b]	606	658
XYL	109	178	462[a]	645	676
XOS + XYL	108	177	500[a]	636	690
SEM[2]	4.170	10.15	15.42	22.17	14.62
P values[3]					
XOS	0.418	0.745	0.504	0.713	0.616
XYL	0.745	0.759	0.171	0.215	0.045
XOS * XYL	0.737	0.967	0.011	0.189	0.078

[a,b] different superscripts with the same column indicate means that are significantly ($P < 0.05$) different

[1] Interaction means from replicate pens of treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] pooled standard error of mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

There was no effect of XOS or XYL supplementation on feed intake when compared to diets that lacked the corresponding additive. When comparing the means for interaction, the diets supplemented with XOS alone had a significant lower intake compared to the other groups during the third week of the trial (Table 3.8). No interaction was observed at any other time point.

Table 3.8: Weekly feed intake (g/bird) of birds fed XOS and xylanase alone and in combination

	D0-8	D8-14	D14-22	D22-28	D28-35
Main effect					
XOS					
0 g XOS /tonne	173	306	665	996	3222
100 g XOS/ tonne	165	293	684	987	3258
XYL					
0 g XYL/tonne	173	306	665	996	1068
100 g XYL /tonne	165	293	684	986	1073
Interaction[1]					
CON	166	308	674[a]	1015	1061
XOS	161	287	621[b]	987	1074
XYL	180	304	656[ab]	978	1062
XOS + XYL	168	299	748[a]	986	1084
SEM[2]	8.40	17.62	25.67	38.35	20.77
P values[3]					
XOS	0.292	0.470	0.463	0.844	0.831
XYL	0.255	0.816	0.055	0.631	0.429
XOS * XYL	0.456	0.854	0.012	0.921	0.866

[a,b] different superscripts with the same column indicate means that are significantly ($P < 0.05$) different

[1] Interaction means from replicate pens of treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] standard error mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

Birds fed on xylanase supplemented diets converted feed more efficiently during the last two weeks although this effect was not significant (Table 3.9). There was no XOS * XYL interaction at any time point for FCR (Table 3.9).

Table 3.9 Weekly feed conversion ratio (FCR) of birds fed XOS and xylanase alone and in combination

	D0-8	D8-14	D14-22	D22-28	D28-35
Main effect					
XOS					
0 g XOS /tonne	1.58	1.72	1.40	1.58	1.63
100 g XOS/ tonne	1.55	1.69	1.48	1.58	1.60
XYL					
0 g XYL/tonne	1.50	1.72	1.42	1.63	1.64
100 g XYL /tonne	1.63	1.70	1.46	1.53	1.58
Interaction[1]					
CON	1.47	1.69	1.38	1.64	1.68
XOS	1.53	1.75	1.47	1.63	1.62
XYL	1.69	1.70	1.42	1.52	1.59
XOS + XYL	1.57	1.69	1.50	1.55	1.58
SEM[2]	0.09	0.09	0.05	0.05	0.03
P values[3]					
XOS	0.70	0.77	0.11	0.84	0.28
XYL	0.18	0.77	0.51	0.054	0.06
XOS * XYL	0.38	0.96	0.13	0.27	0.17

[1] Interaction means from replicate pens of treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] standard error mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

3.4.7 Measurement of mucin layer thickness

There was no significant difference in the main effects or XOS * XYL interaction for thickness of the mucin layer at the measured time points (Table 3.10)

Table 3.10. Mucin layer thickness (µg of alcian blue released per gram of tissue)

	D8	D22	D35
Main effect			
XOS			
0 g XOS /tonne	166	54.3	105
100 g XOS/ tonne	167	48.6	110
XYL			
0 g XYL/tonne	173	54.1	106
100 g XYL /tonne	160	48.8	108
Treatment[1]			
CON	169.9	60.53	98.68
XOS	176.8	47.74	113.6
XYL	162.5	48.14	110.3
XOS + XYL	157.2	49.49	106.4
SEM[2]	13.47	6.965	15.20
P values[3]			
XOS	0.953	0.432	0.708
XYL	0.352	0.464	0.894
XOS * XYL	0.785	0.550	0.910

[1] Interaction means from replicate pens of treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] standard error mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

3.4.8 Gene expression levels in day-35 old broilers

3.4.8.1 Quality and quantity of extracted RNA

The ratio of 260/280 is commonly used as an indicator of the purity of RNA in relation to DNA contamination. A ratio of ~2.0 is generally accepted as pure for RNA. For this trial the extracted RNA from all samples had a 260/280 ratio between 1.95 – 2.09. In addition, the RNA integrity of each sample was assessed using agarose gel electrophoresis. On visualising the gel all the samples showed two distinct intact bands of 28S and 18S rRNA indicating good quality of the extracted RNA (Figure 3.2). In addition, the majority 260/230 ratios were also found to be in the range of 2.2 -2.5 which is used as a secondary measure of nucleic acid purity (See Appendix B)

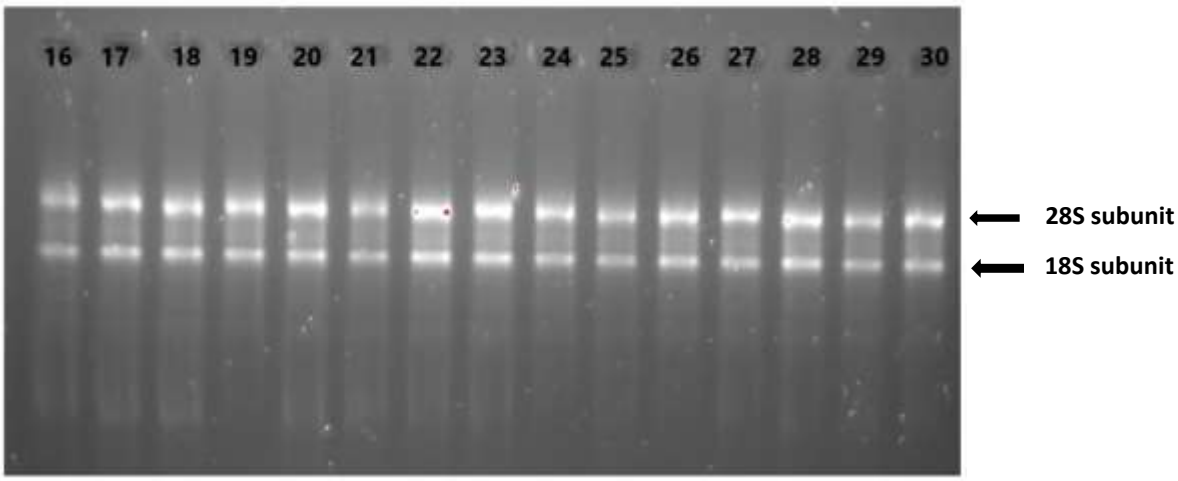


Figure 3.2: An agarose gel electrophoresis image of extracted RNA (pens 16 – 30) to check the quality of RNA. The two bands represent the 28S and 18S subunits of rRNA. Both bands are intact indicating good quality RNA.

3.4.8.2 Effect of XOS supplementation alone and in combination with xylanase on the mRNA expression of gut barrier genes and the short chain fatty acid receptor gene in the ileum of 35-day-old broilers

The gene expression of *MUC2*, *sIgA*, *CLDN1, CLDN5, OCLN, IL-1β* and *FFAR2* are shown in Table 3.11. XOS + XYL interactions were not significantly different for any of the genes studied. Birds in the XOS + XYL group had numerically higher expression of the FFAR2 gene compared to the CON group and numerically lower expression of all gut barrier genes except CLDN5. There was no statistical difference for the main effects for XOS or XYL.

Table 3.11: Effect of dietary inclusion of XOS and xylanse alone and their in combination on relative (to GAPDH) gene expression ratios of ileal mucosa barrier genes and Short Chain Fatty Acid receptor gene of 35-day-old broilers raised under optimal conditions

	MUC2	*sIgA*	*CLDN1*	*CLDN5*	*OCLN*	*IL-1β*	*FFAR2*
Main effect							
XOS							
0 g XOS /tonne	1.197	1.047	1.487	1.085	1.034	1.051	1.919
100 g XOS/ tonne	0.951	0.770	0.291	1.081	1.004	0.966	1.216
XYL							
0 g XYL/tonne	1.084	0.926	1.133	0.990	1.098	1.037	1.217
100 g XYL /tonne	1.065	0.891	0.645	1.175	0.939	0.980	1.918
Interaction[1]							
CON	1.080	1.096	1.946	1.041	1.055	1.043	1.051
XOS	1.087	0.756	0.319	0.940	1.140	1.032	1.383
XYL	1.314	0.997	1.029	1.128	1.012	1.060	2.787
XOS + XYL	0.816	0.785	0.262	1.222	0.867	0.899	1.049
SEM[2]	0.32	0.21	0.73	0.13	0.21	0.14	0.59
P values[3]							
XOS	0.463	0.207	0.126	0.975	0.892	0.549	0.255
XYL	0.954	0.868	0.516	0.169	0.474	0.687	0.256
XOS * XYL	0.451	0.764	0.566	0.455	0.603	0.6	0.104

MUC2 = mucin 2; *sIgA* = secretory immunoglobulin A; *CLDN1* = claudin-1; *CLDN5* = claudin-5; *OCLN* = occludin, *IL-1β* = interleukin-1β, *FFAR2* = free fatty acid receptor-2

[1] Interaction means from 4 pooled replicate pens per treatment. Treatments include: control = no additions (CON), 100 g xylo-oligosaccharide /tonne diet (XOS), xylanase 100 g/ tonne diet (XYL) and combination of xylo-oligosaccharide + xylanase (XOS+XYL)

[2] standard error mean

[3] 2-way Anova XOS+ XYL + XOS*XYL+e

3.4.9 16S rRNA Sequencing of Caecal Microbiota: temporal changes and effects of dietary treatment

3.4.9.1 Sequencing output, pre-processing and taxonomic assignment.

After the initial filtering and adaptors trimming process, a total of 5,678,162 read-pairs with a median of 61,154 read pairs per sample (IQR: 50484 - 71963). Median length of the reads per sample was 300 bp (IQR: 251 – 301). After pre-processing and removal of low-count ASVs as described in section 2.2.4.1.4 of Chapter 2, 2,251,454 amplicon reads from the 93 samples were classified into 674 taxa.

The Number of reads that passed through each step of the DADA2 pipeline are presented in Table 3.12.

Rarefaction curves generated from the ASVs approach a plateau indicating sufficient sequencing depths in all samples (Figure 3.3). Chicken-to-chicken variation was observed in each age-group. The rarefaction curve patterns showed similarity within each age group rather than diet type (Figure 3.3).

3.4.9.2 Alpha Diversity

A steady increase in species richness was observed as the chickens aged, as indicated by Observed ASV and Chao1 (Figure 3.4). Both estimators showed similar patterns reassuring that the sequencing depth obtained was sufficient. The average Shannon and Simpson indices values were lowest in youngest chickens, indicating that the species present were not equally abundant, and reached the highest values at day 35, suggesting that the abundance of the different species was then more even. Kruskal-Wallis tests of Richness, and Evenness indicated that bacterial diversity in chickens significantly differed ($p < 0.001$) from one age-group to another (Figure 3.4A).

None of the alpha diversity indices were statistically different between diets, neither when the diet effect was analysed in the whole dataset (Figure 3.4B) nor when restricting the dietary comparison to subsets of age-groups only and thereby controlling for the age effect i.e there was absence of age x diet interaction (data not shown).

3.4.9.3 Beta Diversity

The overall microbial community structure exhibited significant shifts by age (total variance explained $R^2 = 0.30$, $p < 0.001$) based on PERMANOVA. Betadisper revealed that individual variation in the community structure was significantly greater in younger birds (8-day-old), while the lowest variance was found among 35-day-old broilers ($F = 19.61$, $p < 0.001$). Despite the lack of homogeneity of multivariate dispersion (deviation from centroid), the NMDS plot based on Bray-Curtis dissimilarity matrix still showed age-related clustering, with chickens of the same age clustering more closely together than those from different ages (Figure 3.5A). Nineteen of the 22-day-old broilers shared

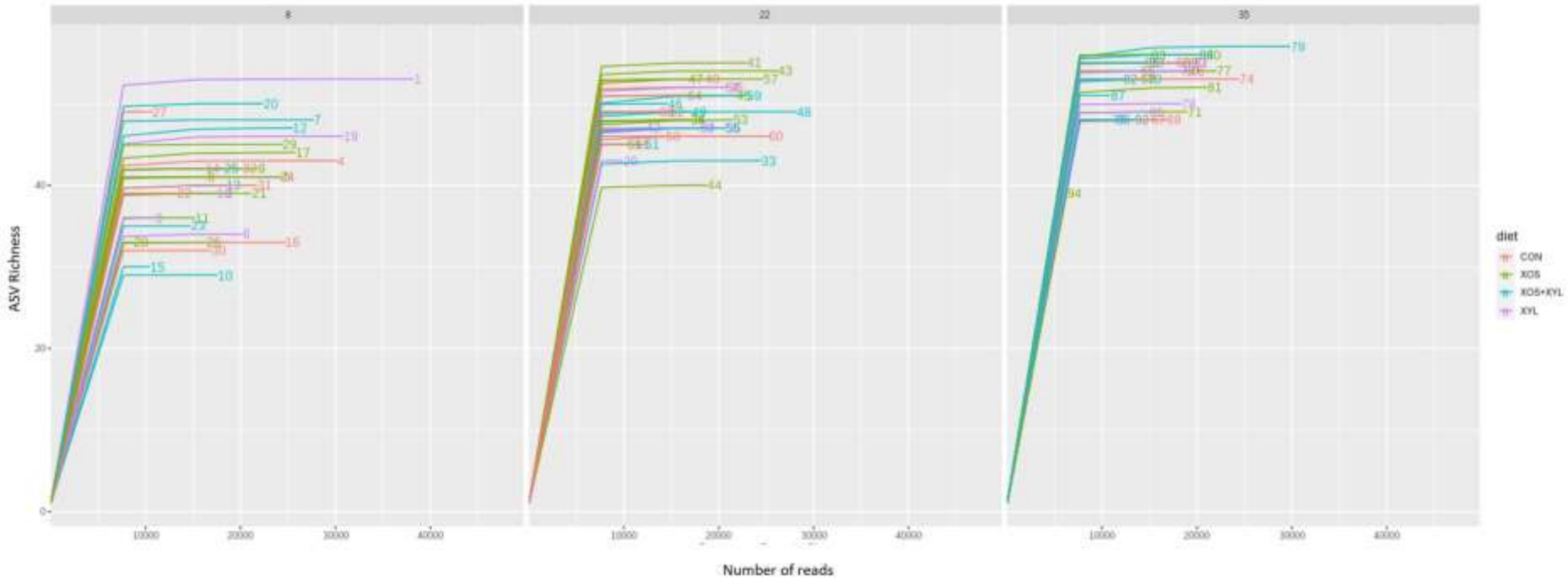
similar community structure with 35-day-old birds and of these 15 also shared a similarity in community structure with 8-day-old birds.

In contrast, diet was not a significant factor either for the whole dataset (R^2 = 0.023, p = 0.782) (Figure 3.5B) or when comparing different diets within age groups (d8 R^2 = 0.068, p= 0.794; d22 R^2 = 0.093, p = 0.565; d35 R^2 = 0.108, p = 0.41). These results indicate that the chickens shared a core set of microbiota in the cecum regardless of the dietary supplementation.

Table 3.12: Number of reads that passed through each step of the pipeline in DADA2

Factor	n	Input		Filtered		Denoised		Merged		Non-chimeric	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
By Age (days)											
8	32	1,944,980	16827	1,733,446	14469	1,689,358	14217	1,330,044	11695	789,596	7,636
22	31	2,004,044	14393	1,798,976	12507	1,740,244	12320	1,276,192	10264	765,730	5,637
35	30	1,728,937	15686	1,546,164	13813	1,484,761	13452	1,050,003	10310	696,128	6,128
By Treatment											
CON	23	62,382	15,320	55,792	13,307	54,003	13,085	40,167	10,842	24,363	6,007
XOS	28	61,316	15,649	54,839	13,661	53,051	13,414	39,292	10,942	24,040	5,977
XYL	19	62,007	15,214	55,460	13,208	53,686	12,964	40,009	10,686	24,541	6,371
XOS+XYL	23	60,822	15,412	54,417	13,469	52,622	13,230	38,867	10,827	23,924	5,991

A



B

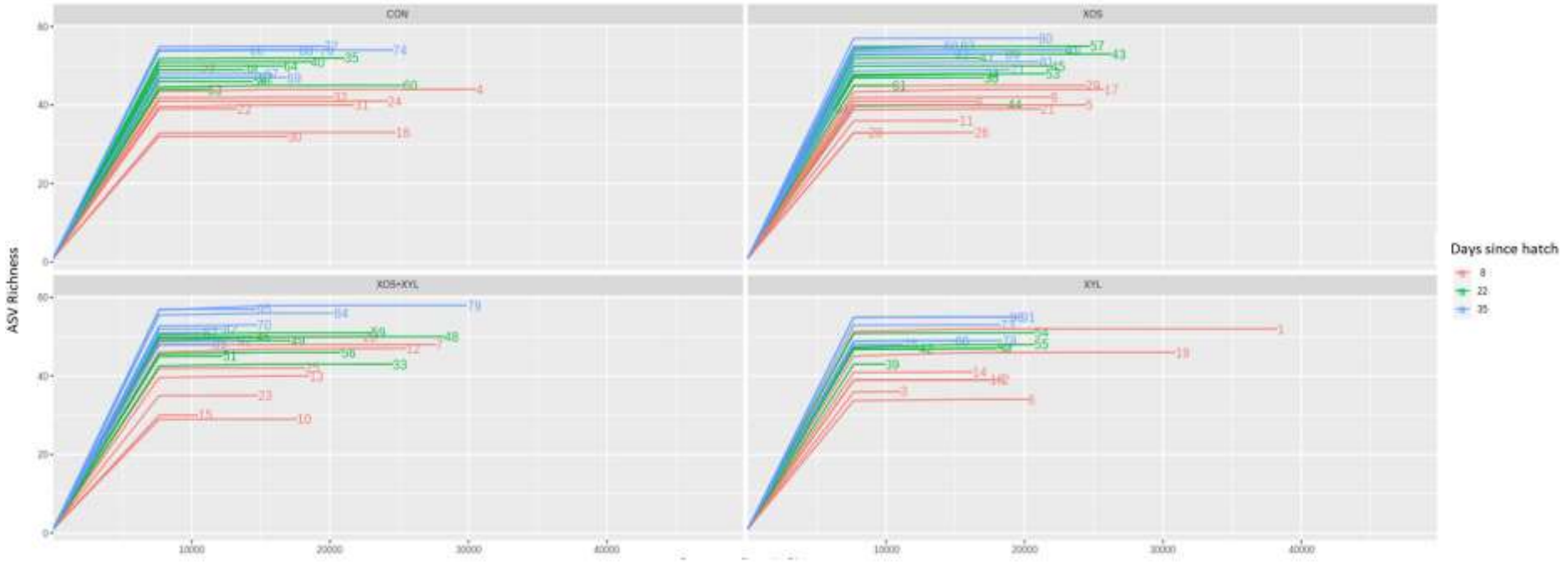
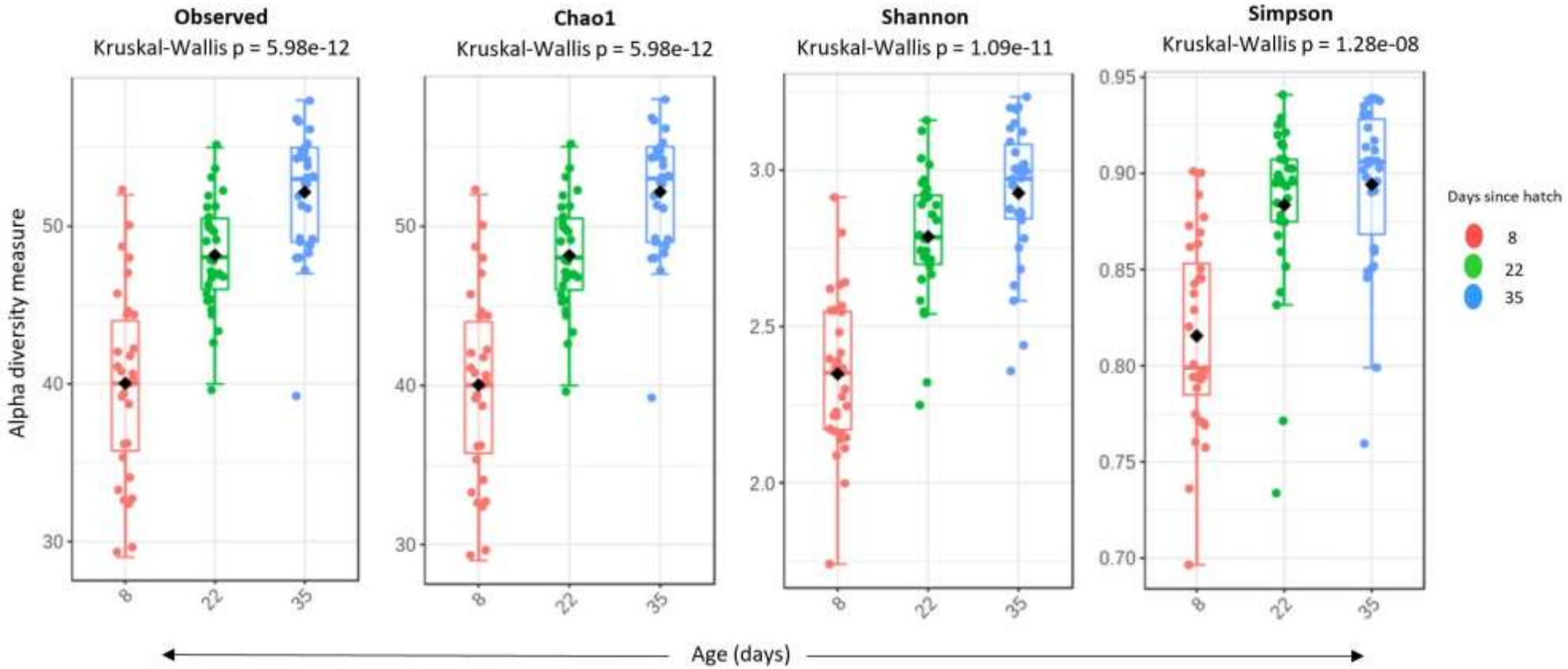


Figure 3.3: Rarefaction curves plotting the number of observed ASVs over the number of sequencing reads per sample according to age (A) and according to diet type (B) (numbers next to the curves are sample numbers in the order of loading on a 96-well plate during Illumina sequencing library preparation)

A)


Observed
Kruskal-Wallis p = 5.98e-12
Chao1
Kruskal-Wallis p = 5.98e-12
Shannon
Kruskal-Wallis p = 1.09e-11
Simpson
Kruskal-Wallis p = 1.28e-08
Alpha diversity measure
Days since hatch
8
22
35
Age (days)

B)

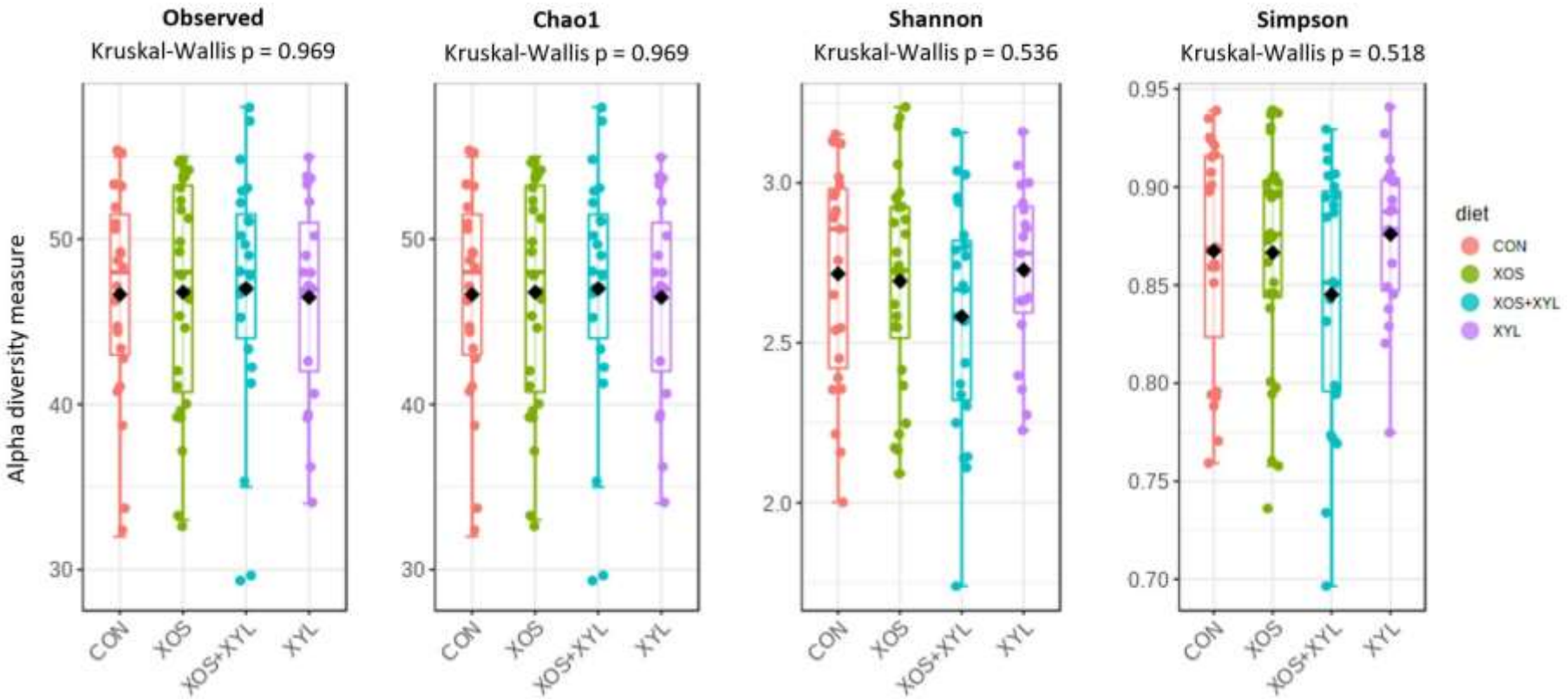
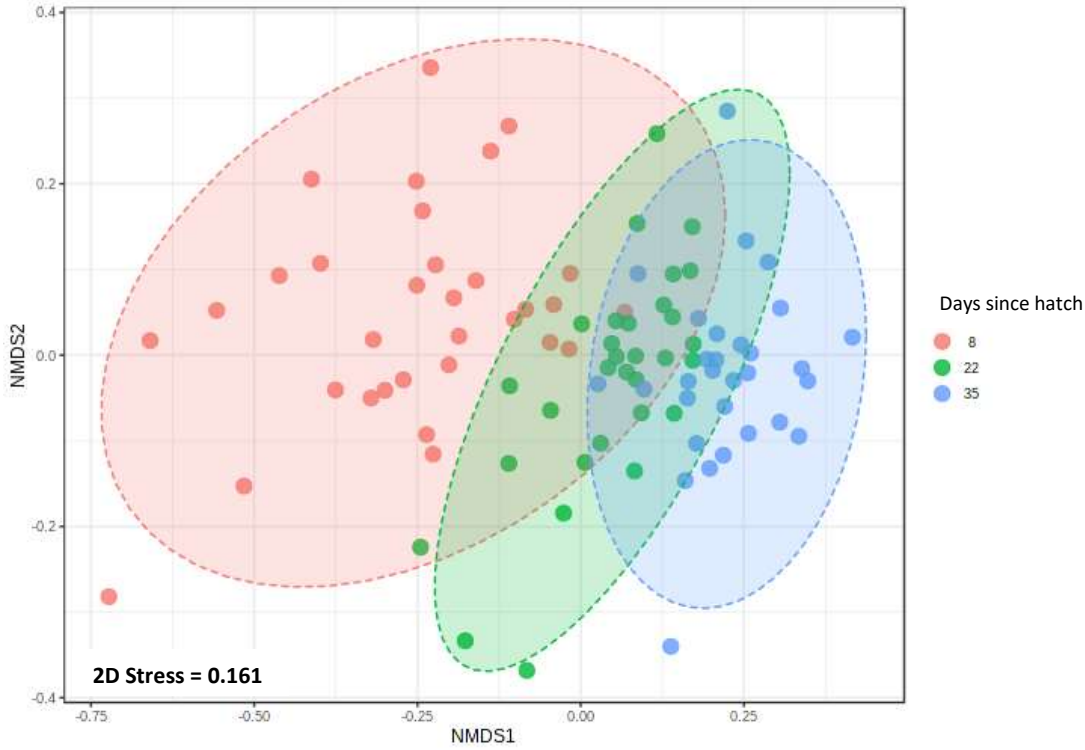
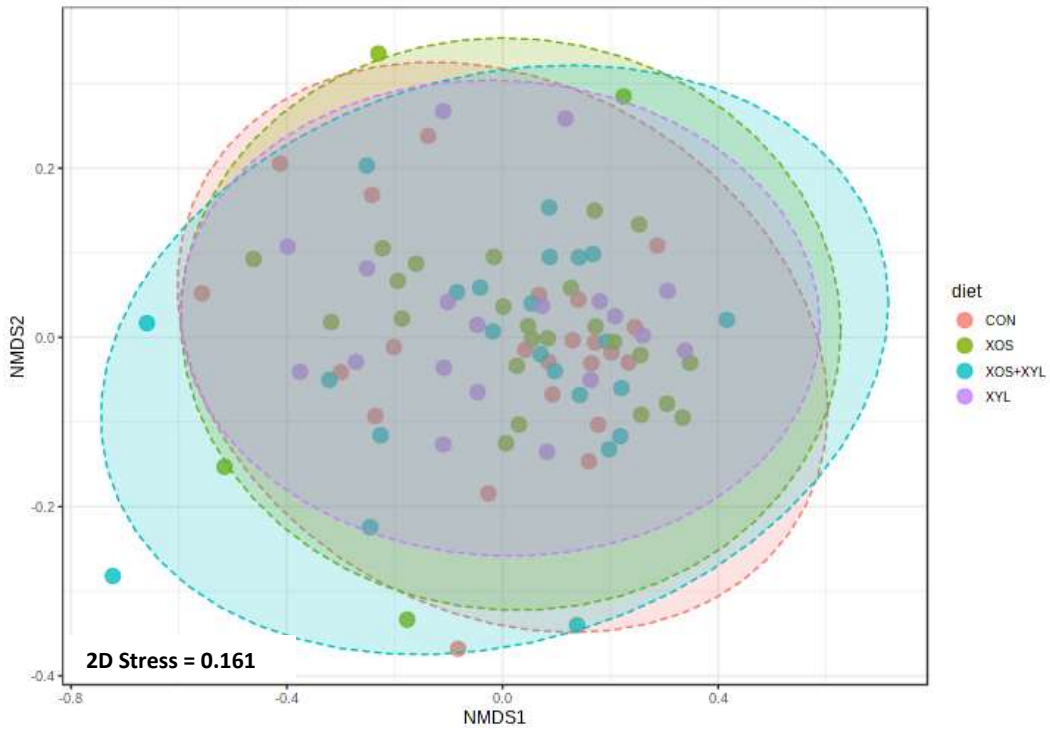


Figure 3.4. Boxplots representing alpha diversity metrics of richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson) grouped according to age (A) and diet type (B). Non-parametric Kruskal-Wallis test for multiple comparisons was conducted. Each point represents the diversity score for a sample and points are colour-coded according to age (3.4A) or diet type (3.4B). The box represents the first (Q1) and third (Q3) quartiles of the distribution and the line within the box marks the median. The whiskers extend from Q1 to Q3 to the last data points and values beyond these whiskers are considered as outliers.

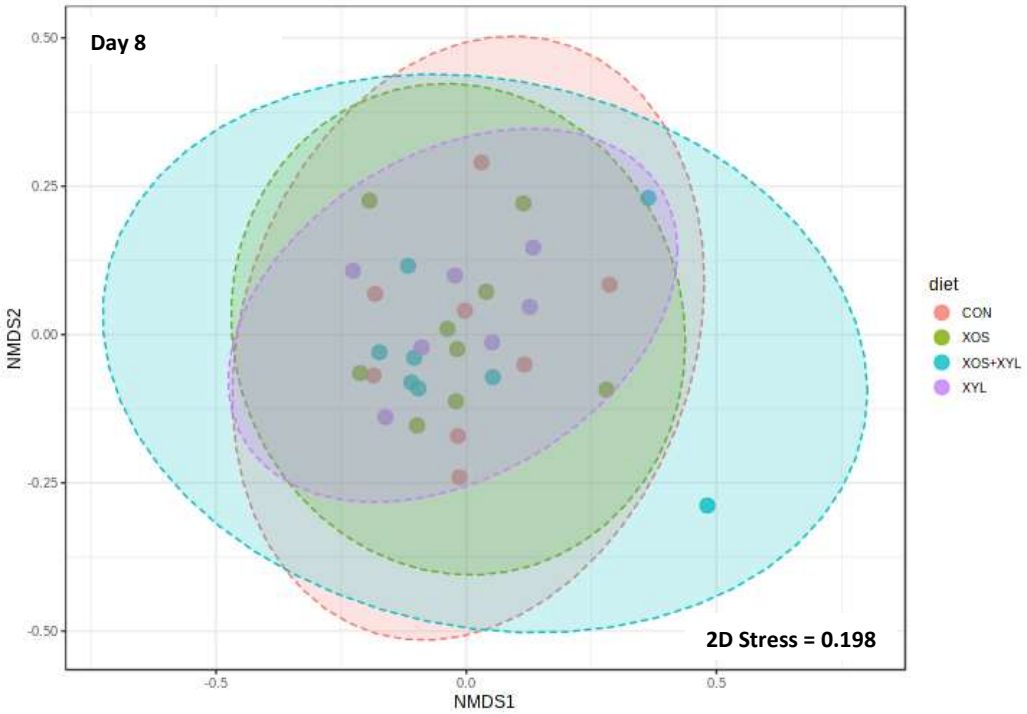
A)


NMDS2
NMDS1
2D Stress = 0.161
Days since hatch
8
22
35

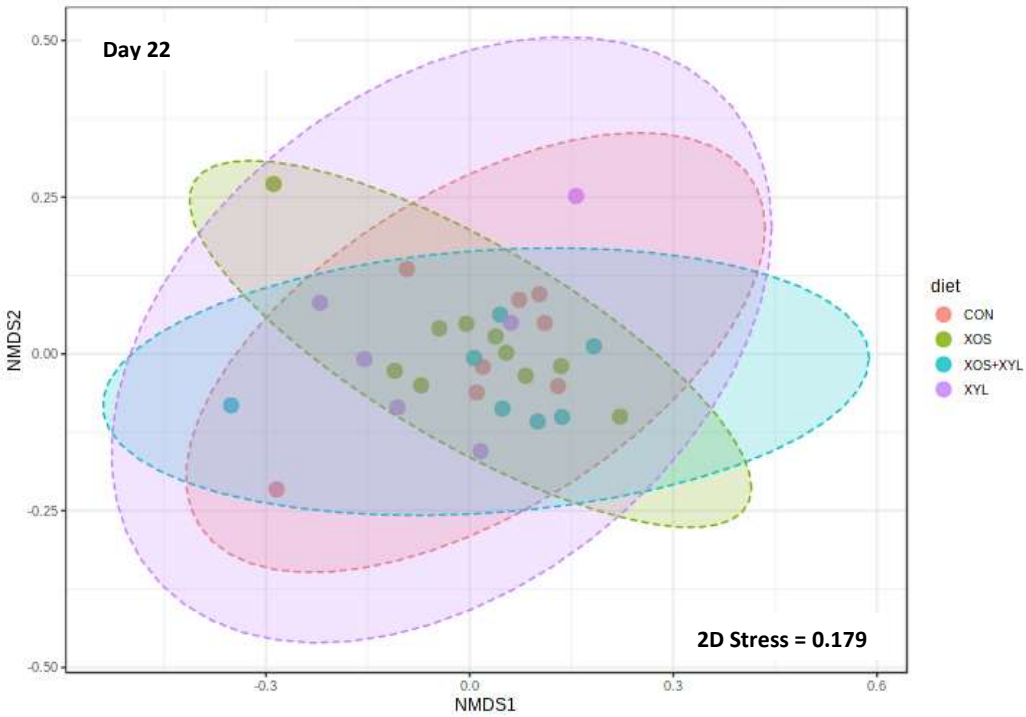
B)


NMDS2
NMDS1
2D Stress = 0.161
diet
CON
XOS
XOS+XYL
XYL

C)


Day 8
0.50
0.25
0.00
-0.25
-0.50
NMDS2
-0.5
0.0
0.5
NMDS1
2D Stress = 0.198
diet
CON
XOS
XOS+XYL
XYL

D)


Day 22
0.50
0.25
0.00
-0.25
-0.50
NMDS2
-0.3
0.0
0.3
0.6
NMDS1
2D Stress = 0.179
diet
CON
XOS
XOS+XYL
XYL

E)

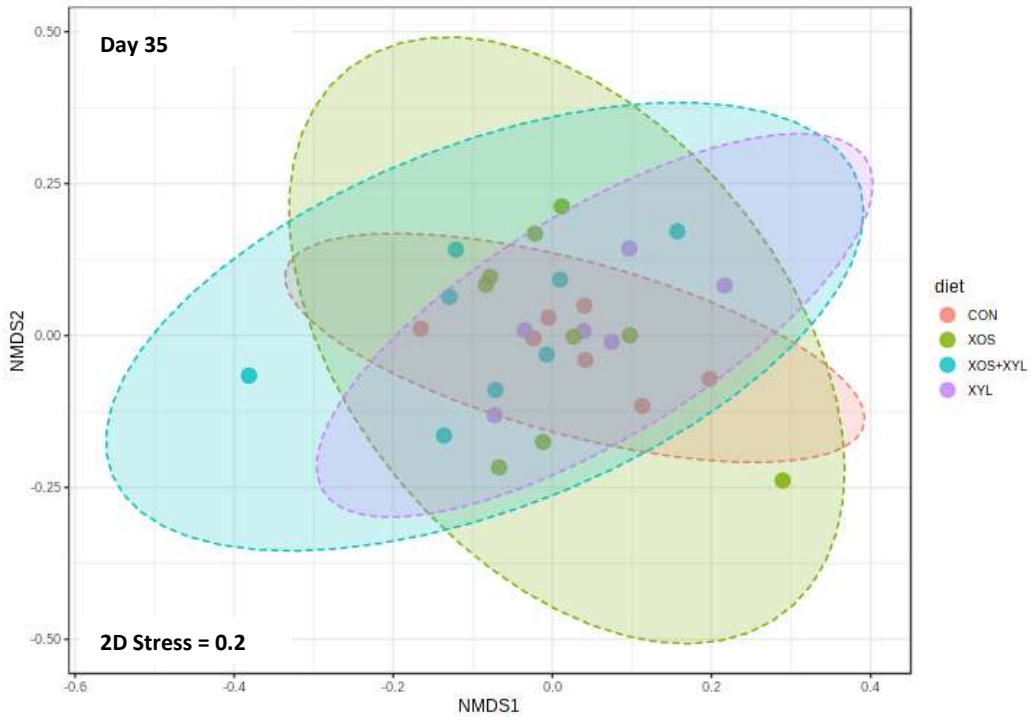


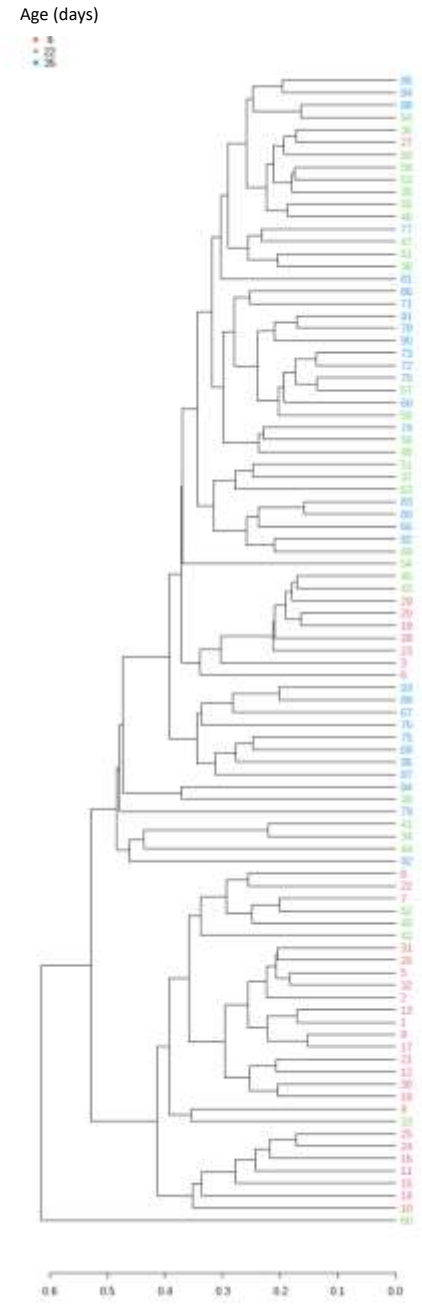
Figure 3.5.: Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity matrix on relative abundance data for age (A) or diet type (B) or diet type at age 8-days (C), 22-days (D) or 35-days (E). Colours indicate age groups (A) or diet type (B, C, D, F). Ellipses indicate 95% confidence intervals of multivariate t-distribution around centroids of the groupings with age (A) or diet (B, C, D, F) as factor.

3.4.9.4 Hierarchical clustering

In agreement with the results observed in NMDS plots, dendrogram of hierarchical clustering revealed that samples from 8, 22 and 35 day-old broilers formed three separate clusters with samples from 22 day-old broilers clustering closer to 35 day-old than to 8 day-old broilers (Figure. 3.6A). On the other hand, samples from 22 day-old broilers grouped with both the 8-day-old and the 35-day-old broilers, with no clear predominant cluster defined.

Again, no diet-related clustering was observed, neither in the whole dataset (Figure 3.6B) nor within subsets of age-groups (data not shown).

A



B

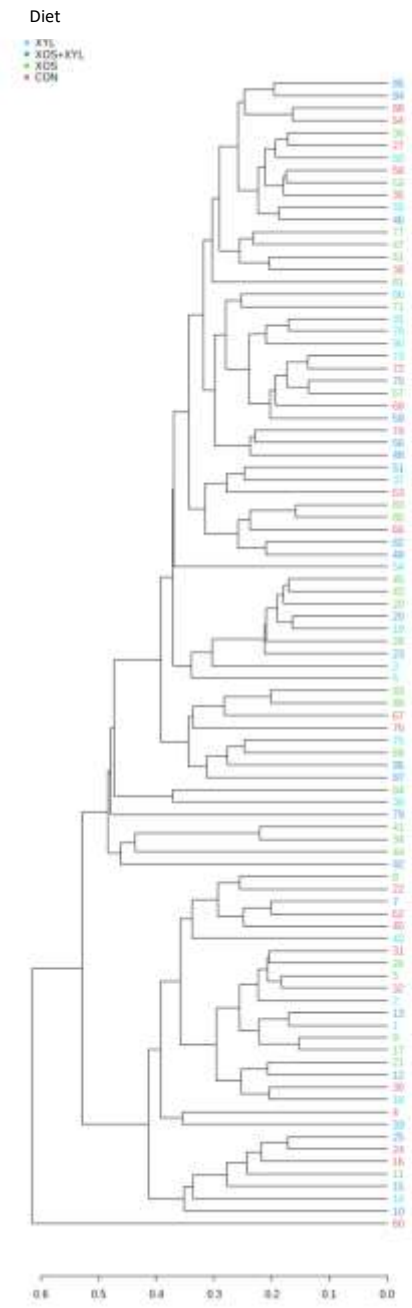


Figure 3.6.: Hierarchical clustering analysis: Dendrogram of Bray-Curtis dissimilarity matrices between samples based on different age groups (A) and diet type (B)

3.4.9.5 Microbial community dynamics

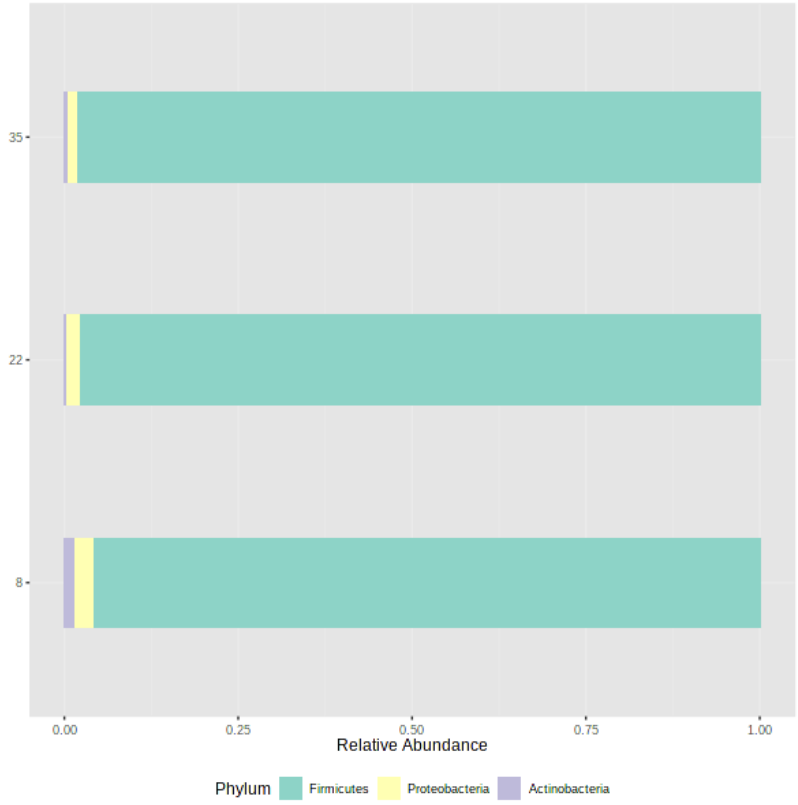
There was no significant difference in the relative proportions of microbial communities according to age or diet at the phylum level when comparing the whole data set (Figure 3.7A & B) or when comparing different diets within age-groups (Figure 3.7C). Microbial taxa consistently present over

time (core microbiome) were represented by 61 ASVs and those present in every treatment group were represented by 71 ASVs (Figure 3.10).

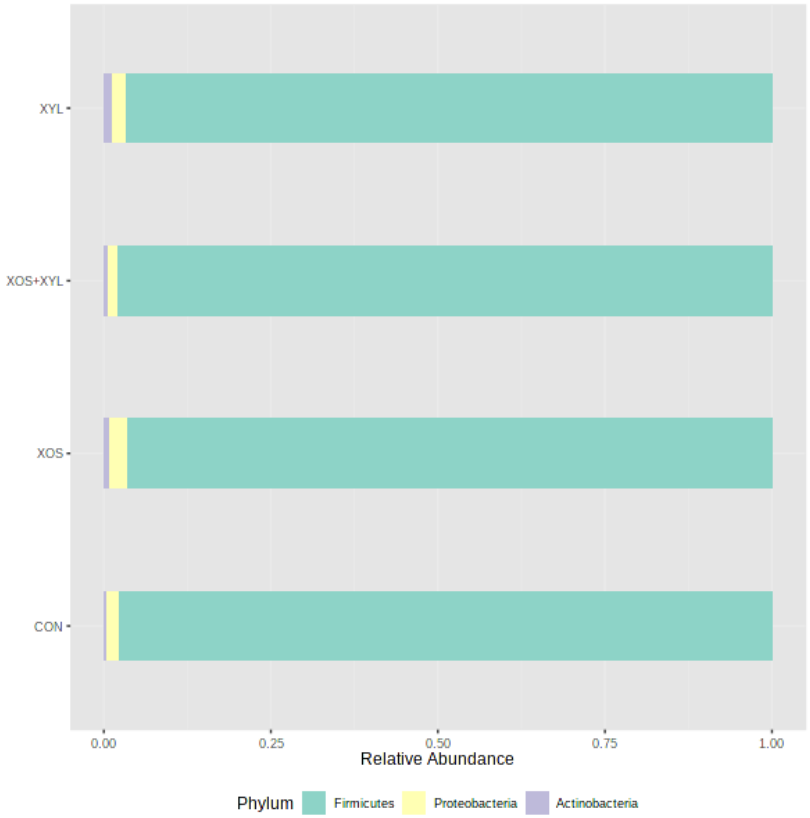
At phylum level, Firmicutes (>95%) formed almost the entirety of the caecal microbiome in all time points and treatment groups together with minor phyla Proteobacteria and Actinobacteria.

There was no significant difference in the proportion of either Firmicutes, Proteobacteria or Actinobacteria due to supplementation of XOS or xylanase, alone or in combination.

A



B)



C)

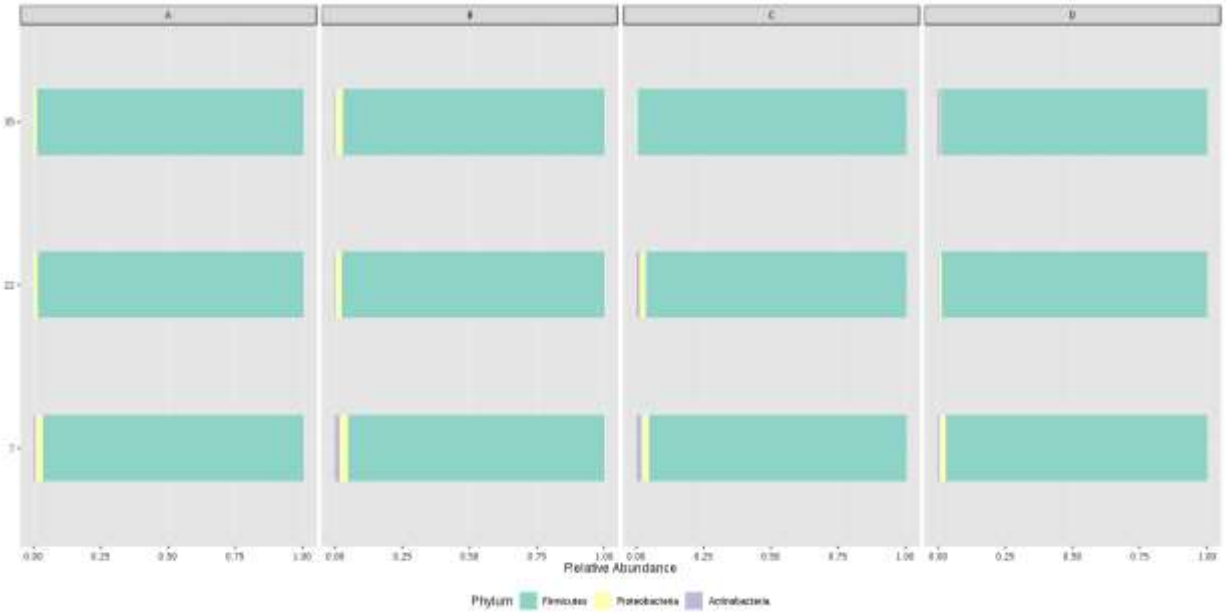


Figure 3.7.: Microbial community composition of chicken caecal content at the phylum level. Stacked bar plots representing relative abundances of the different phyla in all samples according to age (A) and diet type (B) in whole data set and comparing diets within age groups (C)

Firmicutes was dominated by families within the order Clostridiales (Ruminococcaceae and Lachnospiraceae) whose relative abundance varied with age but they formed a part of the core microbiome (Figure 3.11 and Table 3.13). In fact, most of the genera identified at every sampling point and in every treatment group, belonged to the order Clostridiales although there was no significant difference ($p > 0.05$) in the relative abundance of Clostridiales due to the dietary supplementations. Clostridiales was present at important levels throughout the entire trial period but highest proportions were detected in 35 day -old birds (89%). In all age groups this order was composed mainly of families Ruminococcaecae and Lachnospiraceae. While the relative abundance of Ruminococcaceae increased with age (42% at day 8 to 72% at day 35) that of Lachnospiracecae remained fairly steady at all time points (13.6% at day 8, 15.2% at day 22 and 14% at day 35). The relative abundance of Ruminococcaceae was numerically higher in diet supplemented with XOS + XYL while Lachnospiraceae was numerically higher in the xylanase group. *Blautia* was the top genera within the Lachnospiraceae family and was present at all sampling points followed by genera *Fusicatanibacter*. *Subdoligranulum, Faecalibacterium, Ruminococcaceae_UCG_014, Ruminiclostridium_5* of Ruminococacceae were among the top genera and were observed throughout the trial period. In fact, these four genera were identified as biomarkers of at least one of the three age groups (Figure 3.11) but none were significantly different when comparing the effect of treatment.

Lactobacilliales was the next dominant order after Clostridiales in the phylum Firmicutes but unlike Clostridiales their relative abundance almost halved at every sampling point, from 34% at day 8 to 16% at day 22 to 7.5% at day 35. Lactobacillaceae was the most depleted family as Ruminococcaceae and Lachnospiraceae gained importance. *Lactobacillus* genus was present as the core microbiome, was a biomarker of the youngest birds (LDA effect size > 6, Figure 3.11). The relative abundance of *Lactobacillus* was highest in the XOS supplemented group 19%, compared to 16.5% in the control and xylanase groups and 15.6% in the XOS but these differences were not statistically significant. Streptococcaceae (another family of Lactobacilliales) was also a part of the core microbiome with

Streptococcus accounting for nearly 6% of bacteria at day 22. Like Lactobacillus the relative abundance of *Streptococcus* was highest in the XOS supplemented diet.

Enterobacteriales formed all of the Proteobacteria and their relative abundance remained fairly similar throughout the entire trial period. Enterobacteriaceae was a part of the core microbiome and was represented by genera *Escherichia_Shigella* in all age groups. *Escheria_Shigella* was also identified as a biomarker of 8 day-old chicks (LDA effect size > 6, Figure 3.11). Amongst treatment groups, the highest relative abundance of Enterobacteriaceae and thus *Escherichia_Shigella*, was observed in the XOS group (2.5%) while lowest was found in the combination of XOS and xylanase (1.2%).

Bifidobacteriales amongst Actinobacteria were dominant in younger chickens but their relative abundance declined with age and they represented only 0.4% of the bacteria at the end of the trial. Bifidobacteriales was represented by only one genus, *Bifidobacteria* at very low proportions, ~1% in chicks and 0.3-0.4% in older birds. None of the treatment groups significantly enhanced the relative abundance of the beneficial *Bifidobacteria* compared to the control (Control – 0.3%, XOS – 0.7%, XYL – 1% and XOS + XYL – 0.6%)

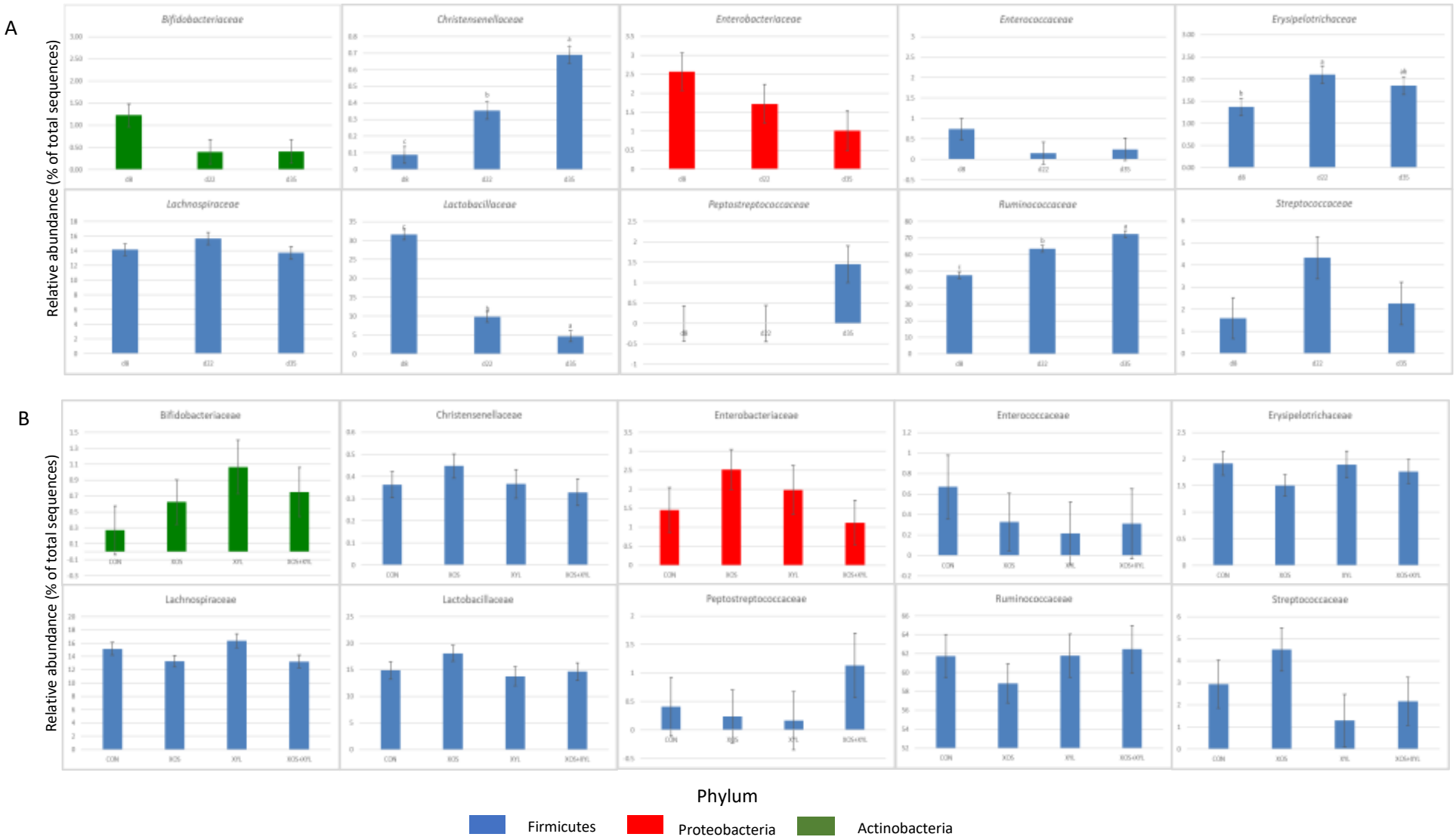
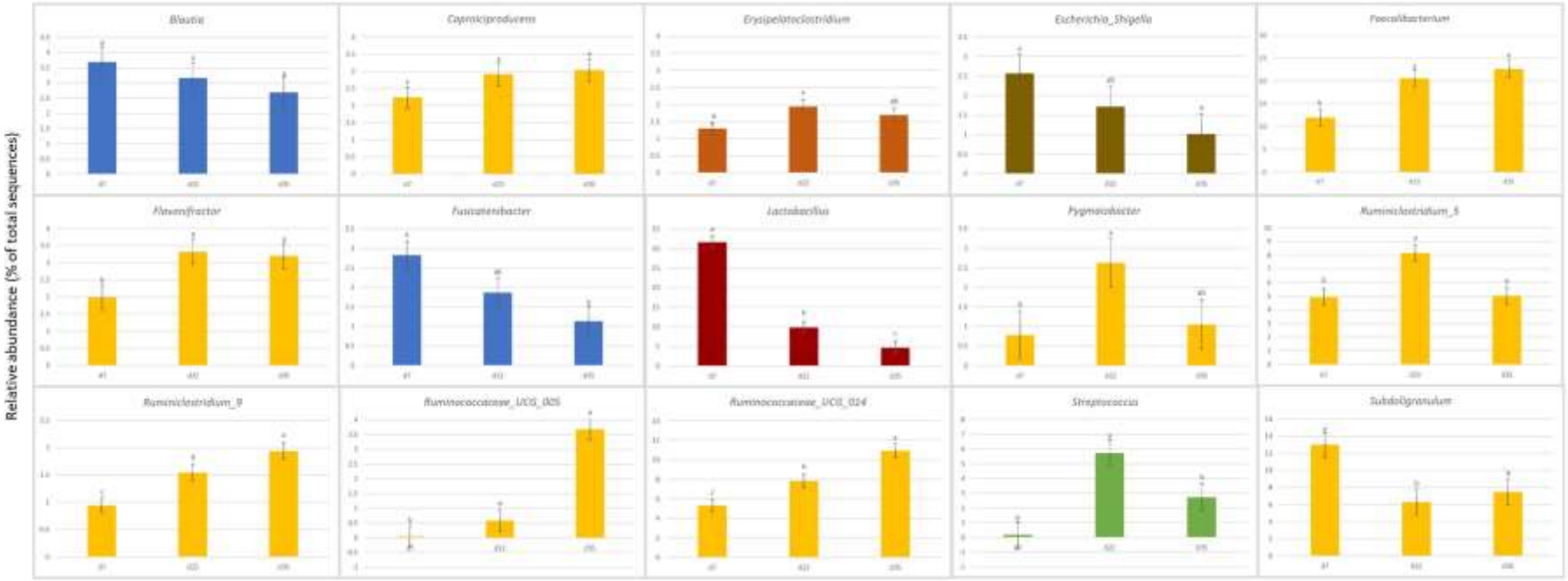


Figure 3.8: Relative abundance of the top 10 families averaged over all samples for age (A) and diet (B)

A


Relative abundance (% of total sequences)
Blautia
Coprococcus
Erysipelatoclostridium
Escherichia_Shigella
Faecalibacterium
Flavonifractor
Fusicatenibacter
Lactobacillus
Pygmaeobacter
Ruminiclostridium_5
Ruminiclostridium_9
Ruminococcaceae_UCG_005
Ruminococcaceae_UCG_014
Streptococcus
Subdoligranulum

B

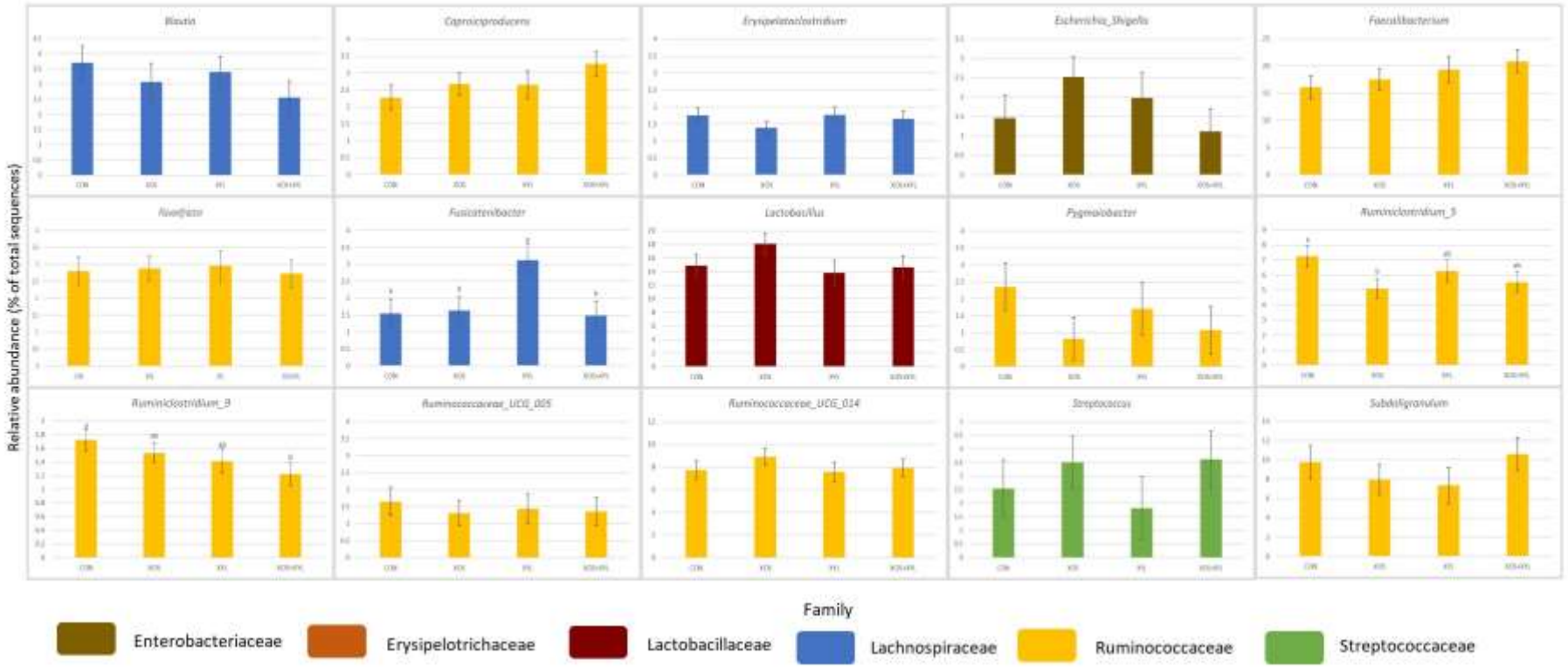


Figure 3.9.: Relative abundance of the top 15 genera averaged over all samples for the age (A) and diet (B) groups

A B

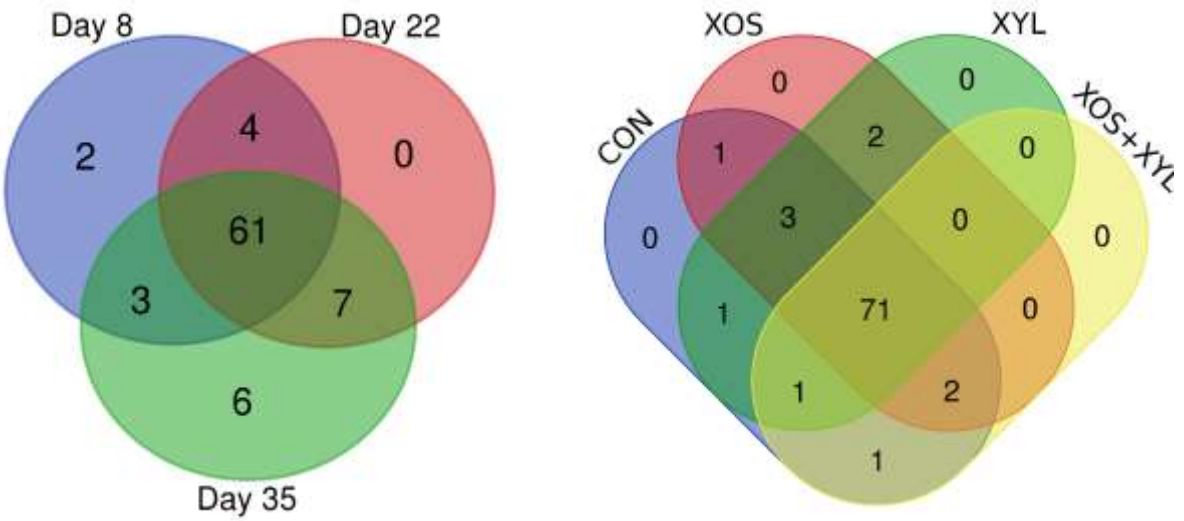


Figure 3.10: Venn diagram illustrating the number of genera unique to each and common between time points (A) and dietary treatments (B). There were no genera unique to each treatment whereas 2 and 6 genera were unique to day 8 and 35 respectively.

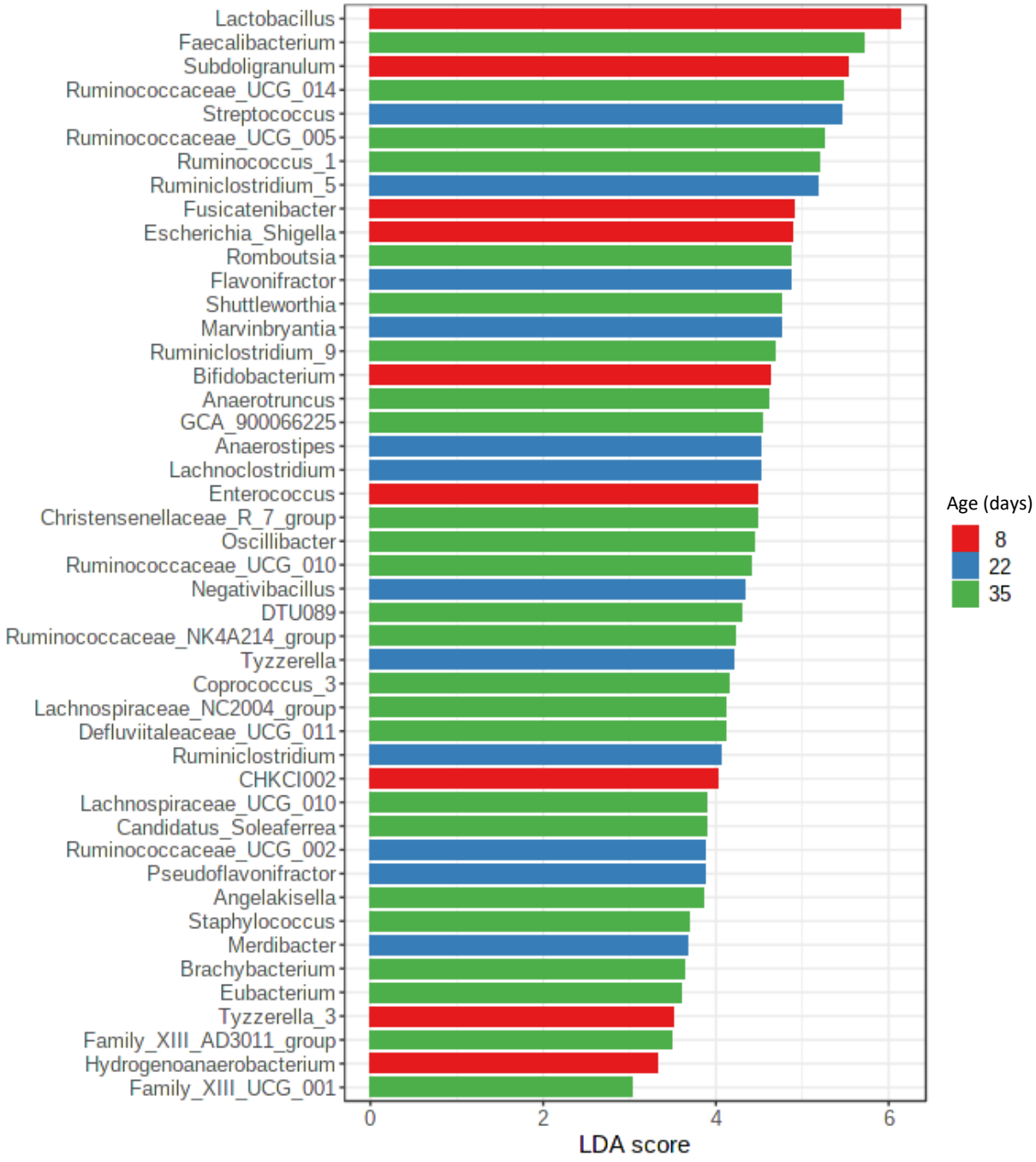


Figure 3.11: Linear Discriminate analysis (LDA) effect size (LEfSe) was used to identify genera that significantly associated with age. Forty-six different genera with LDA scores > 2.0 and p value < 0.05 were identified and are listed in the order of descending LDA scores. LefSe employs Kruskal Wallis rank sum test to detect features with significant differential abundance with regard to the independent variables (age, diet) followed by Liner Discriminant Analysis to evaluate the relevance of effect size of the differentially abundant features

Table 3.13: Microbial taxa consistently present over time (core microbiome) at family and genus level with at least 80% prevalence at each time point. The taxa were sorted in alphabetical order.

Core families (n= 3)	Core genera (n = 8)
Lactobacillaceae	*Blautia*
Lachnospiraceae	*Caproiciproducens*
Ruminococcaceae	*Faaecalibacterium*
	Flavonifractor
	Lactobacillus
	Ruminiclostridium_5
	Ruminococcaceae_UCG_014
	Subdoligranulum

3.4.10 Short Chain Fatty Acids (SCFA) in the caecum: effect of treatment

To further identify whether the observed microbial changes due to dietary treatment also affected the gut function, SCFAs in the caecum were measured. The SCFA, acetate, propionate or butyrate concentrations in the caecum were not affected by dietary treatment at any time point (Figures 3.12, 3.13, 3.14)

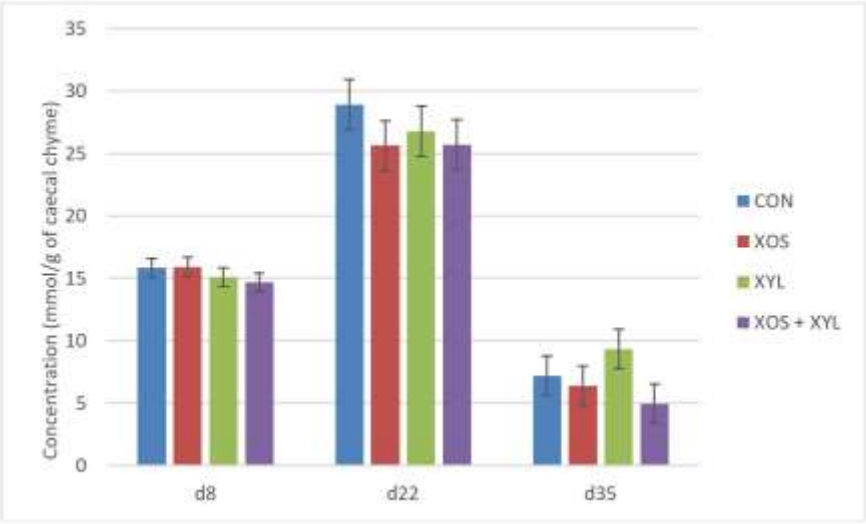


Figure 3.12: Acetate concentrations in the caecum at days 8, 22 and 35

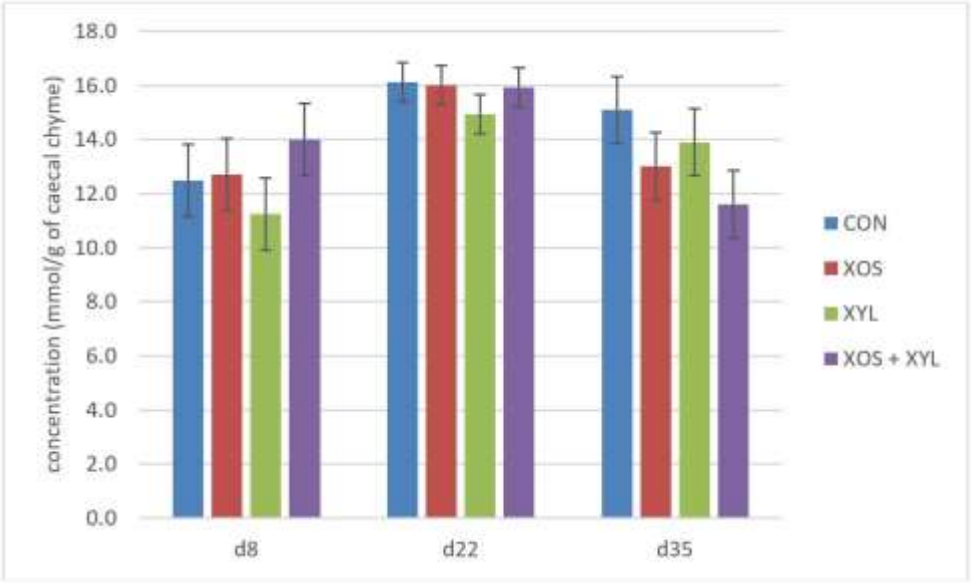


Figure 3.13: Propionate concentrations in the caecum at days 8, 22 and 35

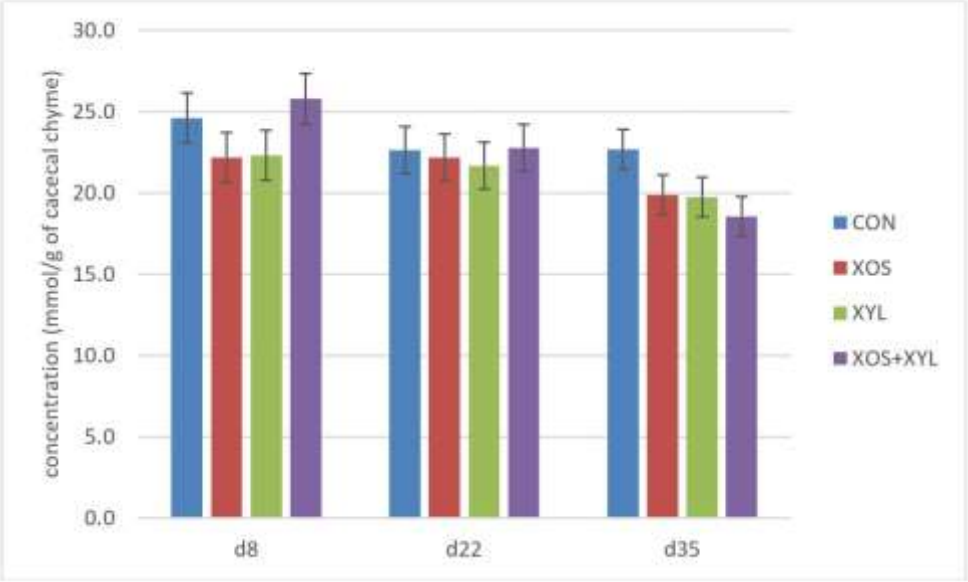


Figure 3.14: Butyrate concentrations in the caecum at days 8, 22 and 35

3.5 Discussion

The aim of this study was to investigate the effects of XOS and xylanase supplementation alone and in combination on performance and gut health parameters of chickens raised under research conditions of high biosecurity, high animal welfare and controlled atmosphere and fed a nutritionally adequate wheat-corn-soya diet.

3.5.1. Effect of diet on performance

While a number of data points for feed intake required exclusion prior to data analysis due to feed spillage; the number of outliers removed is no more than 3 for any diet at any given time point for any measured variable (See appendix C). The removal of 3 or less observations from the total 12 replicates per treatment was deemed to leave sufficient replicants to ensure the final outcome of assessment for that a given point and diet was unaffected, particularly as the loss of data points was broadly balanced for the diets at any given time point.

The body weights and hence the weight gain of XOS fed birds was consistently lower than the other treatment groups throughout the entire trial with a significantly lower weight gain for the entire trial period (d0-35) and between days 14 - 22. The reason for decreased body weights and weight gain of the XOS fed birds is unclear. A possible explanation for this effect could be the absence of xylanase in the XOS diets. The diet fed to birds in this trial was a wheat-based diet. Wheat is rich in arabinoxylans (AX) which is a major anti-nutritional factor for poultry as it increases the viscosity of the digesta causing poor absorption of nutrients and hence decreased performance (Bedford and Schulze, 1998; Bedford, 2000; Aftab and Bedford, 2018). Unfortunately, the digesta viscosity was not measured in this trial. Moreover, a similar decrease in performance of the birds fed the control diet which also lacked supplemental xylanase was not observed, it is therefore uncertain whether the viscosity effect was the responsible factor. Birds fed the xylanase or xylanase together with XOS were heavier than the control birds at the end of the trial period although this difference was not significant. Likewise, there was also no significant difference ($p>0.05$) in the weekly or cumulative FCR of any of the treatment groups when compared to the control. These findings agree with those of Craig et al. (2020, 2019, 2018) and Yuan et al. (2018) who also reported no significant differences in FCR of broilers fed XOS or xylanase supplemented diets. However, it is worthy to note that the diet supplemented with both XOS and xylanase performed better from third week onwards, producing numerically heavier birds, higher weight gain and lower FCR, although this difference was not statistically significant. This

may be due to the action of xylanase on the xylan in the cereal grains thereby quantitatively increasing XOS *in situ*. This may also suggest that when XOS is used without added xylanase, a dose higher than that used in this study (0.1 g/kg) may be needed to achieve significant effects on performance. For example, Zhenping *et al*. (2012), De Maesschalck *et al*. (2015) and Pourabedin *et al*. (2015), reported significant improvements in either body weights or FCR on supplementation with 10 g/kg, 5 g/kg and 2 g/kg XOS respectively. Another reason for the no differences in performance of birds in this trial could be optimal conditions of growth. The trial was conducted in a research facility with high biosecurity, high welfare, absence of disease as evident from the low mortality rate and raised on a nutrient adequate diet. Hence the birds may already be performing to their maximum production potential and addition of the feed additives did not cause further improvement in performance. This is supported by the fact that the average body weights of birds at d35 in all the treatment groups was between 2.0 – 2.1 kg or more which is close to the benchmark of 2.3 kg for birds of this age given by Aviagen (Aviagen, 2019 http://eu.aviagen.com/tech-center/download/1339/Ross308-308FF-BroilerPO2019-EN.pdf). It is important to note that this study uses mash diets resulting in lower overall bird weights compared to industry standards for pellet-fed bird. This is also supported by the fact that some trials conducted at the NTU poultry research unit feeding mash diets to birds had similar body weights and weight gains as seen in this trial (Scholey *et al*. 2018 and 2020)

3.5.2. Effect of diet on diversity and composition of microbiota

Despite the contradictory results in literature regarding the effects of XOS on performance, many studies agree that XOS positively modulates the gut microbiota by encouraging the growth of beneficial bacteria mainly *Lactobacillus*, *Bifidobacteria* and members of Clostridium cluster XIVa (De Maesschalck *et al*., 2015; Pourabedin *et al.*, 2015; Ribeiro *et al*., 2018). To study the gastrointestinal microbiota of chicken, high throughput sequencing of the 16S rRNA genes has been employed in a number of studies. This technique has been used to identify functional diversity (Sergeant *et al.*, 2014) or variability (Stanley, Geier, Hughes, *et al.*, 2013) of the microbiome as well as to identify members of the microbiome to linked to high or low FCR in broilers (Singh *et al*., 2012). However, on the subject

of dietary XOS supplementation previous studies have used either low resolution bacterial detection techniques (Courtin *et al*., 2008; Samanta *et al.*, 2016) or high throughput pyrosequencing using the Roche 454 GS FLX technology (De Maesschalck *et al.*, 2015; Pourabedin *et al*., 2015; Ribeiro *et al.,* 2018). In our study V3-V4 region of 16S rRNA gene was sequenced using the Illumina MiSeq platform to monitor the changes induced in the caecal microbiota by supplemental XOS or xylanase or a combination of the two. In addition, temporal changes in development of the microbiome were also examined. Illumina sequencing is superior over 454 sequencing in generating larger number of sequences and having a stringent quality control in terms of getting an overlap between forward and reverse sequences. The Roche 454 sequencing is also known for its high error rate in the homopolymer region and can have up to 15% sequences produced as a result of *in vitro* or artificial amplification (Lohman *et al.*, 2005; Curtis and Hall, 2009). Illumina does not appear to share these limitations but does tend to have higher error rates at the 3' end of sequences compared to the 5' end (Schröder *et al.*, 2010) and increased single-base errors have been observed in association with GGC motifs (Nakamura *et al.*, 2011). However, algorithms that detect and correct these errors are being developed and incorporated into existing data processing pipelines (Luo *et al.*, 2012).

Based on the phylogenetic diversity of bacterial communities, the number of observed OTUs, and the fact that the communities did not clearly separate between treatment groups, it can be concluded that the dietary treatments did not significantly alter the community membership in the caeca. Similar results have also been reported in chicken (Pourabedin et al., 2015) and pigs (Moura et al., 2007). Firmicutes, Proteobacteria and Actinobacteria were the phyla detected in all samples irrespective of the treatment group. However, an unusual observation is the complete absence of the phylum Bacteroidetes in this trial. This is contradictory to studies in chicken (Pourabedin et al., 2015), rats (Fei *et al.*, 2020) and pigs (Chen *et al.*, 2021) where Bacteroidetes has been one of the dominant phyla in animals fed a XOS supplemented diet. The differences between our and other broiler studies may be due to composition of basal diet, experimental conditions, age at sampling, differences in sequencing technology or differences in primers used. Kers *et al.* (2018) compiled a data of studies for which 16S

rRNA gene amplicon sequencing data of caecal samples was available for two broiler breeds, Ross and Cobb. Their analysis showed that in caecal samples Bacteroidetes were present in all four Cobb studies (100%) and in six out of eight Ross studies (75%). This indicates that absence of Bacteroidetes is not uncommon.

At the family level, there was no significant difference of XOS or xylanase or their combination in the relative abundance of *Bifidobacteriaceae* when compared to the control. The bifidogenic property of XOS is well established from several *in vitro* (Patrícia Moura *et al.*, 2007b; Moniz *et al.*, 2016; Fehlbaum *et al.*, 2018), and animal (Campbell, Fahey and Wolf, 1997; Yu *et al.*, 2015) and human studies (Chung *et al.*, 2007; Finegold *et al.*, 2014). The lack of any difference in the relative abundance of Bifidobacteriaceae may be due to the very low dose of XOS used. Lactobacillacecae and Streptococcaceae and their corresponding genera *Lactobacillus* and *Streptococcu*s were increased in the XOS group compared to control. Previous studies on broilers fed XOS supplemented diet have also significant increases in *Lactobacillus* (De Maesschalck et al., 2015; Pourabedin et al., 2015; Ribeiro et al., 2018). Like *Lactobacillus*, *Streptococcus* belongs to the order Lactobaccillales and the clade of lactic acid producing bacteria. However, as the concentration of lactic acid in caeca was not measured the collective role of these bacteria in lactic acid production cannot be commented on. The Enterobacteriaceae family of Proteobacteria has been denoted as signature of inflammation-associated dysbiosis in the mouse model (Hughes *et al.*, 2017). In the chicken, a negative correlation between performance parameters and Enterobacteriaceae expansion has been reported (Eeckhaut *et al.*, 2016). The increases in the family Enterobacteriacecae and its genus *Escherichia_Shigella* in the XOS supplemented diets may be nullifying the beneficial effects of *Lactobacillus* or *Streptococcus* and may partly explain the lack of improvement in performance in this group. Ruminococcaceae and Lachnospiracecae were the two most dominant families across all samples irrespective of diet type. Although there were no significant differences due to diet in these families and their corresponding genera (*Caproociproducens, Faecalibacterium, Flavonifractor, Ruminoclostridium, Subdoligranulum, Pygmaiobacter*, unclassified genera of Ruminococcacecae and *Blautia* and *Fusicatenibacter* of

Lachnospiracecae) these families have recently drawn the attention of researchers in their role in degradation of complex plant polysaccharide in the human and animal gut. Using genomic data from a large number of sequenced genomes and datasets Biddle et al. (2013) showed that the genomes of Ruminococcaceae and Lachnospiracecae have abundant and diverse carbohydrate active genes encoding glycoside hydrolases and carbohydrate binding modules (CBM) enabling them to degrade a wide variety of polysaccharides. Members of Ruminococcaecae are predominantly butyrate producers as they harbours all genes required for butyrate production from pyruvate and acetyl-CoA (Medvecky *et al.*, 2018). On the other hand *Blautia,* of Lachnospiraceae (identified as the most abundant genera of this family in this study) has been reported to consume H_2 released by butyrate producers and converting it to acetate via acetogenesis (Medvecky *et al.*, 2018). Fermentation of carbohydrates results in production of H_2 and its increased concentration supresses glycolysis (Sergeant *et al.*, 2014). Thus, the presence of Lachnopiracecae and Ruminococcaceae as the most dominant families demonstrates their role the intricate cross feeding mechanisms existing amongst the caecal microbiome members which ultimately supports gut health of the animals.

3.5.3. Temporal changes in microbiota

The temporal development of the caecal microbiota observed in this study is similar to what has been previously reported (Lu *et al.*, 2003; Oakley *et al.*, 2014; Ranjitkar *et al.*, 2016; Ocejo, Oporto and Hurtado, 2019). The diversity and composition of caecal microbiome increased as the chickens grew. Even though the microbiome clustered according to age, there was inter-individual variation observed in the microbial community within birds of same age. However, this variation was mainly due to differences in relative abundance rather than to taxonomic composition. Each age-group showed an age-associated community profile with a transition period at the middle of their lifespan. This is evident from the fact that at 22-day old birds shared the microbiota profile of both 8 and 35 day old birds and there were no unique OTUs at day 22. Contradictory to other reports (Ocejo, Oporto and Hurtado, 2019), the microbial community did not differ at phylum level between age groups and the microbiome in all age groups was dominated by Firmicutes followed by Proteobacteria and

Actinobacteria. A decline in the number of microaerophilic bacteria, genera of families, Lactobacillaceae, Bifidobacteriaceae and Enterobacteriaceae (of Actinobacteria and Proteobacteria) was observed with age along with an increase in anaerobes of Lachospiraceace and Ruminococcaceae which is in agreement with the findings of Ranjitkar et al. (2016) and Richards et al. (2019). The relatively high abundance of members of the families Lachnospiraceae and Ruminococcaceae, the acetate and butyrate producers, in older birds likely associated to the high nutrient requirements for growth during this phase since butyrate is the most preferred source of energy for enterocytes in rapidly growing chickens. Microbiota development is probably a continuum process of microbial communities' succession, where certain taxa are replaced by others as chickens grow. Based on the time points assessed, the stages described here for the whole chicken lifespan clearly showed the evolution from an early immature stage to a mature microbiota.

3.5.4. Effect of diet on mucin layer

In this trial there was no significant difference in the mucin layer thickness between treatments. The mucin adherent layer lines the intestinal tract, providing a vital barrier function, lubrication, and protection to the mucosal surface from mechanical injury and enteric pathogen entry (Tsirtsikos *et al.,* 2012). Secreted from goblet cells in the lining of the gut epithelium. If the layer is too thick mucin is thought to effect nutrient absorption and conversely, if the layer is not adequately thick, then barrier integrity may be compromised (Lea *et al.*, 2012). However, there is no standard for mucin layer thickness in poultry and it may vary from bird to bird with age, genetics, nutritional or disease status and environmental factors. Very few studies in poultry have used mucin layer thickness as a response variable to addition of feed additives (Tsirtsikos, Fegeros, Balaskas, *et al.*, 2012; Tsirtsikos, Fegeros, Kominakis, *et al.*, 2012). These authors reported an increase in thickness with addition and inclusion levels of either probiotic or phytogenic feed additive. But due to differences in method used, a direct comparison with our study is inappropriate. The method used in this study was described by Smirnov et al. (2004) and Thompson and Applegate, (2006) in the investigation of the effect of feed withdrawal on mucin layer thickness. When examining the mucin layer thickness of the control birds (not subject

to feed withdrawal) Smirnov et al. (2004) reported a thickness of 75 µg alcian blue /cm^2 of ileum at 31 days of age while Thompson and Applegate (2006) reported 86 µg alcian blue/ g of ileum at day 43. In this study the control birds at day 35 had a mucin layer thickness of 98 µg alcian blue/ g of ileum and there were no significant differences between treatments. Nevertheless, as the values obtained in this study are similar to those published in literature, it may indicate that the addition of XOS, xylanase or their combination did not have any deleterious effect on the mucin layer.

It was also observed that mucin layer thickness decreases at day 22 and then increases at day 35. This may be due to the developments of the GIT with age. Chicks are exposed to solid feed from the day of hatch or soon after. This may cause the physical removal of the mucin layer. However, as the intestine develops and the numbers of goblet cells that secrete the mucin layer increase with age (Reynolds, Cloft, and Wong, 2020) the thickness of the layer also increases

3.5.5. Effect of diet on caecal SCFA concentrations

The beneficial effects of prebiotics on the host are exerted at least in part due to production of SCFA, particularly butyrate, on fermentation of prebiotics by gut microbiota (Volman, Ramakers and Plat, 2008; Janardhana *et al.*, 2009; Nawaz *et al.*, 2018). There was no significant effect of the dietary treatments on the concentrations of acetate, propionate or butyrate in the caeca at any of the time points measured. These findings agree with those of Pourabedin et al. (2015) who also reported no differences in the concentration of these acids in birds fed either 1 g/kg or 2 g/kg XOS when compared to the control at days 15, 25 and 35. Pourabedin et al. (2015) reported an increase in acetate concentration from day 15 to d 35 but there was no significant difference in the XOS supplemented groups when compared to the control. Similarly, there was no significant effect or age or treatment in the levels of propionate or butyrate in the caecum. Contradictory to Pourabedin et al.'s study, our study reported the lowest levels of acetate in the oldest birds while priopionate and butyrate were highest in the oldest birds. Similarly Craig et al. (2019) also reported no significant differences in concentration of these acids in birds fed either a XOS or xylanase supplemented diet at 14 and 28 days

of age. Conversely, Yuan *et al*. (2018) reported significant increase in acetate and butyrate in broilers fed 2 mg/ kg XOS in their diet on days 21 and 42. These differences may be due to the differences in the source of XOS, composition of the basal diets, levels of inclusion, duration of supplementation or housing management practices. *In vitro* studies have revealed significant increases in at least one of these acids on fermentation of XOS by human faecal microbiota or selected probiotic strains (Yu *et al.*, 2015; Carlson *et al.*, 2017; Fehlbaum *et al.*, 2018; Monteagudo-Mera *et al.*, 2018). The reason for the differences in *in vitro* and *in vivo* studies is that *in vivo* systems are dynamic, and the acids get absorbed by enterocytes or utilized in other metabolic pathways making it challenging to measure the actual quantities produced.

Another possible reason for the no significant differences in concentrations of the measured SCFA may be the absence of bacteria belonging to the phylum Bacteroidetes in the caeca as evident from the 16S rRNA sequencing results. Bacteroidetes is, normally, the most dominant phylum in the gut microbiota after Firmicutes and members of this phylum are glycan degrading generalists capable of degrading a variety of complex plant and host polysaccharides (Martens *et al.*, 2014). Firmicutes, on the other hand are more specialised in their preference for plant storage polysaccharides and oligosaccharides (Rogowski *et al.*, 2015). It is well established that there are interactions and cross feeding mechanisms between members of the gut microbiota (Kelly *et al.*, 2010; Flint *et al.*, 2012). In a simplified model of human gut microbiota in germ free mice, Mahowald et al. (2009) showed how *Bacterioidetes thetaiotaomicron*, a prominent gut Bacteroidete and *Eubacterium rectale*, a Firmicute are able to adapt their substrate utilization, in response to one another and to host dietary changes. They also demonstrated how acetate produced by *B.thetaiotaomicron* could be used by *E.rectale* to generate butyrate. In this trial, there was significant reduction ($p<0.001$) in acetate at d35 compared to days 8 and 22 within each treatment group. This may be due to the absence of the acetate producing Bacteroides observed in this trial or conversion of acetate to butyrate by members of Firmicutes. However, the former is more likely to be the case because if acetate was being produced there would have been corresponding increase in butyrate which was not seen. In fact, the levels of

butyrate within each treatment were similar across the time points taken. Small amounts of acetate seen at day 35 may be due to it being produced in minor amounts by unclassified members of the gut microbiota. However, the butyrate levels were not significantly different at the measured time points. This is may be because of butyrate being produced from lactate generated by the Lactobacilli which was identified as one of the top genera in this study. Lactate is thought to be converted to butyrate by lactate-utilizing-butyrate-producing bacteria in the chicken hind gut (De Maesschalck *et al*., 2015). Unfortunately, lactate was not measured in this trial due to the limitations of the chosen protocol. The beneficial effects of butyrate as a primary energy sources for colonocytes and having immune modulatory, anti-inflammatory properties and anti-pathogenic properties are well documented (Guilloteau *et al.*, 2010; Onrust *et al.*, 2015; Ahsan *et al.*, 2016). Although butyrate was being produced throughout the entire trial period, its quantities were not enough to translate into improved performance.

3.5.6. Effect of diet on gene expression in the ileum

In trial we also investigated the effect of XOS, xylanase and their combination on expression on *MUC*2 glycoprotein, one of the most abundant mucin proteins (MUC2), tight junction proteins, claudin-1 (CLDN-1), claudin-5 (CLDN-5) and occludens (OCLN) as wells secretory IgA (*sIgA*). These genes were chosen as they are considered to be biomarkers of gut barrier health (Chen *et al.*, 2015). In addition, effects of these feed additives on interleukin 1β (IL-1β), pro-inflammatory cytokine and short chain fatty acid receptor (FFAR-2) were also studied. To our knowledge this is the first study to examine the effects of XOS, xylanase or their combination on gut barrier integrity. Gut barrier function is a critical aspect of gut health. As the largest organ in the body, the gut serves as a selective barrier to take up nutrients and fluids into the body, while excluding undesirable molecules and pathogens (Groschwitz and Hogan, 2009; Neu, Sharma and Young, 2010). An optimal gut barrier function is therefore essential to maintain overall health and represents a key line of defence against foreign antigens from the environment. There were no significant differences in expression of the above-mentioned genes in any of the treatment groups. This may be because of absence of any potential threat to the gut barrier

integrity from pathogens or environmental stress as the birds were raised under controlled research conditions and absence of disease as evident by the low mortality rate and no striking different expressions of *sIgA* ir IL-1β between samples. However, there are reports of improvement of gut barrier integrity by feed additives even in the absence of disease. For example, Li et al. found that zinc supplementation in breeder diets improved morphometry, increased the number of goblet cell per villus, and *MUC*2 gene expression, and reduced mRNA levels of proinflammatory cytokines, such as IL-6 and IL-1β in the jejunum of their offspring (Li *et al.*, 2015). On the other hand Palamidi and Mountzouris (2018) reported that birds fed 1 g/kg organic acid blend had significant downregulation of tight junction proteins in the absence of disease yet had improved performance. Similarly, Paraskeuas and Mountzouris (2019) also reported increased expression of *CLDN5* and *MUC2* genes in the absence of disease. Interestingly, like our study, these authors have also reported no significant differences in acetate, propionate and butyrate concentration due to the addition of feed additives. The absence of significant improvements in gut barrier integrity functions may correspond to lack of significant differences in SCFA concentration noted in this trial.

The results of this trial were unexpected, with no significant differences in performance or any of the gut health parameters tested. This does not support the original hypothesis of this research as this implies that there were no real effects of XOS or xylanse supplementation alone or in combination at the dose included. As the birds in this trial were growing under optimal conditions they were possibly performing to their maximum potential. Hence it would be interesting to upscale this study to a commercial setting where birds are exposed to a variety of stressors. Secondly a dose response study will help to determine if a higher dose of XOS is needed to see significant effects of inclusion of this prebiotic.

3.6. Conclusion

Supplementation of either 0.1 g/kg of XOS or xylanase, alone or together, in diets of broilers growing under environmentally controlled conditions with high biosecurity and absence of infection by pathogens does not improve their performance or gut health.

Chapter 4: Effect of XOS on performance and gut health parameters of broilers raised under sub-optimal conditions

4.1 Introduction

In commercial broiler production systems eggs are hatched in hatcheries with no exposure to the mother. It has been hypothesized that in broilers the early development of intestinal bacterial community is shaped rather randomly and is quite heterogenous due to exposure to bacteria from a variety of environmental sources after hatch rather than maternally derived bacteria (Stanley, Geier, Hughes, *et al.*, 2013). The type of production system, organic, free range or conventional can also influence the microbiota composition (Bjerrum *et al.*, 2006; Ocejo, Oporto and Hurtado, 2019). In addition, housing conditions, including biosecurity level, type and quality of litter, humidity and temperature of the poultry house can all affect the composition of the microbiota (Sohail et al., 2015; Torok et al., 2009). For these reasons, early beneficial microbiota colonization via early feeding strategies like *in ovo* feeding which involves experimental injection of small amounts of nutrients into a fertilized egg during incubation or specially formulated post hatch diets can confer health benefits and help maintain a healthy microflora balance (Jha *et al.*, 2019). Prebiotics have been shown to stimulate the microbiome when administered *in ovo* or from hatch. The idea is that prebiotics would provide a substrate for the colonization and growth for beneficial microbiota rather than random colonization by bacteria from the environment. For example, one study showed that inclusion of prebiotics at an early stage of life increased the abundance of *Lactobacilli* and *Bifidobacteria* and suppressed coliforms (Chee *et al.*, 2010). In another study birds fed diet supplemented with mannanoligosaccharide and acidifier immediately post hatch had improved growth performance and development of intestinal morphology and immune response to *C. perfringens* challenge compared to birds whose access to feed and water was denied for 48 hours post hatch (Ao, Kocher and Choct, 2012).

The evidence from several other published studies (described above) has shown XOS to be a promising prebiotic via its mechanism of modulating the gut microflora and providing a substrate for microbial fermentation leading to increased levels of short chain fatty acids in the caeca. However very little is

known about the effect of XOS in influencing the development of the microbial community under sub-optimal conditions since all the previous studies in chickens have been carried out under experimentally controlled and clean environmental conditions. Broiler chickens growing in industrial production systems are often exposed to a wide range of stressors such as overcrowding, changes in temperature, poor feeding, dirty litter that may alter the balanced population of gut microbiota (Sohail *et al.*, 2011a). Cold temperatures in addition to high stocking density is a common environmental factor that affects growth and physiology of broilers on farms in countries with a temperate climate (Pourabedin *et al.*, 2014). Therefore, the effects of a feed additive on gastrointestinal tract and immune system could be more obvious at times of stress (Baurhoo, Phillip and Ruiz-Feria, 2007; Pourabedin *et al.*, 2014). This trial was therefore conducted to investigate if XOS improved gut health under sub-optimal growth conditions in broilers up to 35 days of age.

4.2 Aims

Based on the results of Trial 1, the initial hypothesis of the research described in section 1.8 were rejected. A new hypothesis was thus proposed that the effect of XOS on performance and gut health parameters would be more pronounced in birds raised under challenging environmental conditions

The key aims of this trial were

1) to determine the effect of XOS on performance of birds raised under sub optimal conditions
2) To study the development of caecal microbiome of birds under such conditions
3) To examine the effects of XOS in modulating the caecal microbiome and consequent effects on caecal short chain fatty acid levels and gene expression in the ileum

4.3 Materials And Methods

4.3.1 Bird husbandry

A total of 830 birds were randomised by weight and distributed across the 4 pens on the day of hatch with pen 1 and 2 housing 225 birds each and pen 3 and 4, 190 birds each. The birds were within the weight range of 30 - 40 g and from breeder flocks aged approximately 33 weeks. Sixty birds per pen

were wing tagged using 6 different colour tags (10 birds per colour) and the weight of each tagged bird was recorded. Birds were kept with a stocking density aiming for a commercial stocking density of 30 kg per m^2 at the end of the trial. There were 2 dietary treatments with 2 pen replicates for each treatment. The litter provided was as wood shavings at a depth of 7 cm and was topped up if necessary, to maintain adequate environmental welfare. Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was obtained and is recorded as project ARE716. A typical pen set up in shown in figure 4.1.

Figure 4.1.: A typical pen set up showing the placements of chicks, heating, feed hoppers and drinkers

4.3.2 Experimental diets

The diets were a corn-soya diet and based on the nutritional requirements for the strain of bird. The diet was manufactured as a crumb starter and pelleted grower by Research Diet Services (RDS) according to the formulation described in Table 4.1. A sack of the appropriate feed was placed outside each pen. The study lasted 35 days with a three-phase feeding programme; starter diets were fed

from 1 to 14 days; grower diets were fed from 14 to 28 days and finisher diets were fed from 28 to 35 days. The two dietary treatments were

1) Con (CON)

2) Control + XOS at 100 g per tonne (XOS)

Table 4.1 Formulation of the control diet presented as rates of inclusion (%)

Ingredient (%)	Starter	Grower	Finisher
Maize	56.5	59.2	61.8
Soybean meal	36.6	33.3	29.8
Soya oil	2.4	3.5	4.6
Salt	0.3	0.3	0.31
Limestone	1.58	1.43	1.34
Monocalcium Phosphate	1.44	1.29	1.19
Sodium bicarbonate	0.1	0.1	0.1
Lysine HCL	0.18	0.12	0.13
DL-Methionine	0.31	0.26	0.26
Threonine	0.09	0.05	0.04
Vitamin & Mineral premix*	0.5	0.5	0.5
Quantum Blue 5G (Phytase)	0.01	0.01	0.01

*Premix content (volume/kg diet): Vitamin A 10.000 IU, vitamin D3 2.500 IU, vitamin E 50 mg, menadione 1.5 mg, thiamine 2.0 mg riboflavin) 7.5 mg , vitamin B6 3.5 mg, cyanocobalamin 20 µg, Niacin 35 mg, D-pantothenic acid 12 mg, Choline chloride 460 mg, Folic acid 1,0 mg, Biotin 0.2 mg 40 mg, Fe 80 mg, Cu 12 mg, Mn 85 mg, Zn 60 mg, Iodate 0.8 mg, Se 0.15 mg

4.3.3 Treatment schedule / randomisation plan

There were 2 replicate pens per diet and each replicate consisted of either 230 or 190 birds. Only birds weighing between 30 g and 50 g were placed in the pens. The weight of the 60 wing tagged bird replicates in each pen was recorded on days 0, 7, 14, 21, 28 and 35.

4.3.4 Determined parameters

Proximate analysis (protein, fat, dry matter and ash) of feed was done and Ca and P content was determined in house as described in sections 2.4.1 – 2.4.5 of chapter 2 while 500 g of feed was despatched to an external lab for gross energy and phytase analysis.

Twenty-four untagged birds per treatment on day 7 and 20 untagged birds per treatment on days 21 and 35 were transported to the poultry research unit where they were humanely killed via cervical dislocation. Caeca (one each for microbiome sequencing and determination of short chain fatty acids concentration) and a section of the ileum (for gene expression levels) were collected as described in sections 2.3.1 – 2.3.3 of chapter 2.

4.3.5 Analysis of samples collected from birds

16S rRNA sequencing of caecal microbiota, determination of SCFA in caecal chyme and gene expression in ileal tissue was done according to sections 2.4.1 – 2.4.3 of chapter 2

4.3.6 Statistical analysis of data

For statistical analysis of performance data, the experimental barn was blocked such that pens 1 and 4 were considered as one block and pens 2 and 3 as another. This was because of the location of the pens inside the barn shed. Pens 1 and 4 had their left- and right-hand side walls respectively in direct contact with the external environment whereas pens 2 and 3 were in the centre of the barn with no direct external contact. Each block consisted of a pen each of control and XOS diets with the same number of birds placed in the pens at the start of the trial.

Growth Performance data was analysed using according to the following model:

$$Y(ij) = Mean + B(i) + TRT(j) + E(ij)$$

Y(ij): response variable, B(i): effect of block, TRT (j): effect of treatment (XOS vs. control) and E(ij) is the residual

Relative abundance of microbial taxa, SCFA, gene expression data were analysed according to the model

Y(ij) = Mean + Age(i) + TRT(j) + age*trt (ij) + E(ij)

Y(ij): response variable, Age (ij): effect of bird age, TRT (ij): effect of treatment (XOS vs. control) and age*trt (ij): age-trt interaction and E(ij) is the residual

4.4 Results

4.4.1 Diet analysis

The measured values of macromolecules (dry matter, ash, fat and crude protein) minerals and energy and in the diets are represented in Table 4.2

Table 4.2: Measured values of energy, macromolecules and minerals

Phase	Treatment	Gross energy (MJ/kg)	Ca (g/kg of feed)	P (g/kg of feed)	Crude protein (g/kg of feed)	Dry Matter (g/kg of feed)	Ash (g/kg of feed)	Fat (g/kg of feed)
Starter	Con	16.7	10.2	6.72	227	880	52.3	46.2
	XOS	16.7	10.4	6.71	215	877	52.8	45.1
Grower	Con	17.1	9.28	6.18	204	883	47.9	56.6
	XOS	17.4	8.81	6.30	212	883	48.0	61.0
Finisher	Con	17.0	7.89	5.54	186	882	43.5	68.6
	XOS	17.0	8.42	6.04	190	882	45.4	69.0

4.3.2 Environment

As this trial was conducted on a farm barn there were no environmental control systems in place. Heating was provided in the form of gas-fired canopy brooder heaters, but due to the large area of the shed and lack of control over exchange of heat between the shed and external environment the effect of heating was limited to the area below and in close proximity to the canopy. The temperature of the shed greatly varied depending on the weather for the entire trial duration. Temperature was

monitored at two different locations within the shed and at two levels, one at approximately 1 m height above the floor which gave an indication of the shed temperature and second, at the level of chicks. There were large differences between shed temperature and temperature at bird height as well as fluctuations in minimum and maximum temperatures (Figure 4.2).

In addition, 11 instances of leakage from the drinker lines have been documented on the health record sheets causing wet litter that needed to be scooped out and replaced with fresh one. In addition, damp or poor-quality bedding was documented 5 times. Raking was done once daily from day 10 onwards which again gave an indication of the poor quality of bedding.

A

B

Figure 4.2: Temperature monitoring records showing minimum and maximum temperatures of the shed and temperatures at bird height from thermometers placed pen 1 (A) and pen 4 (B)

4.4.3 Health and Condition

Mortality data shown in Table 4.3 demonstrates an unusually high level of mortality compared to a well-run commercial farm or a trial run at the NTU poultry research unit (where mortality is usually 2-4%). There was no statistical significance in mortality between treatments over the entire trial period, the total flock mortality for the study was 9.87%

Table 4.3: Weekly and cumulative bird mortality

Week	CON	XOS	Total Bird Mortality	% Mortality*
1	13	10	23	2.77
2	6	4	10	1.28
3	13	6	19	2.46
4	6	7	13	1.77
5	9	8	17	2.36
Entire trial period	47	35	82	9.87
P				>0.05**

*weekly mortality = total mortality/no of live birds at the beginning of each week, cumulative mortality = total mortality/ no of birds placed on day 0

**proportion of mortality was compared according to Altman *et al*. (2000) at 0.05 level of significance. p value of all comparisons was >0.05

4.4.4 Bird uniformity

The mean start weights of the wing tagged birds are shown in Table 4.4. The chicks in this trial were obtained from a young flock of breeders, 33 weeks of age. Although the NTU poultry research unit requests for chicks from breeders of age 40 to 45 weeks, the hatchery was not able to provide such chicks on this occasion. This is possibly the reason why the chicks were comparatively lighter. However, there was no statistical significance in the start weight of the chicks between treatments.

Table 4.4 The average weight of chicks on day 0

Treatment	Average Body weight (g)	SEM*
Control	37.7	0.110
XOS	37.5	0.110
ANOVA P value	0.162	

*SEM – stand error mean

4.4.5 Cumulative and weekly bird performance

In this trial, the performance of the birds was assessed based on body weights and weight gain as the design of the trial did not allow the measurement of feed intake of the wing tagged birds only that were used for monitoring the body weights and considered representative of the entire pen. All bird weights fell within the 2x standard deviation (no outliers were found).

The data presented in Table 4.5 shows that the birds fed the XOS supplemented diet were significantly ($p < 0.05$) heavier that than the control group up to 28 days of age but this effect was lost at day 35. Likewise, the weight gain of in the XOS group was significantly ($p<0.05$) higher up to the third week and numerically higher in the fourth (Table 4.6). While the weight gain of the control group was significantly higher in the fifth week ($p<0.01$), there was no significant difference in the weight gain of birds because of dietary treatment when considering the entire trial period (Table 4.6).

Table 4.5: The weekly average weight of chicks

Days	*Treatment*	*Average Body Weight per bird (g)*	*SEM**	*ANOVA P value*
7	*Con*	165.1	1.630	<0.01
	XOS	174.4	1.630	
14	*Con*	439.2	4.718	<0.01
	XOS	465.3	4.569	
21	*Con*	951.4	9.977	<0.01
	XOS	1004.6	9.732	
28	*Con*	1619.4	17.16	0.004
	XOS	1689.4	16.87	
35	*Con*	2438.7	31.40	0.812
	XOS	2431.0	31.18	

*SEM – stand error mean

Table 4.6: Weekly and cumulative body weight gains of birds

Days	*Treatment*	*Body Weight Gain (g)*	*SEM*	*ANOVA P value*
0-7	*Con*	127.3[a]	1.612	<0.01
	XOS	136.9[b]	1.611	
7-14	*Con*	274.3[a]	3.447	<0.01
	XOS	291.4[b]	3.383	
14-21	*Con*	508.6[a]	6.454	<0.01
	XOS	541.6[b]	6.358	
21-28	*Con*	658.5[a]	11.29	0.257
	XOS	677.1[a]	11.16	
28 -35	*Con*	845.3[a]	14.87	<0.01
	XOS	738.3[b]	14.99	
0 - 35	*Con*	2400.9[a]	31.16	0.818
	XOS	2393.6[a]	31.38	

a,b - Means within the same column differ significantly ($P \leq 0.05$).
SEM – stand error mean

4.4.6 Gene expression in the ileum of 35-day old birds

4.4.6.1 Quality and quantity of extracted RNA

As explained in section 3.4.8.1 of chapter 3, the 260/280 ratio was used as an indicator of RNA purity and agarose gel electrophoresis was used to assess the RNA integrity of each sample. For this trial the extracted RNA from all samples had a 260/280 ratio in the range of 2.05 – 2.07. On visualising the gel all the samples showed two distinct intact bands of 28S and 18S rRNA indicating good quality of the extracted RNA (Figure 4.3). In addition, 260/230 ratios were also found to be in the range of 1.9 -2.4 which is used as a secondary measure of nucleic acid purity.

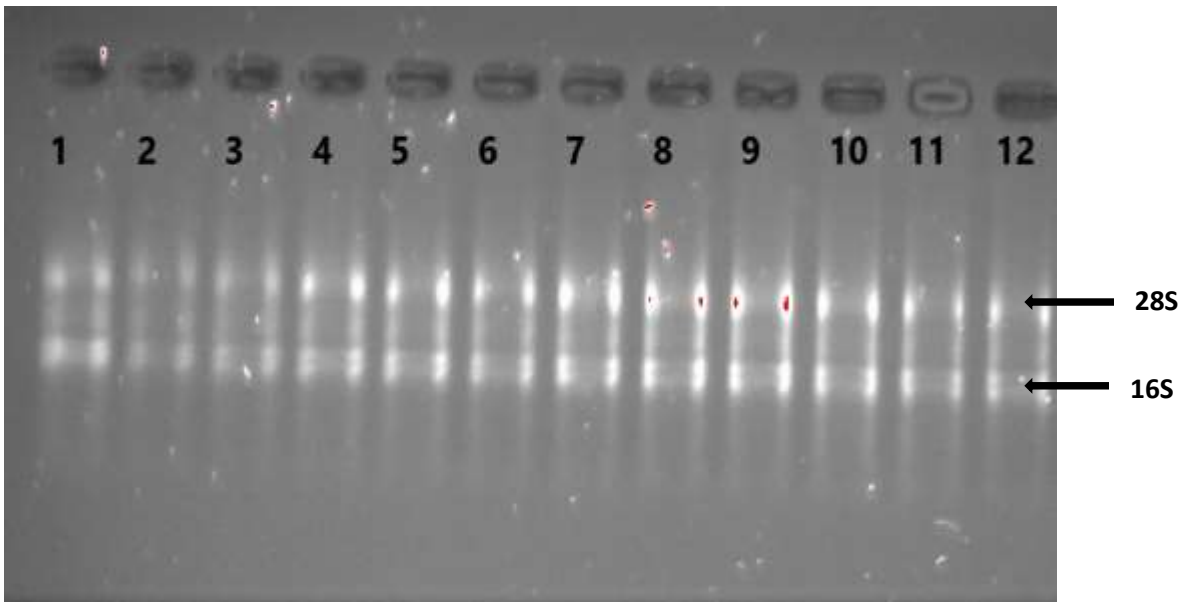


Figure 4.3: An agarose gel electrophoresis image of extracted RNA to check the quality of RNA. Samples in odd number lanes are from the Con group and those in even number lanes are from the XOS group. The two bands represent the 28S and 18S subunits of rRNA. Both bands are intact indicating good quality RNA.

4.4.6.2 Gene expression levels of the gut barrier genes and the short chain fatty receptor gene in the ileum

The gene expressions of *MUC*2, *sIgA*, CLDN1, CLDN5, OCLN, IL-1β and FFAR2 relative to GAPDH are shown in Table 4.7. The XOS supplemented diet resulted in a significant increase in the expression levels of *MUC*2 ($p = 0.033$) and OCLN (0.039). There was no statistical difference in the expressions of the rest of the genes.

Table 4.7 Effect of dietary inclusion of XOS on relative (to GAPDH) gene expression ratio of ileal mucosa barrier genes and Short Chain Fatty Acid receptor gene of 35-day-old broilers

	*MUC*2	*sIgA*	CLDN1	CLDN5	OCLN	IL-1β	FFAR2
Treatment							
CON	1.077[b]	1.341	1.163	1.087	1.075[b]	1.182	1.089
XOS	1.548[a]	2.127	1.063	1.353	1.564[a]	1.068	1.080
Statistics							
SEM	0.121	0.397	0.243	0.152	0.132	0.278	0.147
ANOVA P value	0.033	0.211	0.780	0.263	0.039	0.782	0.968

*MUC*2 = mucin 2; sIgA = secretory immunoglobulin A; CLDN1 = claudin-1; CLDN5 = claudin-5; OCLN = occludin, IL-1β = interleukin-1β, FFAR2 = free fatty receptor-2
a,b - Means within the same column differ significantly ($P \leq 0.05$).
SEM – stand error mean

4.4.7 16S rRNA sequencing of caecal microbiota: temporal changes and effect of treatment

4.4.7.1 Sequencing output, pre-processing and taxonomic assignment.

After the initial filtering and adaptors trimming process, a total of 7,911,264 read-pairs with a median of 141,125 read pairs per sample (IQR: 126,738 – 154,443). Median length of the reads per sample was 251 bp (IQR: 251 – 301). After pre-processing and removal of low-count ASVs as described in methods 4,499,028 amplicon reads from the 57 samples were classified into 1150 ASVs. Number of reads that passed through each step of the DADA2 pipeline are presented in Table 4.8.

Rarefaction curves generated from the ASVs approach a plateau indicating sufficient sequencing depths in all samples (Figure 4.4A). Chicken-to-chicken variation was observed in each age-group. The rarefaction curve patterns showed similarity within each age group rather than diet type (Figure 4.4B).

4.4.7.2 Alpha Diversity

The alpha diversity matrices of Richness (Observed ASVs and Chao1) and Evenness (Shannon and Simpson) showed a slightly different distribution pattern with age. A steady increase in species richness was observed as the chickens aged but the evenness decreased from day 7 to day 21 before increasing at day 35, indicating that one or few species were dominant in 21-day old birds (Figure

4.5A). The average Shannon diversity index was lowest in youngest chickens while the Simpson index was lowest in oldest chickens. The Kruskal- Wallis tests of Shannon and Simpson were contradictory with the latter indicating significant difference in bacterial diversity according to age.

There was no statistical difference in any of the alpha diversity indices when comparing the effect of diets as a whole data set (Figure 4.5B) or when restricting the dietary comparison to subsets of age groups (data not shown).

4.4.7.3 Beta Diversity

There was clear and significant shift in the overall community structure by age (total variance explained $R^2 = 0.48$, $p < 0.001$) based on PERMAOVA and post hoc tests analysis revealed significant differences between each pairwise comparisons between age-groups.

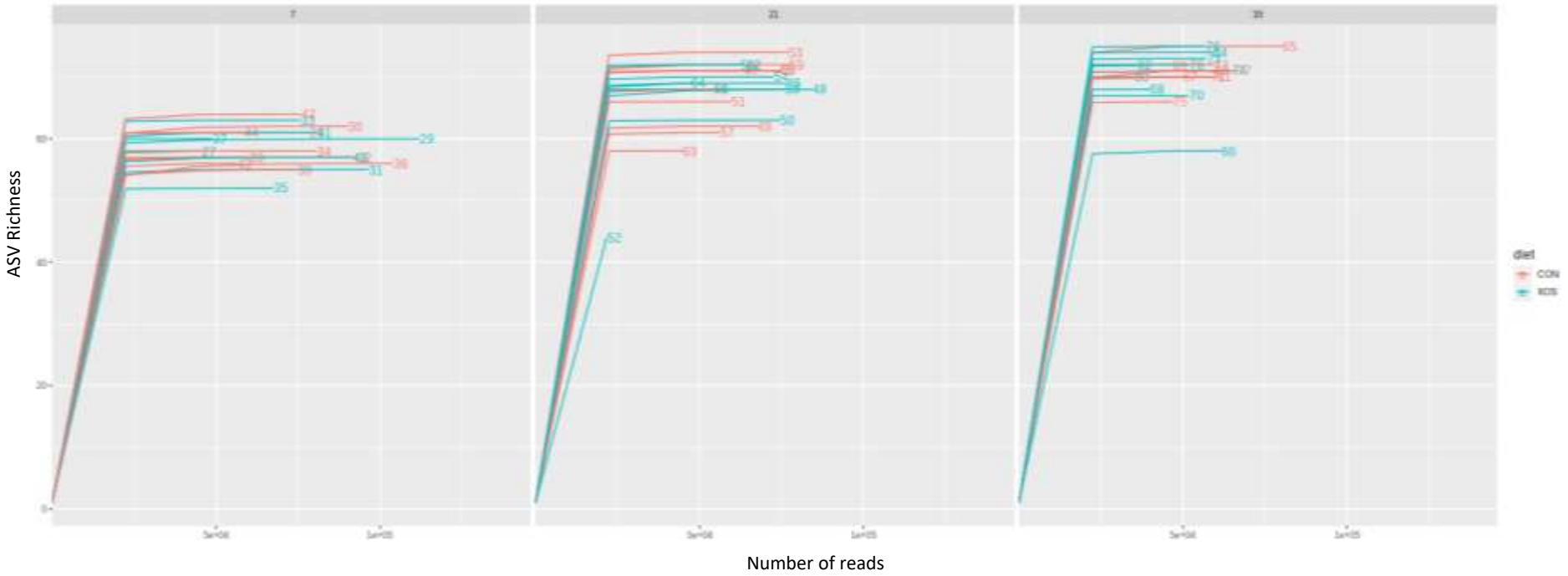
Permutational multivariate analysis of dispersion (PERMDISP) used to test for homogeneity of multivariate dispersions (i.e deviations from centroid) indicated that the individual variation in community structure was greatest in 21-day-old birds and lowest in 35-day-old birds and that microbiome community dispersion between birds of same age group did not significantly vary ($F = 0.10$, $p = 0.748$), reinforcing the age effect in PERMANOVA results. The NMDS plot based on Bray-Curtis dissimilarity also showed age related clustering with birds of the same age clustering more closely together than birds from different age groups (Figure 4.6 B). A vast majority of the 35-day-old birds shared similar community structure with 21-day-old birds.

Distribution around the centroid was greater (according to PERMDISP) within the XOS group compared to the CON indicating greater variation between individuals fed XOS diet although this difference was not significant. There was no significant difference in the microbial community structure between CON and XOS diets neither for the whole data set ($R^2 = 0.009$; p-value = 0.815) (Figure 4.6 B) or nor when comparing diets between age groups ($p > 0.05$) (Figure 4.6 C, D, E). These results indicate that the chickens shared a core set of microbiota in the cecum regardless of the dietary supplementation.

Table 4.8: Number of reads that passed through each step of the pipeline in DADA2

Factor	n	Input		Filtered		Denoised		Merged		Non-chimeric	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
By Age (days)											
7	18	149,199	22,364	149,196	22366	145339	22367	125416	23697	95981	23993
21	20	137,423	30,406	137,423	30406	131786	29118	103801	23627	77972	17399
35	19	130,367	24,438	130,367	24438	124739	23693	99996	19862	63785	12665
By Treatment											
CON	29	138445	26891	138444	26892	133426	26453	109247	24914	79122	22530
XOS	28	138671	27004	138670	27005	133568	26521	109109	24856	78500	22338

A



B

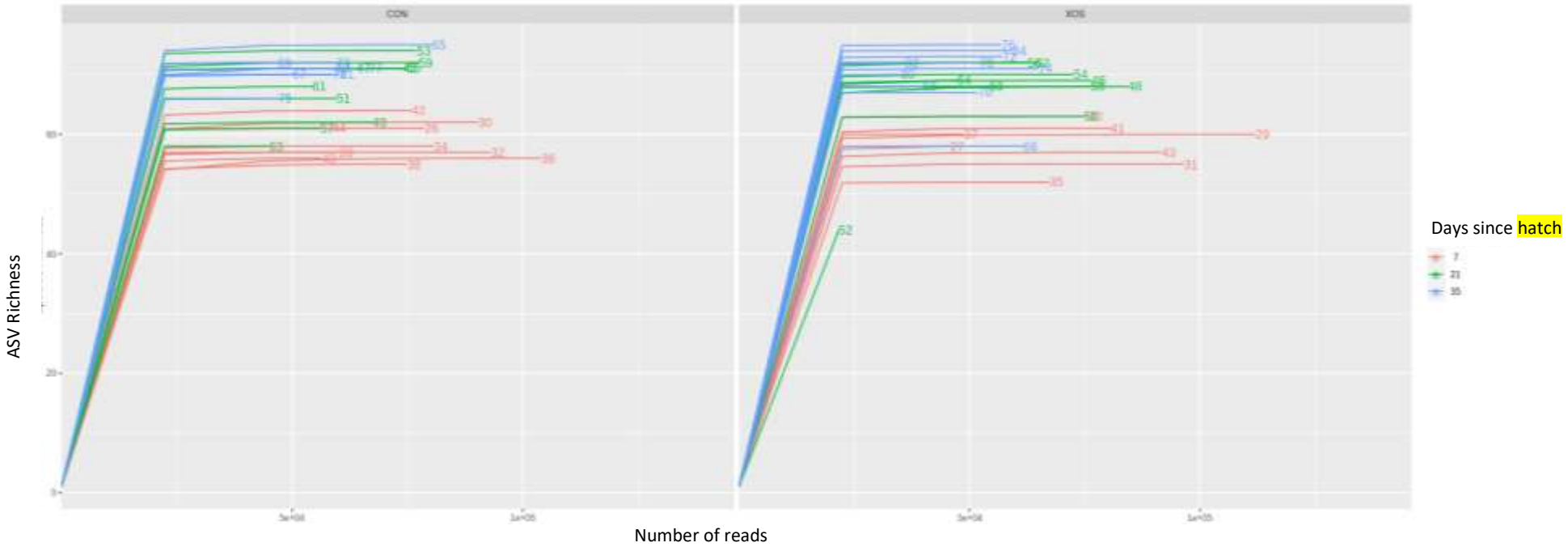
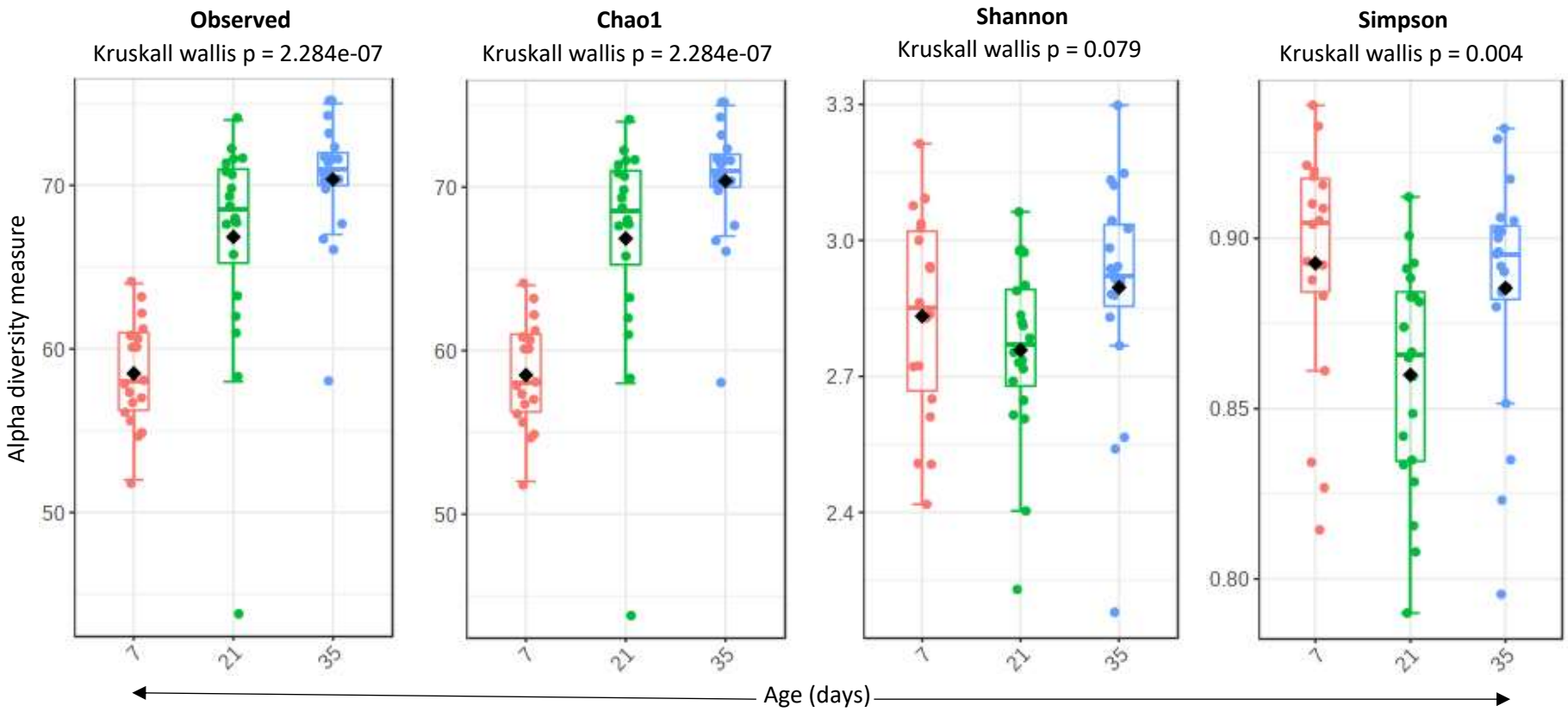


Figure 4.4.: Rarefaction curves plotting the number of observed ASVs over the number of sequencing reads per sample according to age (A) and according to diet type (B) (numbers on the curves are sample numbers in the order of loading on a 96-well plate during Illumina sequencing library preparation)

A


Observed
Kruskall wallis p = 2.284e-07
Chao1
Kruskall wallis p = 2.284e-07
Shannon
Kruskall wallis p = 0.079
Simpson
Kruskall wallis p = 0.004
Alpha diversity measure
70
60
50
3.3
3.0
2.7
2.4
0.90
0.85
0.80
7
21
35
Age (days)

B

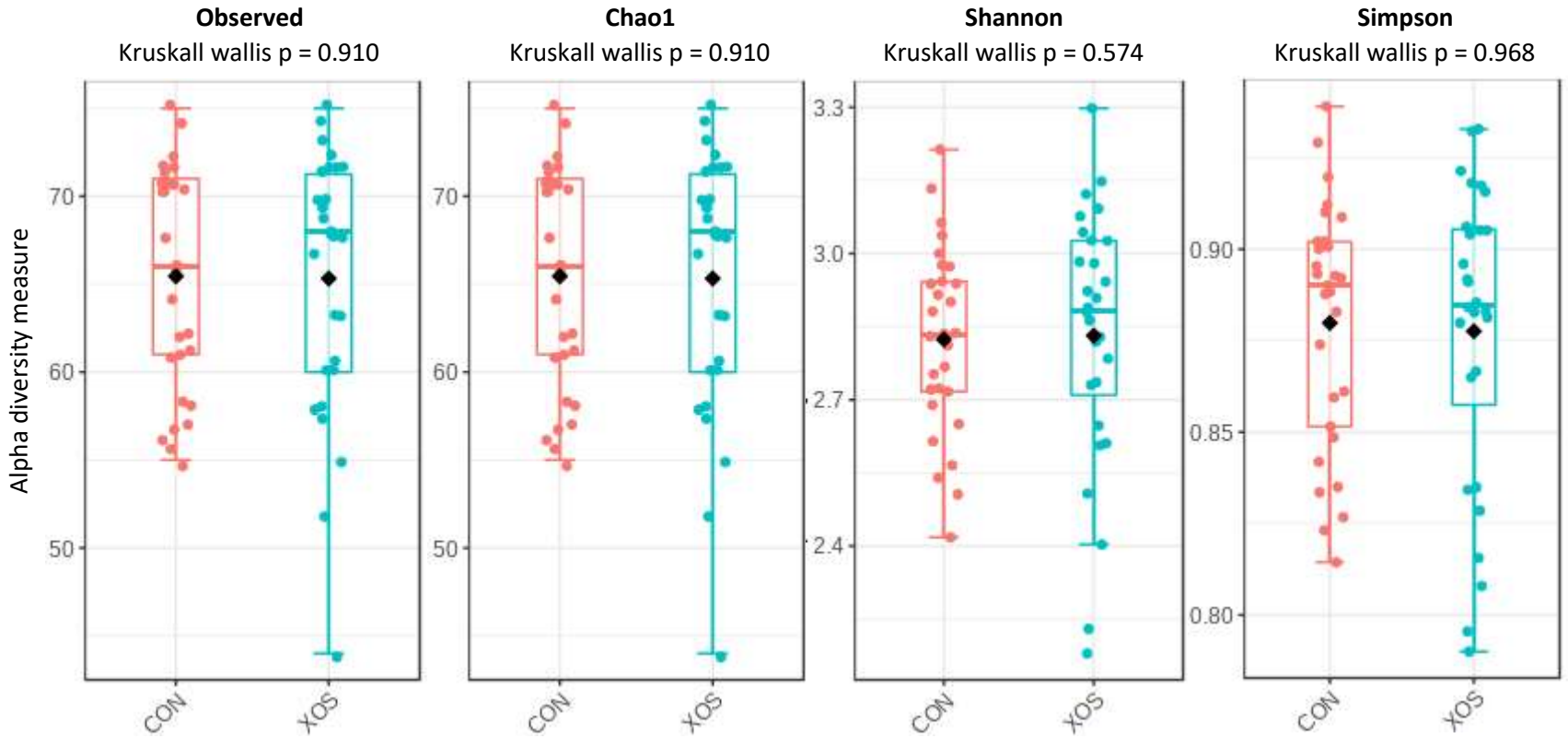
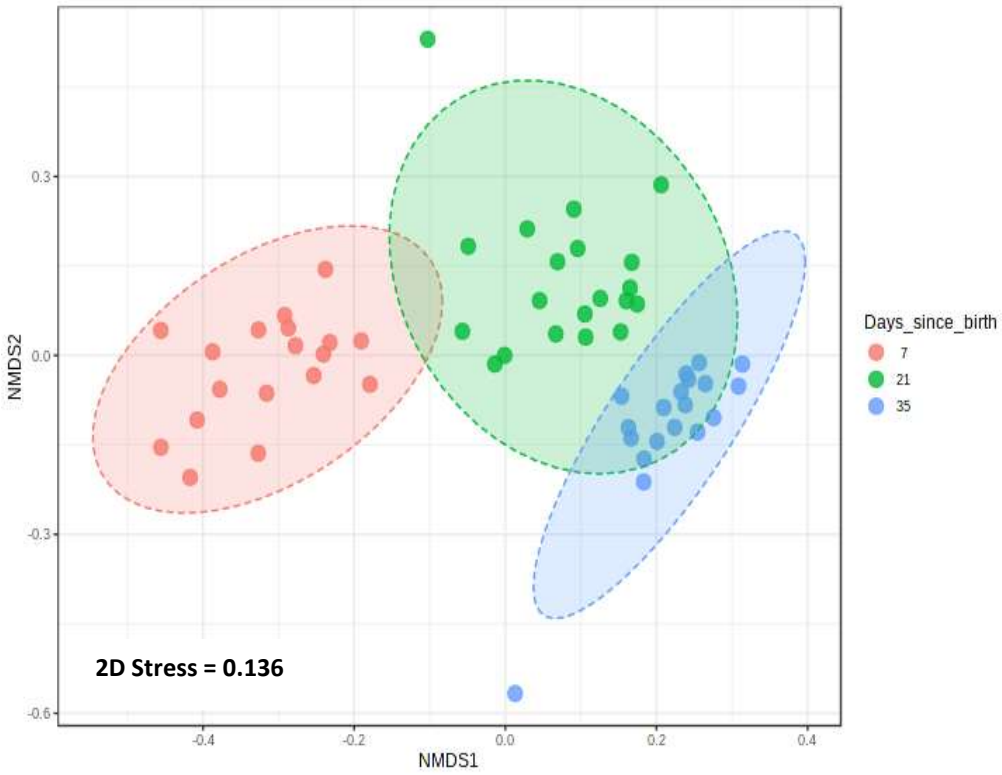
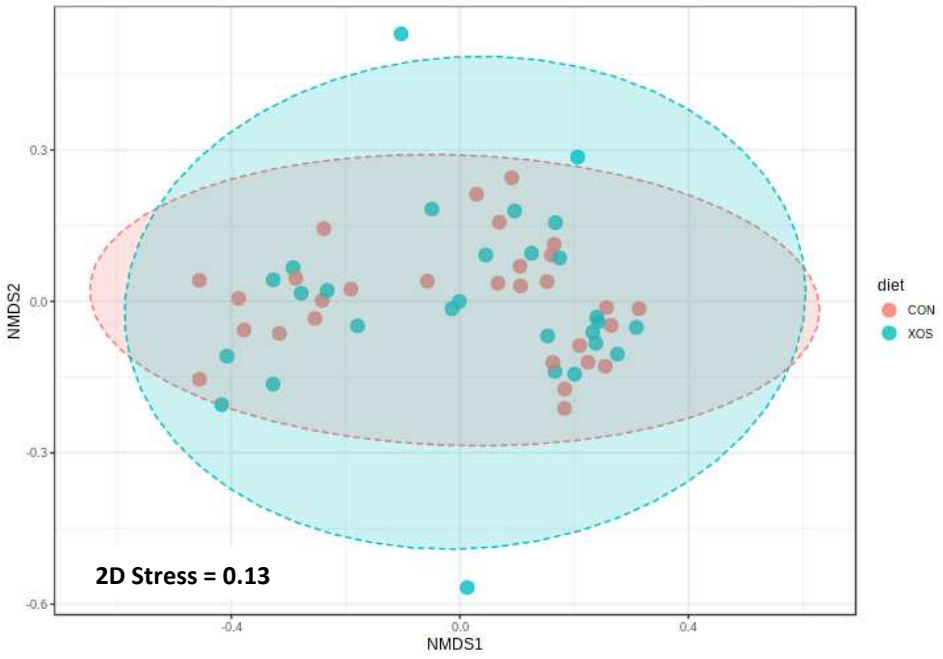


Figure 4.5: Boxplots representing alpha diversity metrics of richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson) grouped according to age (A) and diet type (B). Non-parametric Kruskal-Wallis test for multiple comparisons was conducted. Each point represents the diversity score for a sample and points are colour-coded according to age (4.5A) or diet type (4.5B). The box represents the first (Q1) and third (Q3) quartiles of the distribution and the line within the box marks the median. The whiskers extend from Q1 to Q3 to the last data points values beyond these whiskers are considered as outliers.

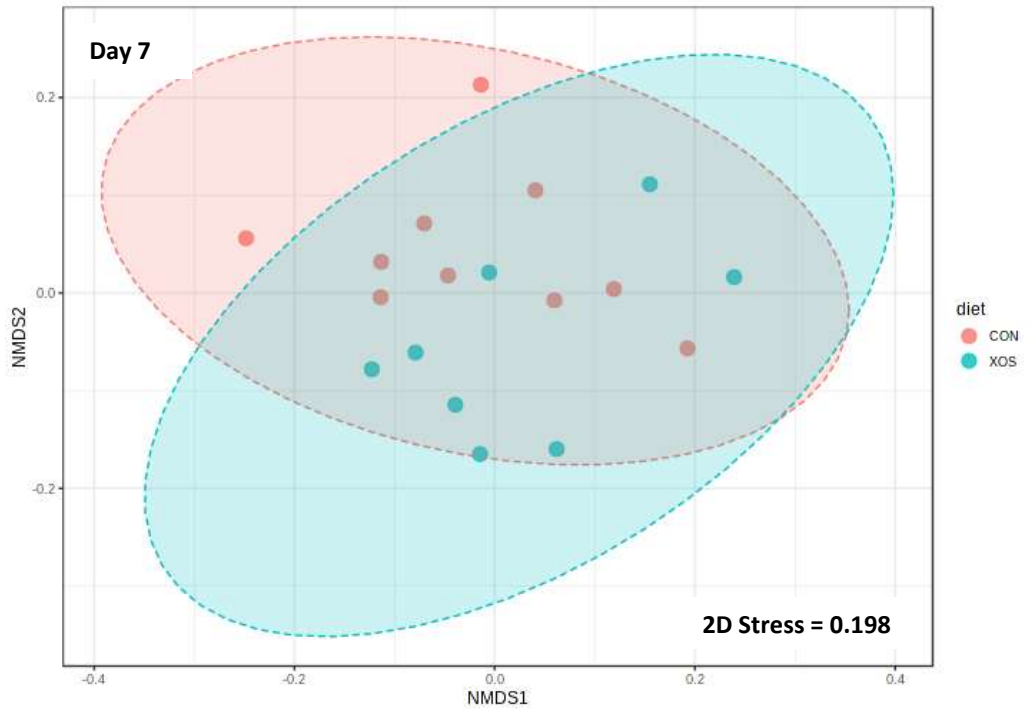
A)


NMDS2
NMDS1
0.3
0.0
-0.3
-0.6
-0.4
-0.2
0.0
0.2
0.4
2D Stress = 0.136
Days_since_birth
7
21
35

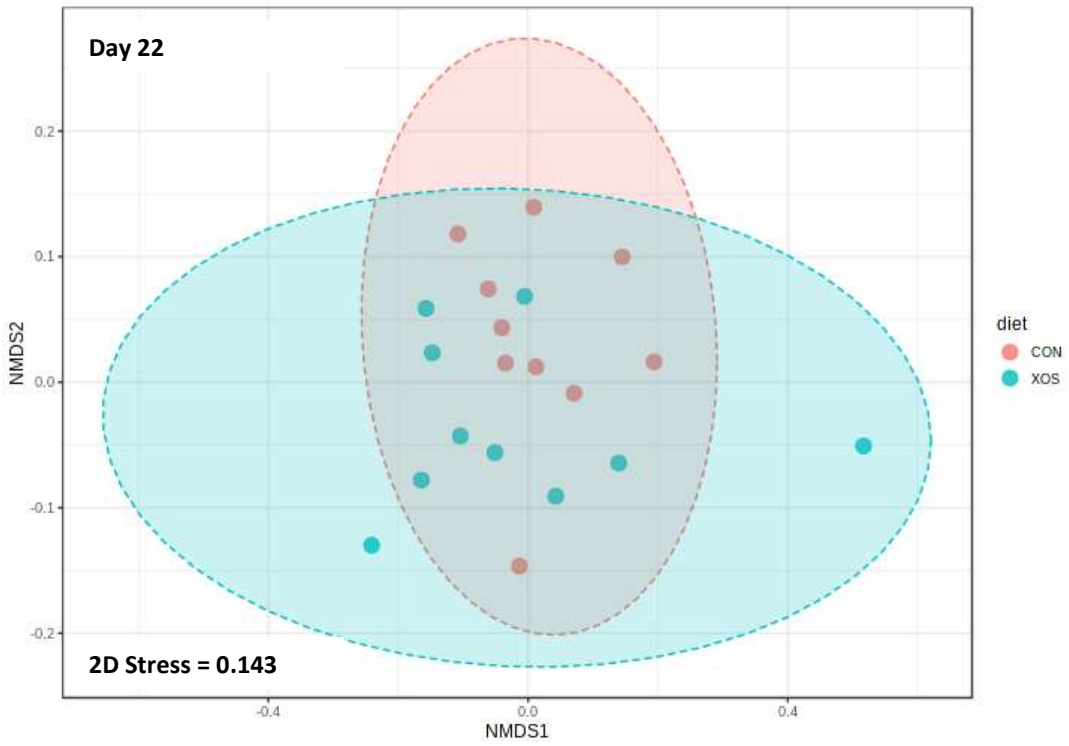
B)


NMDS2
NMDS1
0.3
0.0
-0.3
-0.6
-0.4
0.0
0.4
2D Stress = 0.13
diet
CON
XOS

C)


Day 7
0.2
0.0
-0.2
NMDS2
-0.4
-0.2
0.0
0.2
0.4
NMDS1
2D Stress = 0.198
diet
CON
XOS

D)


Day 22
0.2
0.1
0.0
-0.1
-0.2
NMDS2
-0.4
0.0
0.4
NMDS1
2D Stress = 0.143
diet
CON
XOS

E)

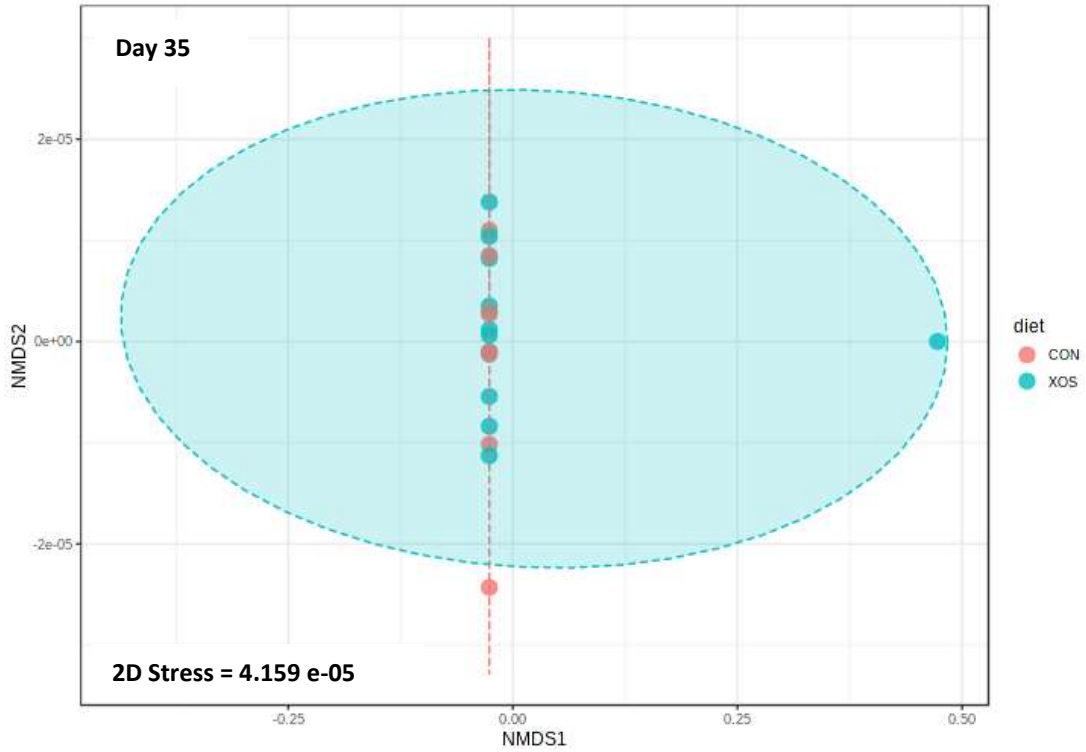


Figure 4.6: Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity matrix on relative abundance data for age (A) or diet type (B) or diet type at age 7-days (C), 21-days (D) or 35-days (E). Colours indicate age groups (A) or diet type (B, C, D, F). Ellipses indicate 95% confidence intervals of multivariate t-distribution around centroids of the groupings with age (A) or diet (B, C, D, F) as factor.

4.4.7.4 Hierarchical clustering

The dendrogram of hierarchical clustering, in agreement with the NMDS plots, showed that samples from the 3 time points formed 3 separate clusters although a couple of samples from day 21 clustered with day 35 and vice versa (Figure 4.7A).

Again, there was no clustering according to diet, neither in the whole data set (Figure 4.7B) nor within age groups.

4.4.7.5 Microbial community dynamics

There was no significant difference in the relative proportions of microbial communities according to age or diet at the phylum level when comparing the whole data set or when comparing diets within

age groups (Figure 4.8). Microbial taxa consistently present over time (core microbiome) were represented by 78 ASVs and those present in every treatment group were represented by 95 ASVs (Figure 4.11).

At the phylum level the microbial communities were dominated by Firmicutes followed by Bacteroidetes at all ages and in all diet types. The relative abundance of Bacteroidetes increased from 17% at day 7 to 30% at day 35. The minor phyla were formed by Proteobacteria and Actinobacteria in 7 and 21-day-old birds and another minor phylum Lentisphaerae appeared in the oldest birds.

Supplementation with XOS caused no significant difference in the relative abundance of any of the phylum either when analysing the whole data set or when examining the effect of diet in different age groups (Figure 4.8C)

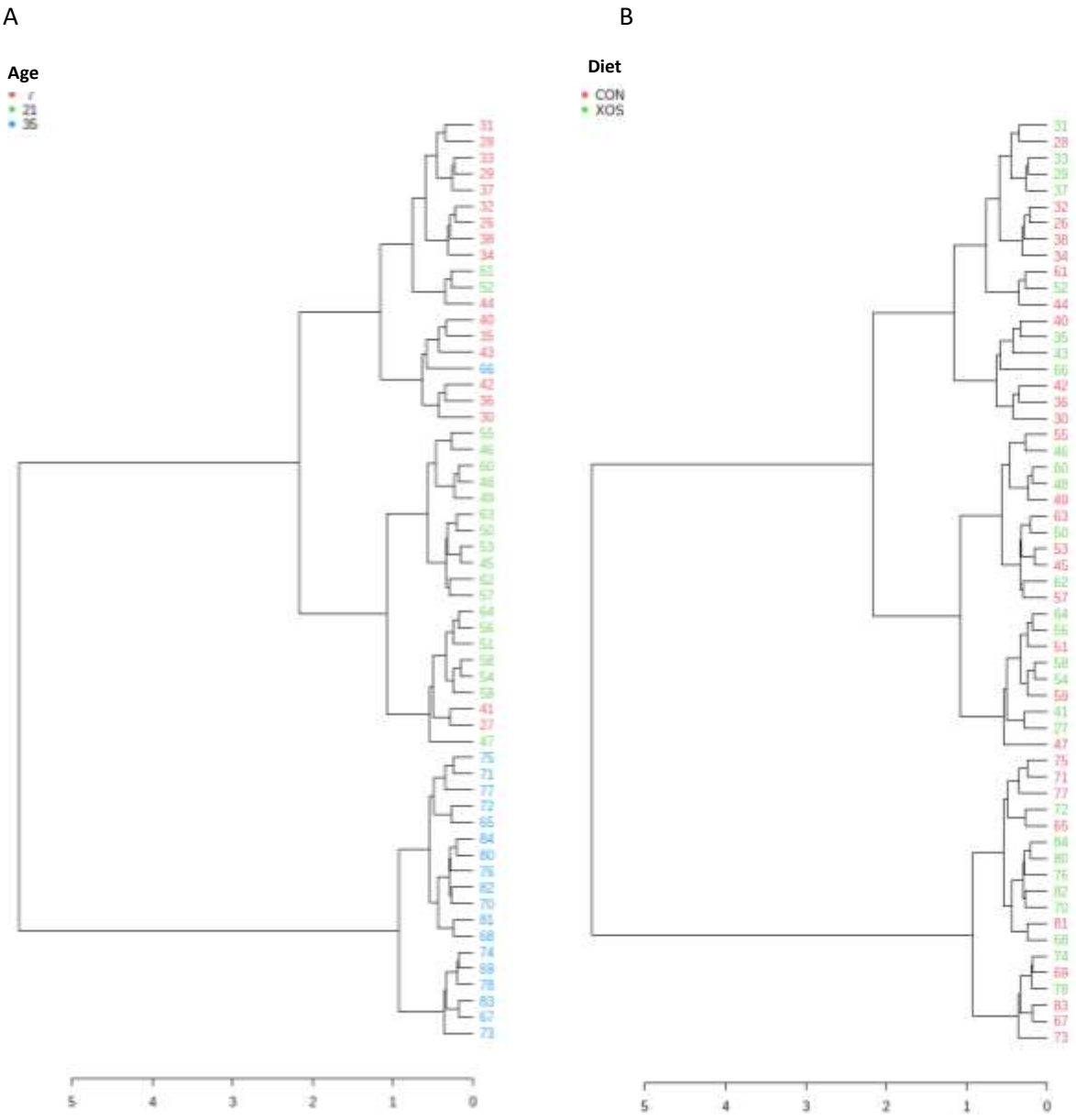
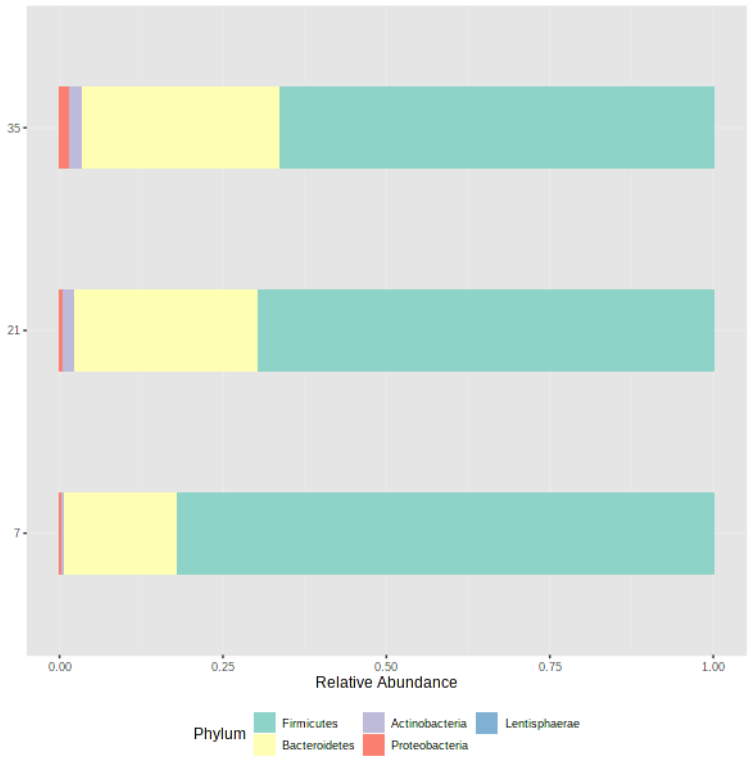
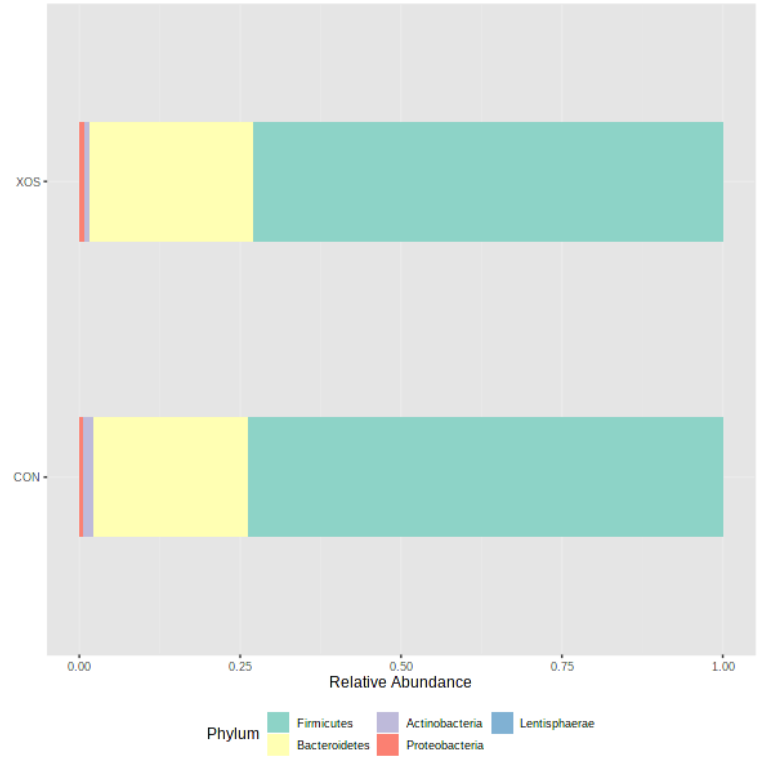


Figure 4.7.: Hierarchical clustering analysis: Dendrogram of Bray-Curtis dissimilarity matrices between samples based on different age groups (A) and diet type (B).

A


35
21
7
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Phylum
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Lentisphaerae

B


XOS
CON
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Phylum
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Lentisphaerae

C

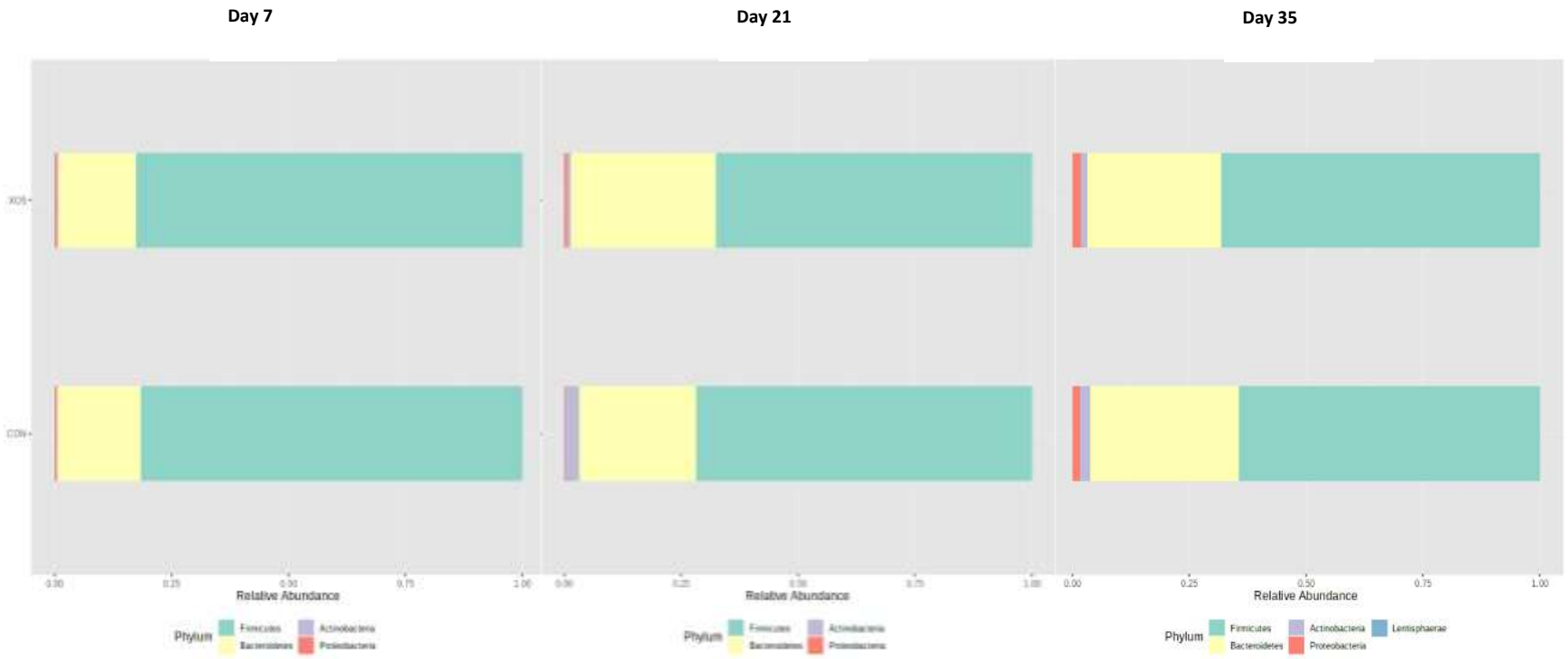


Figure 4.8: Microbial community composition of chicken caecal content at the phylum level. Stacked bar plots representing relative abundances of the different phyla in all samples according to age (A) and diet type (B) in whole data set and comparing diets within age groups (C)

a

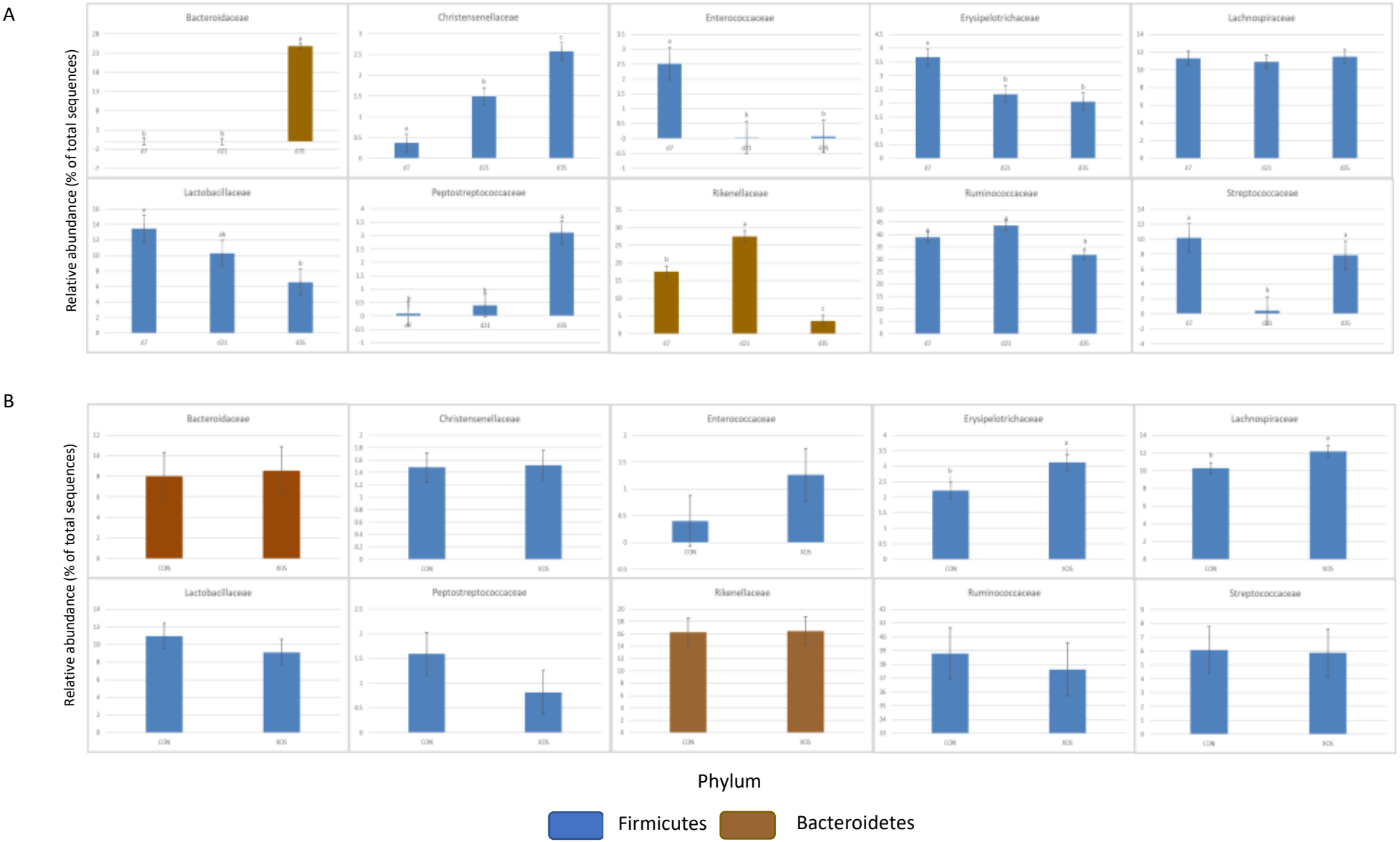
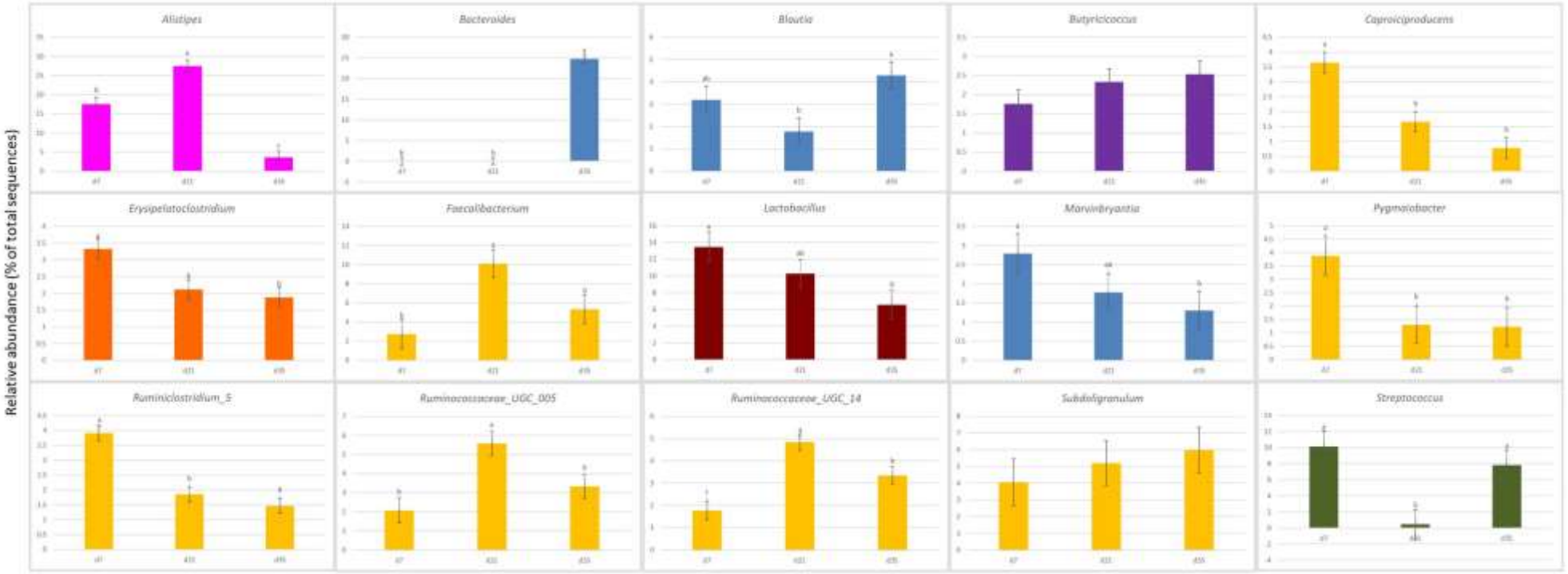


Figure 4.9: Relative abundance of the top 10 families averaged over all samples for age (A) and diet (B). Bars with different letters indicate significant difference ($p \leq 0.05$)

A


Relative abundance (% of total sequences)
Alistipes
Bacteroides
Blautia
Butyricicoccus
Coprococcus
Erysipelatoclostridium
Faecalibacterium
Lactobacillus
Marvinbryantia
Pygmaiobacter
Ruminiclostridium_5
Ruminococcaceae_UCG_005
Ruminococcaceae_UCG_14
Subdoligranulum
Streptococcus

B

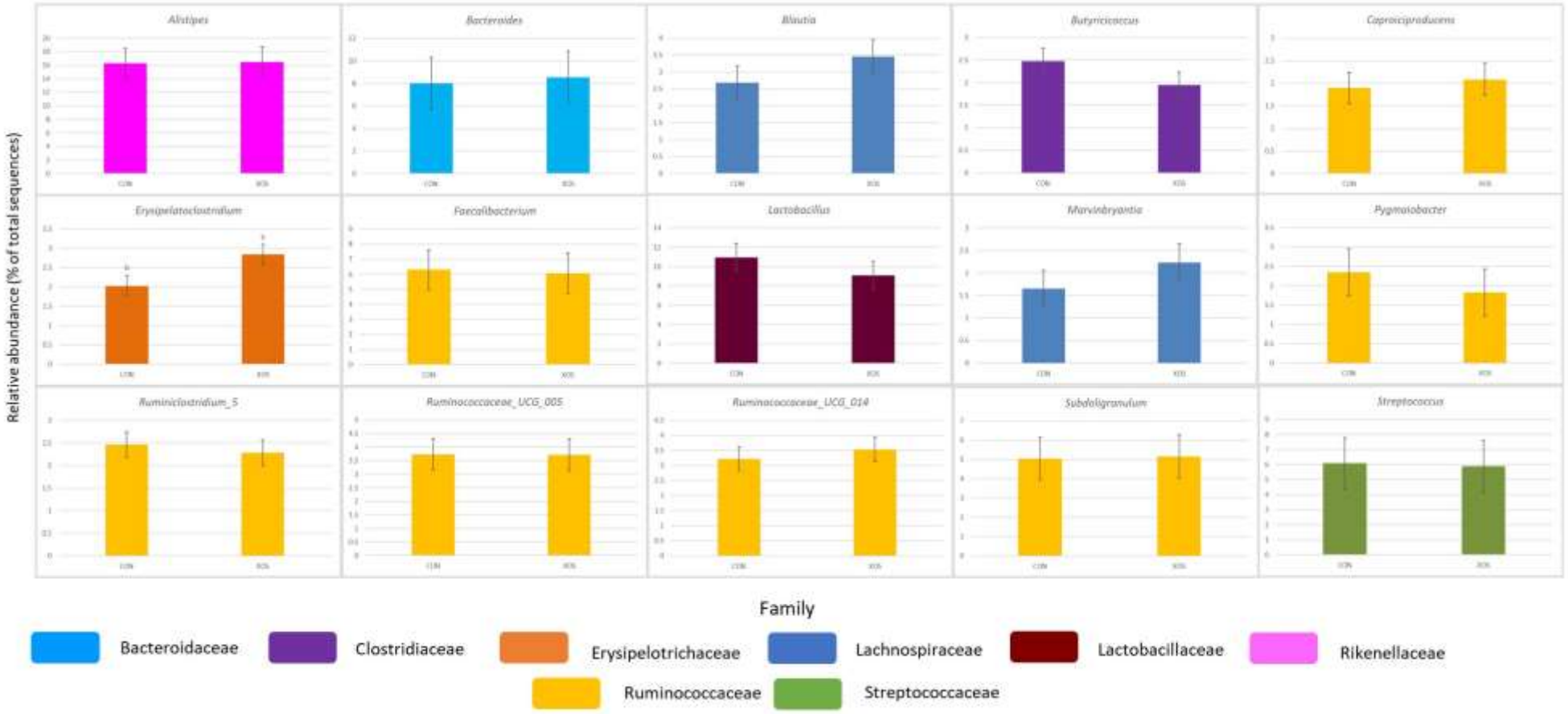


Figure 4.10: Relative abundance of the top 15 genera averaged over all samples for the age (A) and diet (B) group. Bars with different letters indicate significant difference ($p \leq 0.05$)

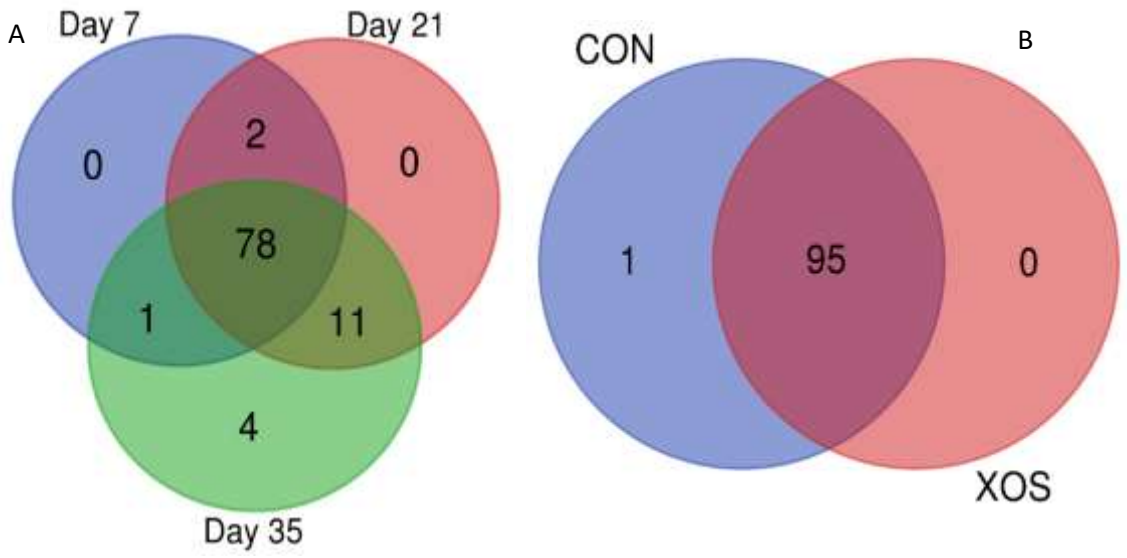


Figure 4.11: Venn diagram illustrating the number of genera unique to each and common between time points (A) and dietary treatments (B)

The order Clostridiales within Firmicutes accounted for 50% or more of the relative abundance at all times points and diet types. In fact, most of the genera identified at every sampling point and diet type belonged to the order Clostriadiales. The highest proportions of Clostriadiales was detected in 21-day-old birds (58%). In all age groups this order was composed mainly of families Ruminococcaecae and Lachnospiraceae and they formed a part of the core microbiome (Figure 4.9A and Table 4.9). There was no significant difference in the relative abundance of Lachnospiraceae at any time point but Ruminococcaceae was significantly lower ($p < 0.001$) at day 35 when compared to days 7 and 21. *Blautia* was the top genera within the family Lachnospiraceae and was present at all sampling points followed by genera *Marvinbryantia* with the former being significantly higher in oldest birds and the latter in youngest birds ($p < 0.05$) (Figure 4.10A). Within the family Ruminococcaceae, the genera were *Faecalibacterium* followed by *Subdoligranulum* which represented 16% and 13% respectively of Ruminococcaceae. Lactobacillaceae of the order Lactobacillales in Firmicutes was the next most dominant family in Firmicutes. There was a steady decline in the relative abundance of Lactobacillacecae as the birds aged with their relative abundance being significantly lower in the oldest birds ($p = 0.02$) (Figure 4.9A). Enterococcaceae another family of Lactobacillales, also showed a sharp decline with age while Streptococcaceae showed a significant decline ($p < 0.001$) at day-21 before

regaining importance at day 35 (10%, 0.4% and 8% at 7, 21 and 35 days respectively) (Figure 4.9A). *Lactobacillus* formed the entirety of the Lactobacillaceae family with the same effect as the family with respective to age. *Lactobacillus* genus was present as the core microbiome, was a biomarker of the youngest birds (LDA effect size > 6) (Figure 4.12 and Table 4.9)

When examining the overall effect of diet, the proportions of Clostriadiales were nearly the same (53%) in both groups. XOS supplemented diet significantly increased ($p = 0.03$) the relative abundance of Lachnospiraceae compared to non-supplemented group (12.2% in XOS vs 10.3% in CON) but caused no difference to the relative abundance of Ruminococcaceae (38.8% in CON vs 37.6% in XOS) (Figure 4.9B). Like Lachnospiraceae, another family Erysipelotrichaceae (order Erysipelotrichales) and its corresponding genera *Erysipeloclostridium* were also significantly higher in the XOS fed birds ($p = 0.01$). There was no significant difference in relative abundance of the family Lactobacillaceae or its genera *Lactobacillus* due to XOS supplementation (Figure 4.9B and 4.10B).

Bacteroidetes was the second most abundant phylum and its abundance increased from 17% at day 7 to 30% at day 35 (Figure 4.8A). The phylum was entirely composed of the order Bacteroidales and within Bacteroidales, families Bacteroidaceae and Rikenellaceae (Figure 4.9A). In fact, Rikenellaceae was the second most dominant family in the data set after family Ruminococcaceae of Firmicutes. Bacteroidaceae sharply increased from nearly 0% at days 7 and 21 to 25% at day 35. At the genus level, *Allistipes* of Rikenellaceae and *Bacteroides* of Bacteroidaceae were significantly higher ($p < 0.05$) at days 21 and 35 respectively and were identified as biomarkers of these ages (LDA effect size > 6) (Figure 4.10A and Figure 4.12). There was no significant effect of diet at family or genus level but a numerical increase in the two families Rikenellaceae and Bacteroidaceae and their corresponding genera was observed in birds fed XOS supplemented diet (Figure 4.9B and 4.10B)

The minor phyla Actinobacteria and Proteobacteria were present at fairly steady levels throughout the entire trial period (0.2 – 2%) (Figure 4.8A) with Atopobiaceae as the mainly family in the former and Enterobacteriaceae in the latter (data not known). Another minor phyla Lentispahaerae made

appearance in the oldest birds but for accounted for less than 1% of the total abundance (Figure 4.8A). Again, XOS supplementation had no effect on the relative abundance of any of the minor phyla or their corresponding taxa either in the whole data set or when analysing the effect of diet in different age groups (Figure 4.8B and C).

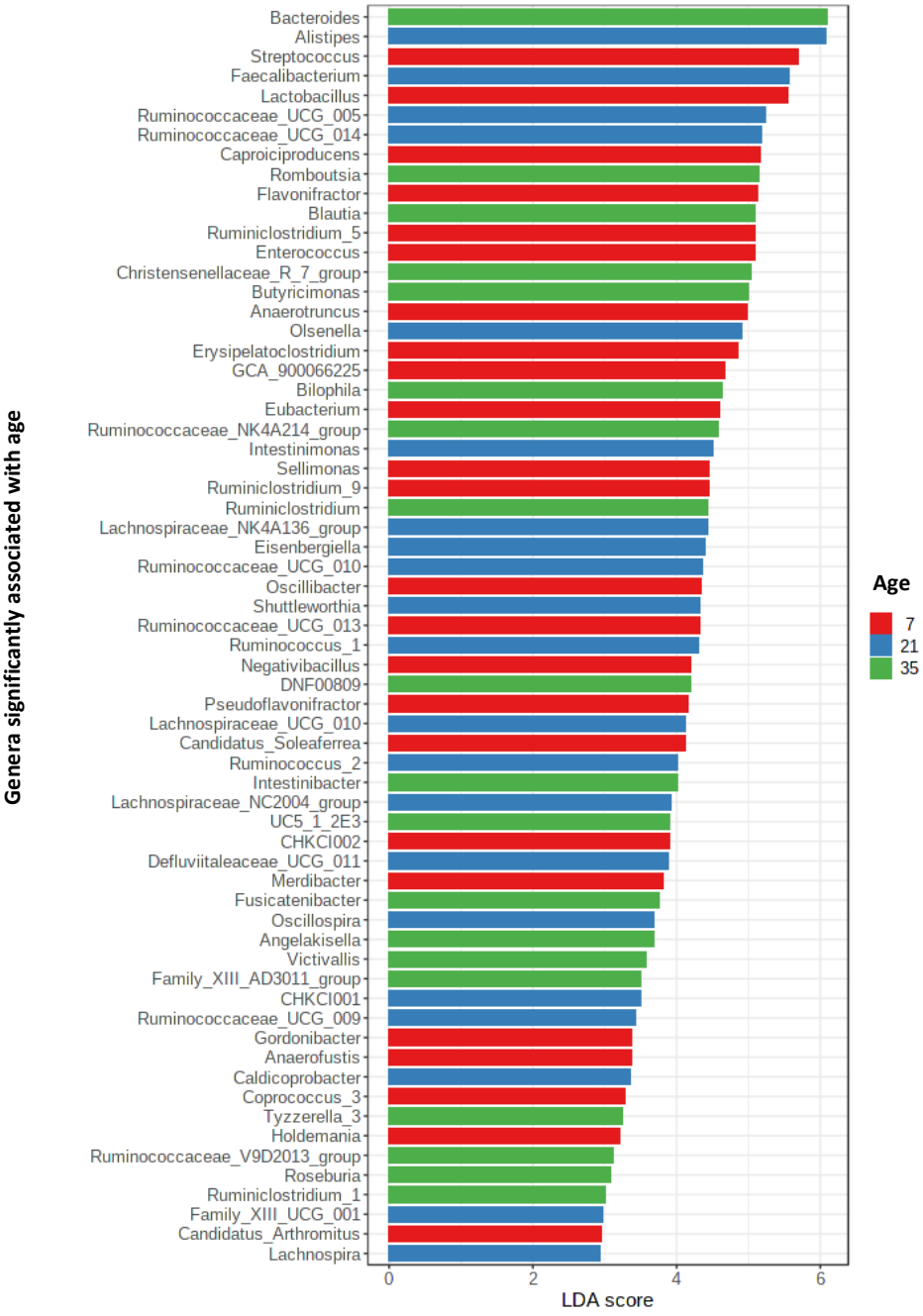


Figure 4.12: Linear Discriminate analysis (LDA) effect size (LEfSe) was used to identify genera that significantly associated with age. Forty-six different genera with LDA scores > 2.0 and p cut off value of < 0.05 (FDR adjusted) were identified and are listed in the order of descending LDA score

Table 4.9: **Microbial taxa consistently present over time (core microbiome) at family and genus level with at least 80% prevalence at each time point.** The taxa were sorted in alphabetical order.

Core families (n= 4)	Core genera (n = 7)
Erysipelptrichaeceae	*Alistipes*
Lactobacillaceae	*Erysipeloclostridium*
Lachnospiraceae	*Lactobacillus*
Ruminococcaceae	*Ruminococcaceae_UCG_005*
	Ruminococcaceae_UCG_014
	Ruminiclostridium_5
	Subdoligranulum

4.4.8 Short Chain Fatty Acids (SCFA) in the caecum: effect of age and treatment

To further investigate the mechanisms of action of XOS in promoting gut health, microbial SCFA namely, acetate, propionate and butyrate concentrations in the caecum were measured.

When diet was analysed in subsets of age groups, XOS fed birds had numerically higher concentrations of each acid at day 21 and statistically higher levels of acetate ($p = 0.021$) and propionate ($p = 0.017$) at day 35 (Figure 4.13, 4.14 and 4.15).

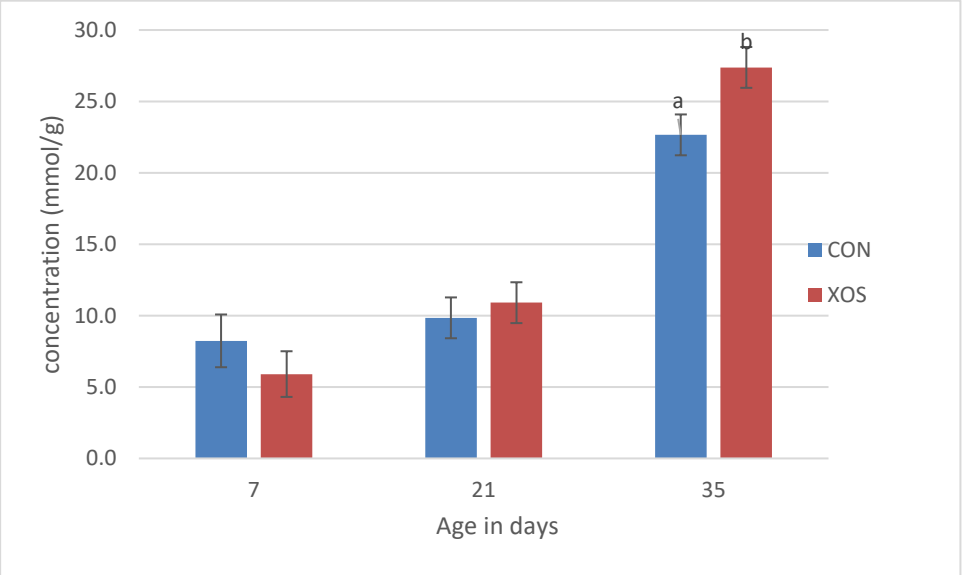


Figure 4.13: Effect of diet on the Acetate concentration in the caecum of broilers across all ages. Bars with different letters indicate significant difference.

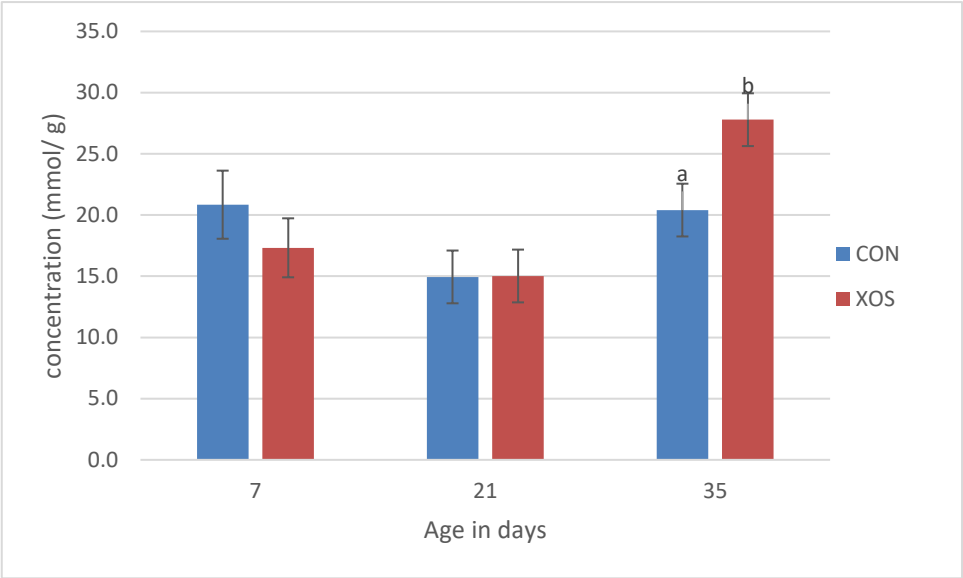


Figure 4.14: Effect of diet on the Propionate concentration in the caecum of broilers across all ages. Bars with different letters indicate significant difference.

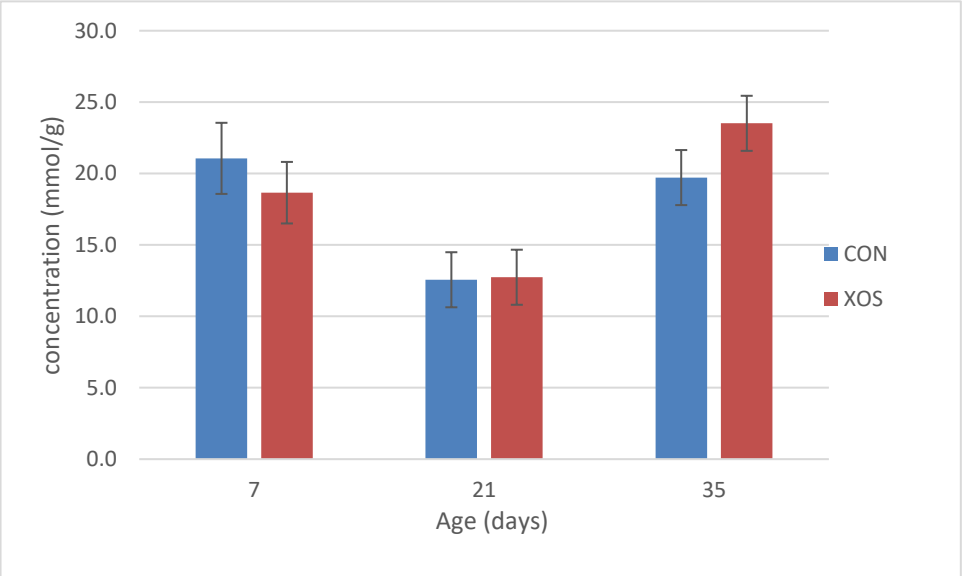


Figure 4.15: Effect of diet on the Butyrate concentration in the caecum of broilers across all ages

4.5 Discussion

In a trial conducted at the poultry research unit, it was seen that XOS did not improve the performance of birds alone or in combination with xylanase presumably because the birds were not subject to any challenge and were already performing to their maximum potential in a controlled environment and raised on a mash diet. The aim of this study, therefore, was to investigate the effects of XOS

supplementation on performance and gut health parameters of broilers raised under conditions of poor hygiene, limited temperature control and limited biosecurity.

4.5.1. Effect of diet on performance

In this trial the birds experienced temperatures significantly higher and lower temperatures than the recommended temperatures for age for Ross308 broilers. In the addition there were large differences between day and night temperatures. Broiler chicken is a homeotherm that can live comfortably only in a relatively narrow zone of thermoneutrality, 18- 21^{o}C over four weeks of age. The changes in environmental temperature below and above the thermal comfort zone can have a negative effect on bird performance. We hypothesised supplementation of XOS would ameliorate the detrimental effects of the environmental stressors on performance, intestinal microflora and gut barrier integrity. The XOS fed birds were significantly heavier than the control birds up to 28 days of age with significantly higher weight gain up to 21 days. Several studies have examined the effect of feed additives on performance of poultry under environmental stress or sub-optimal conditions. These studies suggest that nutritional supplementations of vitamins and electrolytes (Lin *et al.*, 2002; Ahmad and Sarwar, 2019), probiotics (Zulkifli *et al.*, 2000; Bilal *et al.*, 2021) , prebiotics (Houshmand *et al.*, 2012; Slawinska *et al.*, 2020), organic acids (Açikgöz, Bayraktar and Altan, 2011; He *et al.*, 2020), phytogenic feed additives (Khosravinia, 2016; Ruff *et al.*, 2021) or management practices (Abbas *et al.*, 2007) are beneficial to mitigate the detrimental effects of environmental stress on performance of chickens. To our knowledge this is the first study examining the effect of XOS on broilers grown under sub-optimal conditions. Based on a meta-analysis of broiler trials evaluating the effect of mannan oligosaccharides, Hooge (2004) suggested that feed additives may show greater improvements in performance under conditions of stress. However, in this study the improvements in body weights due to XOS were not seen in 35-day old birds. This may be because the birds in the XOS group were towards the end of their exponential growth phase at day 28 as a result of the faster biomass gain compared to the control birds. The growth rate of most living organisms resembles a sigmoidal trajectory with respect to time. This implies that the growth rate increases sharply at the

initial stage, reaching a peak, and then decreases to zero when the weight or biomass of the organism reaches its maximum. Therefore, in this study, whilst inflection point of the XOS fed birds was perhaps near 28 days, the growth rate of the control birds was still in its exponential phase. Marcato et al. (2008) estimated the body weight and nutrient deposition parameters of Ross and Cobb broiler breeds using Gompertz equation and found that the highest growth rate or inflection point for live weight of male Ross broilers occurred at 42 days. The earlier peak in growth rate of the XOS fed bird in this trial may be due the supplementation of XOS while the birds in the study by Marcato et al. (2008) were fed a diet free of additives. Another reason for the lack of performance improvement at day 35 could be adverse temperatures between days 20 and 30. During this period the birds experienced high temperature of 30 – 33^{o}C and low temperatures of 11 – 13^{o}C coupled with high relative humidity of 70%. It is therefore evident that the birds were under heat stress. Heat stress results in reduced feed intake and decline in production efficiency (Lara and Rostagno, 2013). It is believed that heavier birds are more susceptible to heat stress (Petracci and Cavani, 2011). As the XOS fed birds were heavier than the control birds up to 28 days of age, therefore reduction in weight gain was perhaps more pronounced in this group.

4.5.2. Effect of diet on diversity and composition of caecal microbiota

Like the previous trial, the effect of XOS on caecal microbiota was studied by sequencing the V3-V4 region of the 16S rRNA gene using the Illumina MiSeq platform. There were no significant differences in the α-diversity metrices of richness or evenness due to XOS supplementation. Neither was there any diet related clustering (β diversity) as revealed by the dendrogram and NMDS plots. This suggests that XOS did not alter the overall structure of the caecal microbiota. Some studies have shown that diet and feed additives (Apajalahti et al., 2004; Stanley et al., 2013; Pourabedin et al., 2014) are common factors that influence the gut microbiome diversity, composition, and structure, but opposite results have also been reported (Oakley *et al.*, 2014; Thibodeau *et al.*, 2015; Ranjitkar *et al.*, 2016). Firmicutes and Bacteroidetes were the most dominant phyla in all samples and XOS did not alter the levels of these phyla at any time point. These phyla are considered to be the two most abundant

bacterial phyla in the cecum of broilers and their importance in host metabolism has been highlighted (Kers *et al.*, 2018). An increased ratio of Firmicutes/Bacteroidetes has been shown to be associated with obesity in humans and mice due to the increased energy harvesting capacity of members of the Firmicutes phylum (Turnbaugh *et al.*, 2006, 2009).

XOS supplementation significantly increased the relative abundance of two families, Erysipelotrichaceae and Lachnospiraceae. Erysipelotrichaceae is a relatively new family that was first described by Verbarg et al. in 2004 (Verbarg *et al.*, 2004). Erysipelotrichaceae have been isolated from the faeces, oral cavity, and GIT of mammals (Kim *et al.*, 2019) but, most members of Erysipelotrichaceae have not yet been cultured, and their genomic and functional characteristics have not thus far been elucidated (Kaakoush, 2015). Few studies have reported the members of this family to be associated with host metabolic disorders and inflammatory diseases. For instance, Martinez et al. (2009) observed a sharp association between the presence of Erysipelotrichaceae and host cholesterol metabolites. Fleissner et al. (2010) found Erysipelotrichaceae in increased abundance in mice that were fed a high-fat diet. Kaakoush (2015) and Nagao-Kitamoto et al. (2016) found that in human and animal models the gut levels of Erysipelotrichaceae change during the development of Inflammatory bowel disease. Moreover, some members of Erysipelotrichaceae are highly immunogenic and broad-spectrum antibiotic producers (Nagao-Kitamoto *et al.*, 2016). In chronic HIV infections the relative abundance of *Erysipelotrichi* was positively correlated with TNF-α levels (Dinh *et al.*, 2015). An unclassified Erysipelotrichaceae was shown to have a stronger ability to bind with IgA than other members in the gut microbiota (Palm *et al.*, 2014). However, there is no data on the functional capabilities of Erysipelotrichaceae to metabolize carbohydrates. Very recently, Wu et al. (2021) isolated five novel strains of Erysipelotrichaceae from commercial pigs and provided the first insights into their genome. Based on the functional classification of coding sequences by annotating them to the KEGG database, the authors found that in these strains, 50% of the genes were related to metabolism of carbohydrate, nucleotide and amino acids. Using whole gemome sequencing and functional prediction they predicted the presence of polysaccharide utilization loci (PUL) in the

genomes of 30 strains of Erysipelotrichaceae. Most of these strains were predicted to degrade carboxymethylcellulose, xylan, beta-glucan, and lichenan that are plant polysaccharides. Therefore, the enrichment of Erysipelotrichaceae in the XOS fed birds may indicate their possible role in breakdown of complex plant polysaccharides.

Like, the previous trial described in chapter 3, Ruminococcaceae and Lachnospiracecae were two of the most dominant families across all samples, Lachnospiraceae being significantly and Ruminococcaceae being numerically abundant in the XOS fed birds. The role of these families and their corresponding genera in degradation of dietary polysaccharides including xylan has been discussed under section 3.5 of chapter 3.

Unlike the previous trial where there was complete absence of Bacteroidetes, their relative abundance in this trial was approximately 25% in both, control and XOS groups. Under Bacteroidetes, the family Bacteroidaceae and genus *Bacteroides*, which were numerically enhanced in the XOS group, harbours a very broad saccharolytic potential with some strains being to target dozens of complex glycans. Much of the glycan degrading and import machinery within Bacteroides genomes are encoded within clusters of coregulated genes known as polysaccharide utilization loci (PULs). Species of Bacteroides isolated from the human gut microbiota have shown to harbour an extensive xylan degrading system that can respond to different forms of the xylose polymer (Rogowski *et al.*, 2015). This explains the increase in the abundance of *Bacteroides* in the XOS fed birds. Another genus of Bacteroidaceae, Alistipes which was also numerically enhanced in the XOS supplemented diets, was shown to have a positive correlation with broiler performance (Torok *et al.*, 2011). Alistipes were found to be significantly increased in broilers fed an amorphous cellulose supplement (De Maesschalck *et al.*, 2019). Although not much is known about their ability to degrade xylan or utilize xylose, their enrichment in a cellulose supplemented diet reflects their ability to utilize complex plant polysaccharides. *Alistipes* produce succinate as their major end product of fermentation (Rautio et al., 2003; Reichardt et al., 2014) that can deliver energy in 2 discrete ways. Firstly, succinate can be

taken up directly by chicken intestinal cells through a sodium-dependent transport system (Kimmich et al., 1991) and then further introduced in the Krebs cycle. Secondly, many other *Bacteroidetes* bacteria can utilize succinate and convert it into propionate after decarboxylation, which seems to be the most important route for propionate formation (Reichardt et al., 2014). Propionate in turn can be used as an energy source by the epithelial cells and is known to have health-promoting effects, including an anti-inflammatory activity, which may influence performance (Hosseini et al., 2011; Vinolo et al., 2011).

Contradictory to the trial described in chapter 3, the abundance of lactic acid producing genera, *Lactobacillus* and *Streptococcus* non significantly decreased in the XOS fed birds. This may be because of the large abundance of Bacteroidetes producing mainly acetate and propionate as the end products of carbohydrate metabolism (Venegas *et al.*, 2019). Lactobacillus under in vitro conditions was unable to grown on medium containing acetate and also did not co-metabolize it with glucose (Jyoti, Suresh and Venkatesh, 2003). This may indicate that the presence of metabolites produced by other gut microbiota may deter the growth of Lactobacillus.

Many studies have investigated the impact of heat stress on the intestinal microbiota of broilers either with (Abdelqader and Al-Fataftah, 2016; Al-Fataftah and Abdelqader, 2014; Sohail et al., 2015; Song et al., 2014) or without feed additives (Burkholder *et al.*, 2008; Shi *et al.*, 2019). Most of these studies agree that heat stress reduces the viable counts of beneficial *Lactobacillus* and *Bifidobacterium* in the small intestinal contents and while probiotics were successful in increasing the counts of these bacteria, butyric acid supplementation had no significant effects on the microbiota (Abdelqader and Al-Fataftah, 2016; Al-Fataftah and Abdelqader, 2014; Song et al., 2014). Although a direct comparison between these studies and the present trial is not possible due to lack of a control group of birds raised under optimum temperature conditions. A comparison, however, could be made between the microbiota of the XOS fed birds and that of the control birds at day 35 to get an indication of the effect of XOS under heat stress considering that the birds experienced hot temperature spells between 16

and 27 days. The were no significant differences in the overall community structure at either phylum, family or genus level due to supplementation of XOS at day 35. However, the abundance of several families and genera were found to be numerically higher in the XOS group. These include families, Christenellaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobaccillaceae and Streptococcaceae and genera *Blautia, Erysipeloclostridium, Lactobacillus, Marvinbryantia, Ruminoclostridium* and unclassified genera of Ruminococcaceae. The lack of significant differences was perhaps because the administered dose of XOS was insufficient to cause significant increases in beneficial bacteria.

4.5.3. Effect of diet on caecal SCFA concentrations

The concentration of acetate increased with age in both CON and XOS groups but at day 35 acetate was significantly higher in the XOS supplemented diet. On the other hand, the concentration of propionate and butyrate decreased at day 21 before increasing again day 35. And at the final time point, propionate was significantly higher while butyrate was numerically higher in the XOS fed birds. Pourabedin et al. (2015) also reported numerically but not statistically higher concentration of these acids in birds fed either 1 g/kg or 2 g/kg XOS when compared to the control at days 15, 25 and 35. Similarly, Yuan et al. (2018) reported significant increase in acetate and butyrate in broilers fed 2 mg/ kg XOS in their diet on days 21 and 42. The increases in SCFA concentrations in the XOS fed birds may partially explain the improvements in body weights of these birds up to 28 days. The significance of SCFAs particularly butyrate is poultry nutrition is well established. Several studies have attempted to improve bird performance by increasing SCFAs levels in the GIT either via direct supplementation of these acids in the diet or by addition of pre or probiotics or enzymes in feed that increase SCFA *in situ* (Immerseel and Buck, 2004; Berni Canani *et al.*, 2011; den Besten *et al.*, 2013; Onrust *et al.*, 2015; Walugembe *et al.*, 2015; Ahsan *et al.*, 2016; Moquet *et al.*, 2016; Deepa *et al.*, 2018). It is still ambiguous, though, that increase in SCFAs at day 35 in this study does not translate into improvements in body weights and weight gains at this age. Many studies have examined the effect of pre and probiotics in alleviating the detrimental effects of environmental stress in broilers, but these studies have not investigated SCFA in the caecum as a response variable to the supplement (Al-

Fataftah and Abdelqader, 2014; Sohail et al., 2015; Song et al., 2014a; Pourabedin et al., 2014; Sohail et al., 2011; Ashraf et al., 2013). However direct supplementation of butyrate, its acid or salt in the diet of chickens subjected to high temperatures was shown to have positive effects on either performance, meat quality, liver function and gut morphology and integrity (Abdelqader and Al-Fataftah, 2016; Abdelqader *et al.*, 2017; Lan *et al.*, 2020). In addition, butyrate also has proven beneficial effects on intestinal barrier function (Willemsen *et al.*, 2003; Wang *et al.*, 2012), oxidative status (Zhang, Gao, *et al.*, 2011; Sunkara, Jiang and Zhang, 2012), cell growth and differentiation (Blottiere et al., 2003; Le Blay et al., 2000) and is an immunomodulator (Zhang, Jiang, *et al.*, 2011; Zhou *et al.*, 2014; Xu *et al.*, 2016). It was therefore thought to be interesting to examine if XOS improved the gut integrity function studied in this indirectly via SCFAs.

4.5.4. Effect of diet on gene expression in the ileum

In this study XOS significantly improved the expression of mucin (MUC2) glycoprotein and one tight junction protein, occludin (OCLN) in 35 day-old birds. Expression of another tight junction (TJ) protein, claudin 5 (CLDN5) and secretory IgA was numerically increased while that of a pro-inflammatory cytokine, IL-1β was decreased. The integrated intestinal barrier including junctional complexes (TJ, adheren junctions and desmosomes) which seal the adjacent epithelial cells, mucin layer and *sIgA* residing within the mucin layer is of immense importance to the epithelial integrity and cell function (Song et al., 2013). The epithelium is permeable to nutrients and macromolecules but provides an effective barrier against luminal antigenic agents, such as bacteria, toxins, and feed-associated antigens. Impairment of this barrier function leads to increased permeability to luminal antigens, which gain access to subepithelial tissues and result in inflammation, malabsorption, and potentially systemic disease (Moeser et al., 2007). However, no study till date has been published the effect of XOS on intestinal integrity in broilers raised under sub-optimal conditions and experienced temperatures much lower or higher than the recommended temperatures for Ross308 broilers on the vast majority of the days. Hyperthermia leads to disruption of intestinal epithelial integrity mainly by affecting the TJs and adheren junctions (AJ). Damage to TJs and AJs facilitates the transfer of luminal

toxins or pathogens into the lamina propria, that harbours numerous immune cells thus activating and exaggerating the inflammatory reactions, which may further worsen the intestinal damage (Lian *et al.*, 2020). Heat stress also decreases the expression of the mucin-2 glycoprotein (Zhang *et al.*, 2017). The upregulation of *MUC*-2, TJ proteins and *sIgA* and decreased expression of IL-1β in XOS fed birds in this study may indicate the role of XOS in ameliorating the detrimental effects of environmental stress on gut barrier integrity. It is noteworthy that the levels of acetate and propionate and butyrate are also enhanced in the XOS fed birds at day 35. Wang et al. (2012) reported that butyrate upregulates TJ protein Claudin1 in intestinal epithelial cell lines. In another study butyrate reduced the expression of IL-1β in macrophage cells stimulated with LPS of *Salmonella typhimurium* (Wang *et al.*, 2012). This also agrees with a study on neonatal pigs which demonstrated that supplementation of sodium butyrate in the diet led to down regulation of IL-1β (Wang *et al.*, 2012). These evidence, together with the results of SCFAs, suggest that XOS supplementation increases SCFAs which in turn regulate the expression of proteins involved in gut barrier integrity.

The above results support the hypothesis that XOS improves performance and gut health parameters of broilers raised under sub-optimal conditions. They also support the results from other studies which demonstrate that the effect of feed additives may be more pronounced under challenging conditions. In future studies it would be noteworthy to investigate the effects of XOS on biomarkers of heat stress such as heat shock protein (HSPs) that bind to and protect misfolded cellular proteins, a typical sign of heat stress and the cellular redox defence system proteins (glutathione, glutathione peroxidase, superoxide dismutase, and haem oxygenase 1) which are activated in response to reactive oxygen species induced by heat stress.

4.6. Conclusion

XOS improves performance and mitigates the detrimental effects of heat stress on microbiota and gut integrity possibly via improvements in SCFA concentrations.

Chapter 5: Effect of XOS on the carbohydrate breakdown capacity of the caecal microbiota

5.1. Introduction

Monogastric diets inevitably contain substantial amounts of xylans or arabinoxylans, the non-starch polysaccharides that form the cell wall of cereal grains such as wheat, maize or barley that make up 50 – 60% of the monogastric diets. It is well documented that monogastric farm animals (chickens and pigs) do not produce the enzymes needed to breakdown the arabinoxylans. Hence there has been significant interest in monogastric nutrition to enhance the arabinoxylan degrading or fibre fermenting microbiota in the GIT to curtail dysbacteriosis and improve energy extraction from the fibre portion of feed. The term stimbiotic (STB) has been recently presented and was defined by González-Ortiz et al. (2019), as "*an additive that stimulates a fibre-degrading microbiome resulting in an increase in fibre fermentability even though the additive itself contributes little to short chain fatty acid production.*" The concept of STB is that, they are not quantitatively fermented by the microbiome like other prebiotics, but instead they enhance the fermentation of fibre that is already present in the diet (Bedford, 2019). For example, supplementation of broiler diets with 0.1 g/kg and piglet diets with 0.2 g/kg XOS improved performance. From an energy contribution viewpoint, 0.1 g XOS only contributes 0.3 kcal/kg of energy to the diet thus highlighting the mechanism cannot consist of quantitative fermentation alone (Liu et al., 2018; Ribeiro et al., 2018). The results presented in chapter 4 of this thesis also showed that 0.1 g/kg XOS significantly increased body weights of broilers raised under sub-optimal conditions up to 28 days of age. Many commercialized prebiotics used in the animal industry such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and mannan-oligosaccharides (MOS) are all understood to be quantitatively fermented into SCFA. However, dietary supplementation with XOS or *in vivo* creation of XOS in the GIT via the addition of supplemental xylanases likely result in insignificant increments in SCFA directly but significant increments indirectly by preferentially stimulating the growth and activity of beneficial bacteria such as *Bifidobacterium* and other lactic acid producing bacteria and lactate consuming-butyrate producing bacteria in the hind gut of monogastric animals (Cho *et al.*, 2020).

5.1.1. Analysis of proteins expressed by caecal microbiota using Mass-spectrometry

In order to study the STB effect of XOS, its effect on enzymes expressed by the microbiota was examined using Mass-spectrometry. The hypothesis being, for XOS to act as a STB, it would need to up regulate the fibre or complex polysaccharides degrading machinery of the caecal bacteria. Mass-spectrometry is a highly sensitive technique to analyse complex cellular proteomes and low-abundant proteins (Tyers and Mann 2003). Proteomics in general can link genotype to phenotype through a wide-ranging determination of gene or cellular function at the protein level. MS-based proteomics approach is a powerful tool to characterise the structure and function of proteins. To date, MS-based instruments (mass spectrometers) have been employed for proteomic analyses such as protein primary sequencing, post-translational modifications, protein-proteins interactions and secretome (proteins that are secreted extracellularly) (Mishra 2010). Generally, mass spectrometers have three main components (Figure 5.1). First, is the ionisation source, which converts peptides or proteins mixture into positively and negatively charged ions based on the nature of a mixture. Second, is the mass analyser, which sorts and separates ions based on their mass to charge (m/z) ratio. And finally the detector, which detects ions sorted by the mass analyser (Lemière 2001). The development of soft-ionisation methods such as MALDI (matrix assisted laser desorption ionisation) and ESI (electrospray ionisation) has enabled the proteomics field to accurately measure peptide masses and identify their sequences.

5.1.1.1. Mass spectrometry based proteomics

Modern proteomics is dominated by mass spectrometry based approaches which are able to sequence protein and peptides and match against known proteomes using a technique called “tandem mass spectrometry”(tandem mass spectrometer, 2008). An in depth review of mass spectrometry as it related to proteomics is out of the scope of this thesis; briefly:

A mass spectrometer ionises the sample as it is introduced into the instrument and by separation of the charged ions prior to detection by time or space, the *m/z* or “mass to charge ratio” can be

calculated and ultimately the mass of the analyte which will give useful information. By means of fragmentation of peptide ions between two areas of "separation" or ion-filtering, the amino acid sequence of peptides can be elucidated (see Figure 5.1).(Cañas Montalvo *et al.*, 2006; Han, Aslanian and Yates, 2008)

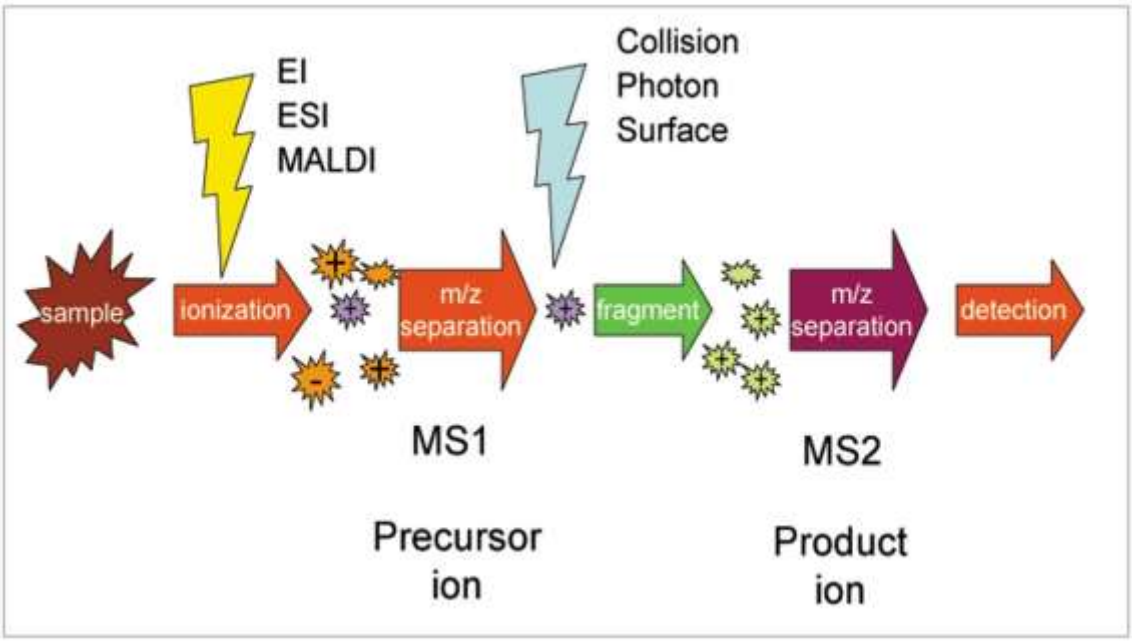


Figure 5.1: Principle of tandem mass spectrometry, showing ionisation in the source, and fragmentation between MS1 and MS2.

5.1.2. Proteomic approaches for sequencing and characterisation of proteins

Protein sequencing can be achieved either by analysing proteins in their intact from or by digesting them into peptides. Although modern MS instruments can measure the molecular weight of whole proteins, it is still very difficult to sequence them in this form, especially high molecular weight or hydrophobic or complex protein mixtures (Chait 2006). Moreover, the sensitivity of analysing intact proteins is not as high as sensitivity of analysing peptides. For this reason, proteomic analysis using digested proteins is highly preferred over that of intact proteins (Feist and Hummon 2015). There are three main proteomic approaches to sequence and identify proteins, namely, bottom-up approach, top-down approach and middle-down approach.

5.1.2.1. Bottom – up (BU) proteomics

Bottom-up proteomics involves proteolytic digestion of proteins before analysis by MS instruments. The term bottom-up implies that the information about the constituent proteins is reconstructed from individually identified fragment peptides released from the protein through either enzymatic (eg. trypsin or chymotrypsin) or chemical (eg. cyanogen bromide) proteolysis. The proteases cleave the amino acids at specific amino acid residues to give peptides of 7 – 20 amino acids, an ideal length for MS analysis. By this strategy, the identity of a protein is deduced by unequivocal detection of peptides that have unique sequences for that protein (Zhang et al., 2013)

5.1.2.2. Top- down (TD) proteomics

This approach can characterise intact proteins from complex biological systems. It involves identifying proteins in complex biological mixtures without prior digestion into their corresponding peptide species. Proteins are extracted from cell or tissues lysates and following protein separation they are typically ionised by ESI and trapped in a Fourier transform ion cyclotron resonance or orbitrap mass spectrometer. Fragmentation for tandem MS is accomplished by electron capture dissociation or electron transfer dissociation. The use of this approach is limited to studying and detecting post-translational modifications and isoforms of proteins (Catherman, Skinner and Kelleher, 2014).

5.1.2.3. Middle-down proteomics

The MD approach is an emerging method for sequencing proteins by combining the principles of both BU and TD approaches. Proteins are neither completely digested as in BU nor analysed intact like TD method, rather they are incompletely digested in polypeptides of 20 -100 amino acids. This length is significantly longer, resulting in fewer and a less complex mixtures of peptides than in the BU approach. Thus, the percentage of sequence coverage is increased high enough to identify proteins and characterise their post-translational modifications (PMTs) and isoforms which cannot be achieved with the BU approach (Pandeswari and Sabareesh, 2019).

5.1.3. Shotgun and Targeted proteomics

5.1.3.1. Shotgun proteomics

Shot gun proteomics is a discovery method that gives a broad overview of proteins expressed in a sample (McDonald and Yates III, 2002). This method is highly efficient in identifying entire proteomes in any biological sample but is rarely used to analyse specific molecules within a cell or tissue. It has been designed to provide complex answers for somewhat non-specific questions (Faria *et al.*, 2017), for example, identifying a significant number of proteins that are upregulated or downregulated in response to environmental conditions or treatments. However, its use will be inadequate to know the main cause for the up- and down-regulation of the proteins (Martins-De-Souza *et al.*, 2010; Castagnola *et al.*, 2012). This methods also suffers the drawback of being unable to identify low abundant proteins because it is based on the analysis of fragmented peptides that are usually identified relative to the abundance of each protein in a sample, thus only the highly enriched proteins will be identified (Wang *et al.*, 2017). However, this does not mean that the shotgun approach is inappropriate or time-consuming in nature. This approach opened avenues for other methodologies such targeted proteomics that can selectively investigate the proteome (Faria *et al.*, 2017).

5.1.3.2. Targeted Proteomics

Targeted proteomics is a question-based or hypothesis-based method that is frequently used in proteomic studies to accurately answer specific questions more rapidly compared to the shotgun approach (Marx, 2013). Targeted approach is mainly used to monitor or analyse a selection of proteins whose identity is already known. Therefore, by using a targeted approach, it is necessary to choose proteotypic peptides (PTPs) that have distinct amino acid sequences which constantly determine a specific protein in a given proteome investigated by MS (Domon and Gallien, 2015). PTPs serve as a signature for the protein of interest and are traced throughout the experimental run on MS. Thus, the selection of PTPs is the most critical step in the targeted approach, which requires exhaustive knowledge about the protein of interest (Gallien, Duriez and Domon, 2011). There are two main

strategies employed in targeted proteomics for selection of PTPs, selection reaction monitoring (SRM) also known as multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM).

In the SRM strategy, a panel of peptides is chosen to quantify the protein of interest and analysed on a mass spectrometer, typically a triple quadrupole mass analysers in which the first mass analyser is used a mass filter with a narrow isolation window to isolate a specific peptide precursor ion generated from the selected peptide (Ebhardt, 2014). The isolated precursor ion is then further fragmented to make smaller fragment ions, and one of the generated fragments ions is monitored by the subsequent mass analyser set to filter a specific m/z value. The dual selection of the peptide precursor ion and fragment ion by the tandem mass analysers is called a transition process which is highly specific and produces highly sensitive measurements for proteins of interest (Picotti and Aebersold, 2012).

In contrast, PRM method (such as from a Q-TOF instrument) simultaneously analyses all the fragments ions generated from the preselected peptide precursor ion (Borràs and Sabidó, 2017). In PRM method, the first MS is set to a narrow isolation window to filter a peptide precursor ion of interest. The selected peptide precursor ion is then fragmented, and the resulting fragment ions are collectively analysed by the next mass analyser set to screening mode (Bourmaud, Gallien and Domon, 2016). Then chromatographic peaks resulted from fragment ions are analysed to determine the best fragment ions for peptide identification and quantification. Therefore, PRM is more flexible method than SRM owing to its wide spectrum analysis of fragment ions rather than monitoring of a single fragment. This method generates highly sensitive and specific data with dynamic range for peptide and protein quantification (Rauniyar, 2015; Borràs and Sabidó, 2017). Thus, the PRM method can be specifically applied for monitoring low-abundant proteins (Faria *et al.*, 2017). However, the need for extremely sensitive and specific biomarkers for targeted analysis restricts its clinical application (Faria, et al. 2017).

5.1.3.3. Data Independent Acquisition

Recent advances in quantitative methodologies such as Data Independent Acquisition (DIA) allow for comprehensive, unbiased analysis and quantitation of digested peptides (Doerr, 2014). In contrast to targeted methods where you must know exactly what you are looking for and narrow coverage but excellent data completeness, of the well-established Data Dependent Acquisition which gives very broad coverage but is plagued with "missing values" DIA gives broad coverage and good data completeness (Wolf-Yadlin, Hu and Noble, 2016). One variation of DIA is called "SWATH" (Ludwig *et al.*, 2018) which is a backronym for "Sequential Window Acquisition of all Theoretical mass spectra"

5.2. Aims

In this study we analysed the effect of XOS administration on proteins expressed by caecal microbiota. The hypothesis of this study was that the microbiota will sense the presence of XOS in the caecal chyme and increase the expression of proteins involved in XOS degradation.

5.3. Material and Methods

5.3.1. Sample collection

Caeca were collected and stored as described in chapter 2, section 2.3.1 from birds raised on the farm barn (trial 2- Oligo13). Caeca from day 35 old birds only was used for analysis considering that the caecal bacteria would have had maximum exposure to the supplemented XOS.

5.3.2. Bacterial Extraction

Bacterial cells were extracted from the ceacal contents following the method described by Tang *et al.* (2014). Briefly, 1.5 – 2.5 g of caecal content was resuspended in 10 ml sterile phosphate buffered saline (PBS) containing 0.1% w/v Tween 80 in 50 ml tubes. The samples were spun at 300 x g at 8^{o}C to separate the bacteria from the cecal digesta and supernatant was collected in fresh 50 ml tubes. Fresh sterile PBS with 0.1% w/v Tween 80 was added to

the sample tubes and the tubes were vortexed to resuspend the pellet. This cycle was repeated four times to gather bacterial cells. Approximately 40 ml supernatant was obtained from each sample. Finally the pellet was discarded and the cells in the supernatant were pelleted by centrifugation at 14000 x g for 20 min at 4^{o}C. The recovered cells were washed three times in 50 ml PBS plus 0.1% Tween 80 through resuspension and centrifugation and stored at -20^{o}C.

5.2.3. Protein Extraction And Quantification

The bacterial pellet was lysed by adding 1 ml of lysis buffer containing 8 M Urea in 50 mM Tris, and 1 µl 1% ProteaseMAX^{TM} (ProteaseMAX™ Surfactant:50mM NH_4HCO_3) (Promega, USA). The pellet was mixed by vortexing. The tubes were then placed in a sonic bath three times for 1 min with 30 sec intervals on ice. The tubes were then centrifuged at 14000 x g for 10 min at 4^{o}C. The concentration of proteins in the supernatant was determined using Bicinchoninic Acid (BCA) assay kit (Sigma-Aldrich, UK) using bovine serum albumin as standard according to manufacturer's instructions. The protein concentration in all samples was normalized to 1.5 – 2 µg/µl before submitting them to Dr. Boocock's lab at The John Van-Geest Cancer Research Centre, Nottingham Trent University, Clifton Campus.

5.3.4. Protein digestion and clean-up

50 ug of protein (approximately 25 uL) from each sample was transferred to individual 1.5 mL lo-bind microtube (Eppendorf, UK) the protein lysate solution was then dried down in a vacuum concentrator (Eppendorf, uk) at 60 C until just dry. Each tube was then reconstituted in 5% SDS in 50 mM triethylammonium bicarbonate (TEAB) at pH 7.5. The sample tubes were then placed in a sonicating water bath for 10 min and centrifuged for 4 min at 13,000 g to remove insoluble matter. The proteins were then reduced and alkylated as follows. 1 uL of 0.5 M dithiothreitol (DTT) was added to each tube and then incubated at 56 C for 20 minutes on a shaking thermomixer. After cooling to room

temperature, 2 uL of 0.5 M iodoacetamide (IAA) was added and the tubes incubated in the dark for 15 min at room temperature.

The proteins were then digested using the Protifi S-Trap protocol (Protifi, USA). To each sample, ~12% aqueous phosphoric acid was added at 1:10 for a final concentration of 1.2% phosphoric acid (2.8 uL to each tube containing 28 uL) this acidifies the proteins which allows binding to the S-trap. To each tube 185 uL of S-Trap binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB and pH 7.1) was added to precipitate the proteins. The tube contents turn slightly cloudy at this point. An S-Trap "micro" was placed in a clean lo-bind 1.5 mL tube and the sample pipetted on gently to the top of the S-Trap (216 uL). The S-trap/microtube was then centrifuged in a benchtop Eppendorf microspin centrifuge for 4 min at 4000 x g until the suspension has passed through into the S-trap and microtube. The protein suspension now trapped on the S-Trap was then washed by adding 150 uL of S-Trap binding buffer and spinning at the same parameters as previously to flow through. This was repeated 3 times to wash of contaminants, salts, detergents etc.

The S-trap was then transferred to a clean lo-bind 1.5 mL microtube ready for digestion with trypsin. Trypsin solution was added to the S-Trap. 50 mM TEAB at pH 7.5-8.0 was added to the vial of lyophilised sequencing grade Promega trypsin (V5111, Promega, UK). 5 ug trypsin (1:10 trypsin:protein ratio) in 20 uL was then added to each S-trap carefully without leaving an air bubble. A plastic syringe was then used to gently add positive pressure to push the trypsin solution into the S-Trap so it almost all entered the S-Trap filter material (leaving a 1 mm layer on top). The S-Trap was then gently capped and placed in a thermomixer at 47 C for 90 min (not shaking). Peptides were then eluted from the S-Trap by sequential addition and centrifugation to the microtube (as previously) of 40 uL 50 mM TEAB followed by 40 uL 0.2% formic acid and finally 35 uL of 50% acetonitrile containing 0.2% formic acid. Samples were then dried down in a vacuum concentrator at 60 C until dry and then reconstituted in 30 uL 5% acetonitrile/0.1% formic acid and transferred to a high recovery plastic HPLC vial for mass spectrometry analysis.

5.3.5. Mass spectrometry analysis

Samples were analysed on a Sciex TripleTOF 6600 mass spectrometer coupled in line with an Eksigent ekspert nano LC 425 system running in micro flow (5 µL/min) mobile phase B (100% acetonitrile + 0.1% formic acid) over mobile phase A (0.1% formic acid). Samples were analysed in two different modes; 1) 3 injections of a pool of all samples in Information Dependent Acquisition or IDA (also known as Data Dependent Acquisition/DDA) to generate a list of protein/peptide identifications to use a spectral/ion library for subsequent SWATH analysis. 2) individual samples in SWATH-MS mode (also known as Data Independent Acquisition or DIA). In brief, 4 uL of reconstituted sample was injected and trapped onto a YMC Triart-C_{18} pre-column (5 mm, 3 µm, 300 µm ID) at a flow rate of 10 µL min mobile phase A 100% for 2 min. The sample was then eluted off the trap column by valve switching and running a gradient and onto a YMC Triart-C_{18} analytical column (15 cm, 2 µm, 300 µm ID) that was in line with the Sciex TripleTOF 6600 Duospray Source using a 50 µm electrode in positive mode, +5500V. The following linear gradients were used: for SWATH, mobile phase B increasing from 3% to 30% over 38 min, 30% to 40% over 5 min, 40% to 80% over 2 min for wash and re-equilibration (total run time 57 min). For IDA, mobile phase B increasing from 3% to 30% over 68 min, 30% to 40% over 5 min, 40% to 80% for column wash and re-equilibration over 2 min (total run time 87 min). Data independent acquisition was performed using 100 variable SWATH windows (optimised previously on complex cell lysate sample types) (TOFMS m/z 400-1250) 25 ms accumulation time; 2.6 s cycle and IDA with a top 30 ion fragmentation (TOFMS m/z 400-1250) followed by 15 s exclusion using rolling collision energy, 50 ms accumulation time; 1.8 s cycle.

5.3.6. Data processing

Spectral library generation, alignment and fold change analysis were performed using PeakView 2.1 software (SCIEX, Framingham, USA) and the SWATH microapp. In brief, IDA data were searched using ProteinPilot 5.02 (iodoacetamide alkylation, biological modifications emphasised in a thorough search) against the Uniprot unreviewed all bacteria database (October 2019, uniprot.org). The group file output from ProteinPilot was imported as an “ion library” into the SWATH microapp in PeakView

2.1 and the SWATH files opened for each sample. Initially the SWATH data and the library will not match due to the different retention times for the peptides between the IDA runs generating the protein identifications and the SWATH runs due to the different gradients from IDA (87 minutes run and SWATH (57 min run). Alignment of the spectral library to the SWATH files was carried out using nine endogenous peptides present in all samples (from protein collagen triple helix repeat) following alignment the expected (from IDA runs) retention time of each peptide matched the observed (SWATH) retention times very closely (Table 5.1).

Table 5.1.: Nine endogenous peptides present in all samples used to align the 57 minute SWATH data files with the 87 minute IDA files used to generate the library. Post alignment shows minimal differences between the expected retention time (library) and the observed retention time (SWATH files) indicating a successful alignment across the chromatographic run.

n	Peptide sequence	charge	confidence	retention time (min) expected	m/z	time observed
1	AAGSEANVEEAIGNYVK	2	99	30.62	861.42	30.57
2	SQVEGVADYVFENTFPIQAIR	3	99	42.65	795.07	42.24
3	NDSNWPFGLPGAYDMHSEK	3	99	34.11	722.32	34.12
4	NDAVGSALNSDNLK	2	99	21.45	709.35	21.43
5	LFAAIPGVAEFK	2	99	36.21	631.86	36.60
6	SENFAHMYDVTPADAK	3	99	24.89	599.27	24.77
7	PIADFTGGAALK	2	99	26.65	580.82	27.00
8	KPYADNSTVGGSEVAK	3	99	13.89	541.61	13.48
9	GETGATIVPK	2	99	16.55	486.77	16.80

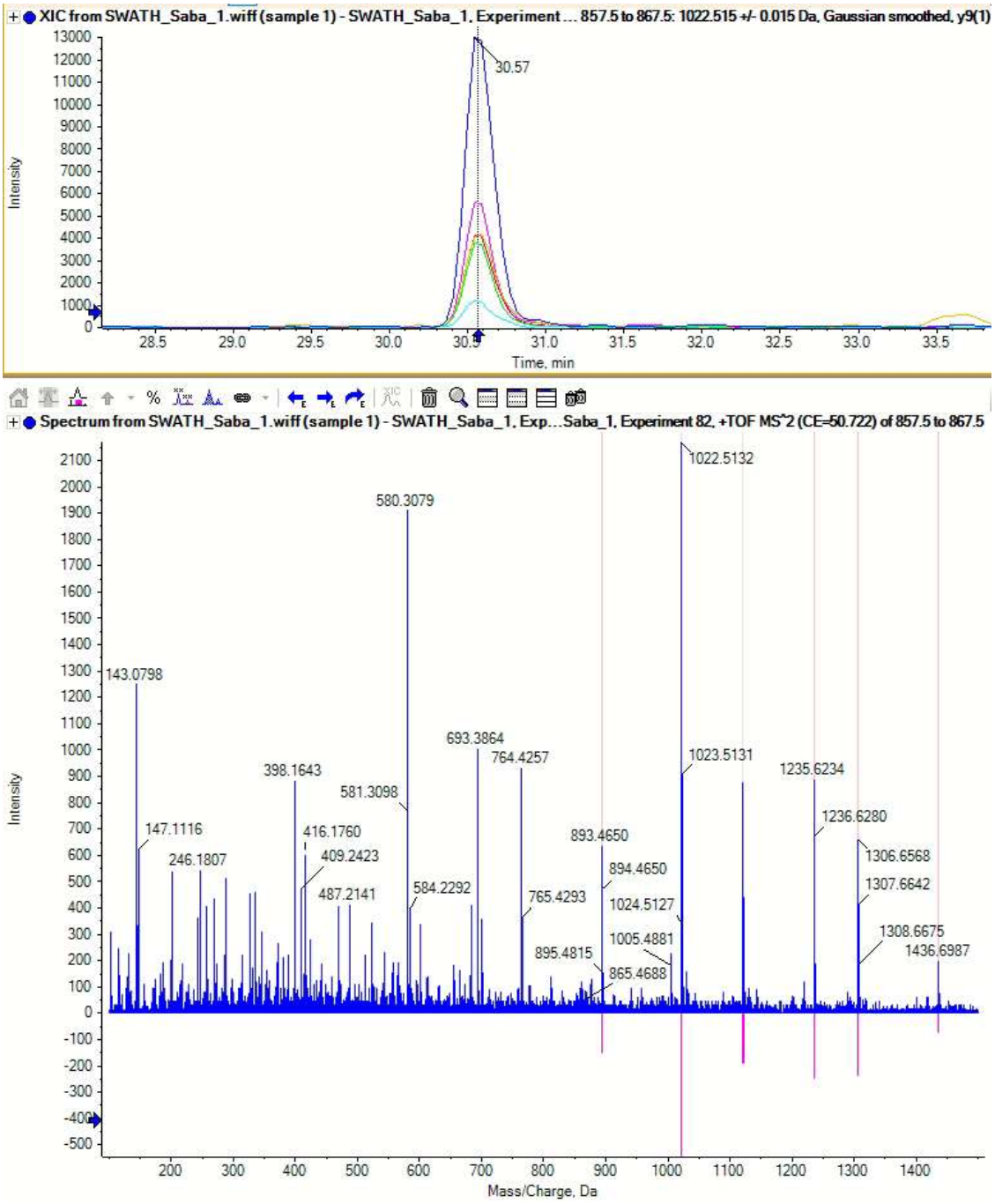


Figure 5.2. Example SWATH extraction of alignment peptide 1 from Table 5.1 (AAGSEANVEEAIGNYVK) showing all fragment ions expected (pink lines, bottom frame), and the matching fragments plotted over time in the opposite pane. Apex of all fragment peaks align perfectly in retention time off the HPLC column indicating they must all belong to the same peptide. Matching with the SWATH library identification of peptide following protein database search). Once all 9 peptides were assessed for quality, alignment calibration was run, R-squared value >0.99.

The aligned data were then processed in PeakView to generate quantitative data based on peak areas for each protein using the following summed parameters: 12 peptides per protein, 6 transitions per peptide at a peptide confidence threshold of 97%. A false discovery level of 5% (peptide) was used and modified peptides excluded. An XIC width of 30ppm was used and the retention time window finally set to 5 min. The processed data were exported (Peak Areas) into MarkerView 1.2 (SCIEX) which was used to carry out a comparative analysis of the peak areas per protein and a T-Test and fold change analysis carried out. Results were then plotted as log2FoldChange vs P-value and an interest list of significantly changed proteins generated with a cut-off of Log2FoldChange ±0.3, p-value <0.05. Data was not corrected for FDR due to the high variation and relatively low number of replicates (Pascovici *et al*., 2016)

Significantly changed protein data were exported into Morpheus (Broad Institute; https://software.broadinstitute.org/morpheus/) to generate a heatmap and carry out hierarchical clustering analysis. Clustering analysis used the following parameters: One minus pearson correlation, linkage method "complete", cluster by "rows and columns", with no grouping.

5.4. Results

5.4.1. Proteomic mass spectrometry

ProteinPilot search of the 3 injections of the pooled sample identified 421 protein groups at 1 % False Discovery Rate (FDR) from 2477 peptides and 6508 spectra. Generating a spectral library in PeakView (SWATH microapp) and removing shared and modified peptides resulted in a library of 418 unique proteins for SWATH quantitation, with 382 being deemed good enough quality after processing (Appendix D). The protein with the highest coverage and most peptides identified was Collagen triple helix repeat (20 copies), species BACUN (Bacteroides uniformis) from which its peptides were also used for library alignment.

5.4.2. Protein Quantitation of SWATH Data

Following analysis with Markerview Software (SCIEX), and generation of a volcano plot of Log2FoldChange vs p-value (Figure 5.3 full and 5.4 zoomed), after threshold cut-offs for significance an “interest list” of proteins that have changed was generated (Table 5.2).

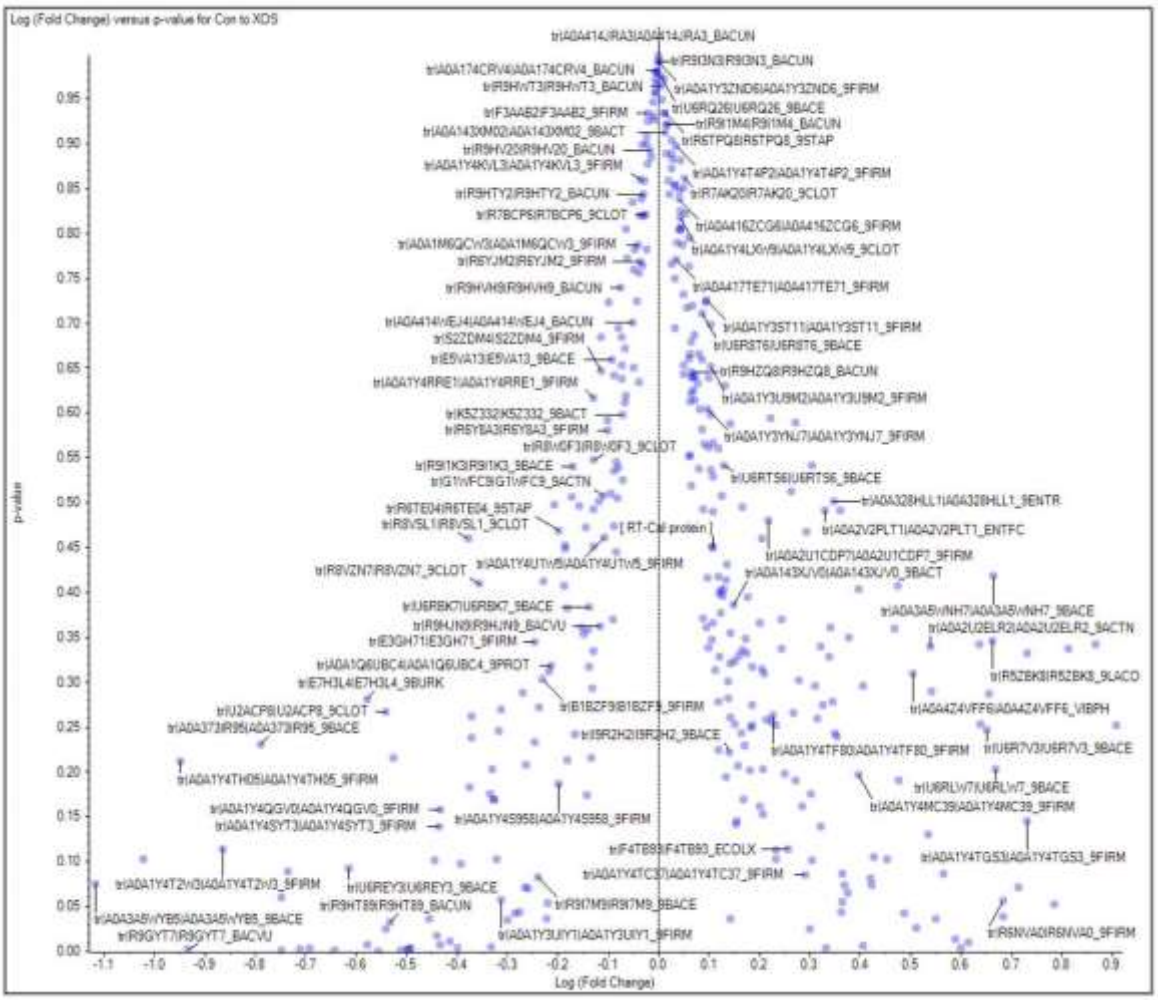


Figure 5.3. Volcano plot of quantified proteins (indicated by their Uniprot id) compared in Con and XOS. T-Test compared “Con to XOS”. All negative fold changes indicated the protein is DOWN in Con. All positive fold changes indicate protein is UP in Con.

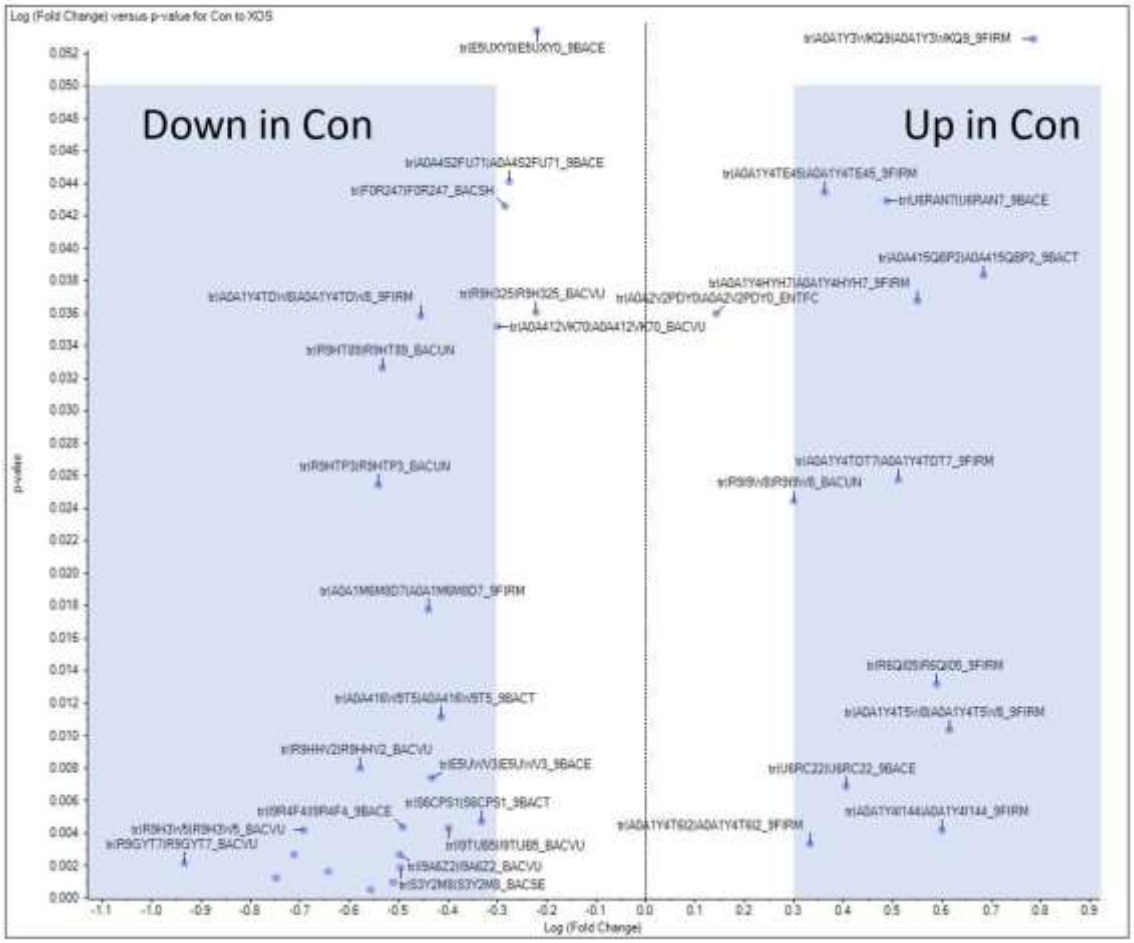


Figure 5.4. Zoomed volcano plot of quantified proteins compared in Con and XOS showing the cut-offs and proteins of interest. T-Test compared "Con to XOS". All negative fold changes indicated the protein is DOWN in Con. All positive fold changes indicate protein is UP in Con.

Table 5.2. shows proteins that were up or down in XOS. The vast majority of the changed proteins were membrane proteins, predominantly cell outer membrane proteins. 29 proteins were significantly different between the 2 groups. Of these, 20 proteins were significant higher in the XOS group. Of the 20, 9 proteins are part of the Sus system (including Sus C, Sus E and the biopolymer transport complex ExbB) in *Bacteroides* spps. A number of uncharacterised proteins and protein domains of unknown function (DUF) were also higher in XOS. Alkaline phosphatase, single-stranded DNA-binding protein, pyruvate phosphate dikinase and outer cell membrane proteins involved in transport function were among the proteins that were lower in XOS.

Table 5.2.: Significantly changed proteins between Con and XOS (<> Log2FC 0.3 and p < 0.05)

	Uniprot ID	Protein ID	Predicted in	Change	Fold change
1	tr|A0A174K9C2|A0A174K9C2_BACVU	Cell surface protein (phosphate dikinase)	Bacteroides uniformis	Higher in XOS	-0.748
2	tr|R9HTP3|R9HTP3_BACUN	SusC/RagA family TonB-linked outer membrane protein	Bacteroides massiliensis B84634	Higher in XOS	-0.542
3	tr|U6RC22|U6RC22_9BACE	SusC/RagA family TonB-linked outer membrane protein	Bacteroides sp. 3_1_40A	Lower in XOS	0.405
4	tr|E5UWV3|E5UWV3_9BACE	DUF3869 domain-containing protein	uncultured bacterium	Higher in XOS	-0.433
5	tr|S6CPS1|S6CPS1_9BACT	TonB dependent receptor (generic)	Bacteroides stercoris CC31F	Higher in XOS	-0.332
6	tr|S3Y2M8|S3Y2M8_BACSE	Uncharacterized protein	Bacteroides uniformis dnLKV2	Higher in XOS	-0.496
7	tr|R9HT89|R9HT89_BACUN	SusE domain-containing protein	Bacteroides dorei CL03T12C01	Higher in XOS	-0.533
8	tr|I9QUZ5|I9QUZ5_9BACE	SusC/RagA family TonB-linked outer membrane protein	Bacteroides vulgatus dnLKV7	Higher in XOS	-0.712
9	tr|R9HHV2|R9HHV2_BACVU	Biopolymer transporter ExbB	Bacteroides vulgatus CL09T03C04	Higher in XOS	-0.579
10	tr|I9U4W6|I9U4W6_BACVU	OmpA-like domain-containing protein (generic functions)	Bacteroides vulgatus dnLKV7	Higher in XOS	-0.512
11	tr|R9H3W5|R9H3W5_BACVU	Uncharacterized protein	Faecalibacterium sp. An122	Higher in XOS	-0.691
12	tr|A0A1Y4T5W8|A0A1Y4T5W8_9FIRM	Alkaline phosphatase (generic function)	Bacteroides vulgatus CL09T03C04	Lower in XOS	0.615
13	tr|I9IW03|I9IW03_BACVU	SusC/RagA family TonB-linked outer membrane protein	Bacteroides massiliensis B84634	Higher in XOS	-0.558
14	tr|U6RCI3|U6RCI3_9BACE	SusC/RagA family TonB-linked outer membrane protein	Faecalibacterium sp. An121	Higher in XOS	-0.642
15	tr|A0A1Y4TE45|A0A1Y4TE45_9FIRM	BMP family ABC transporter substrate-binding protein	Faecalibacterium sp. An121	Lower in XOS	0.362

16	tr|A0A1Y4TDW8|A0A1Y4TDW8_9FIRM	Purine-nucleoside phosphorylase	Bacteroides vulgatus CL09T03C04	Higher in XOS	-0.456
17	tr|I9A6Z2|I9A6Z2_BACVU	DUF5017 domain-containing protein	Bacteroides vulgatus	Higher in XOS	-0.498
18	tr|A0A412VK70|A0A412VK70_BACVU	TonB-dependent receptor	Bacteroides vulgatus dnLKV7	Higher in XOS	-0.300
19	tr|R9GYT7|R9GYT7_BACVU	Uncharacterized protein	Butyricimonas virosa	Higher in XOS	-0.933
20	tr|A0A415QBP2|A0A415QBP2_9BACT	SusC/RagA family TonB-linked outer membrane protein	Bacteroides vulgatus CL09T03C04	Lower in XOS	0.685
21	tr|I9TUB5|I9TUB5_BACVU	SusC/RagA family TonB-linked outer membrane protein	Anaeromassilibacillus sp. An200	Higher in XOS	-0.399
22	tr|A0A1Y4HYH7|A0A1Y4HYH7_9FIRM	Uncharacterized protein	Bacteroides dorei CL02T12C06	Lower in XOS	0.550
23	tr|I9R4F4|I9R4F4_9BACE	SusC/RagA family TonB-linked outer membrane protein	Odoribacter sp. AF15-53	Higher in XOS	-0.491
24	tr|A0A416W9T5|A0A416W9T5_9BACT	DUF4136 domain-containing protein	Faecalibacterium sp. CAG:82	Higher in XOS	-0.415
25	tr|R6QI05|R6QI05_9FIRM	Single-stranded DNA-binding protein (basic function)	Faecalibacterium sp. An121	Lower in XOS	0.590
26	tr|A0A1Y4TDT7|A0A1Y4TDT7_9FIRM	Preprotein translocase subunit YajC (translation)	Anaerotignum lactatifermentans DSM 14214	Lower in XOS	0.512
27	tr|A0A1M6M8D7|A0A1M6M8D7_9FIRM	C4-dicarboxylate transporter, DctM subunit (DctM – part of Tripartite ATP-independent periplasmic (TRAP) transporters)	Faecalibacterium sp. An121	Higher in XOS	-0.441
28	tr|A0A1Y4T6I2|A0A1Y4T6I2_9FIRM	Pyruvate, phosphate dikinase (gluconeogenesis)	Anaeromassilibacillus sp. An200	Lower in XOS	0.332
29	tr|A0A1Y4I144|A0A1Y4I144_9FIRM	Uncharacterized protein		Lower in XOS	0.600

Heatmap map generation and hierarchical clustering showed that the samples clustered clearly between CON and XOS with respect to the most changed proteins (Figure 5.5)

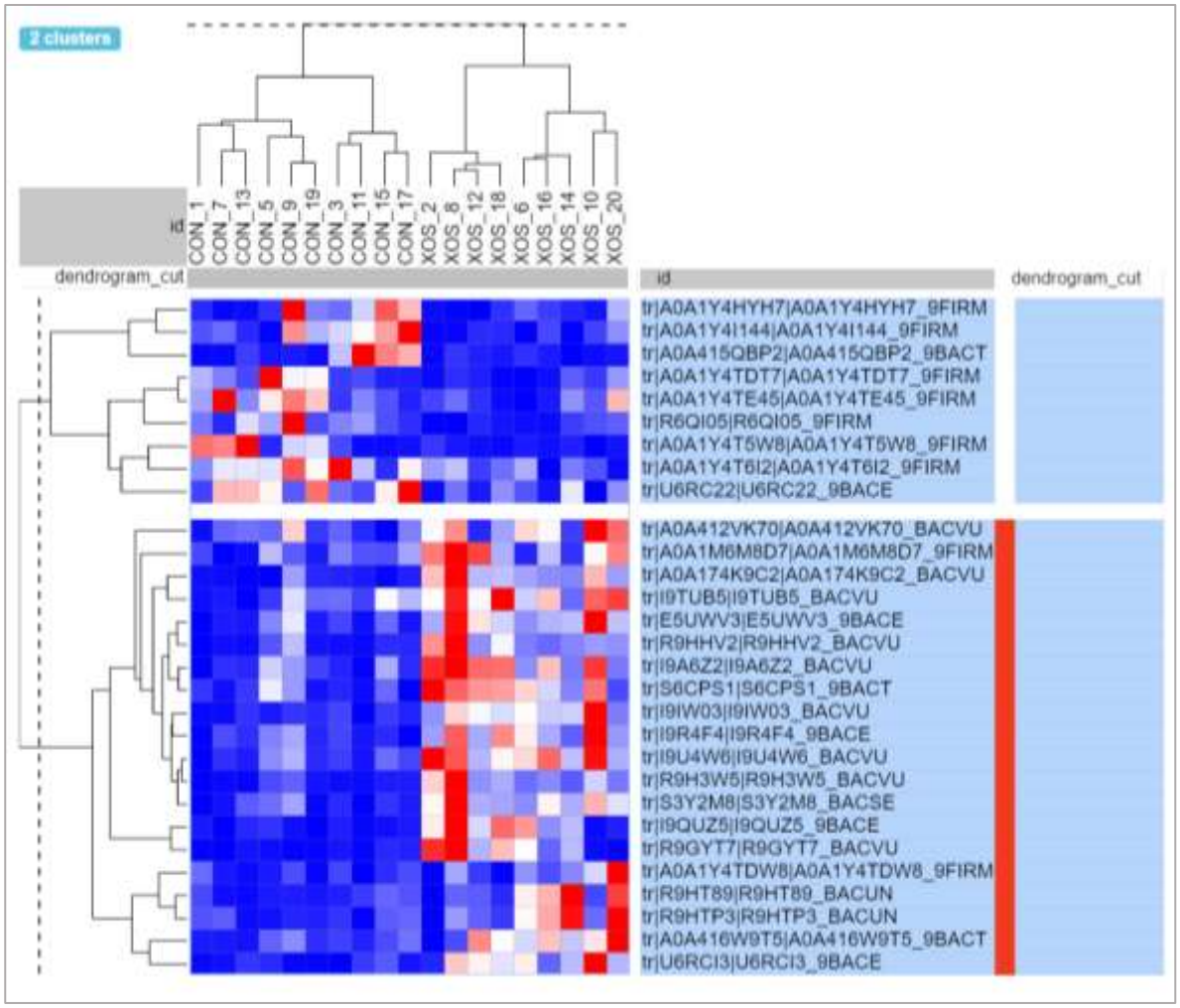


Figure 5.5. Hierarchical clustering heatmap showing proteins significantly changed expression levels in the Con and XOS individual samples.

It was not possible to carry out pathway or enrichment analysis of the proteins that were significantly changed. The protein names (when converted to gene name or gene ID) were not recognised by Reactome or other pathway analysis software. Proteins were ID mapped using Uniprot.org and the columns for gene ontology were added (Table 5.3).

Table 5.3. Proteins with increased expression in XOS showing documented gene ontology (Uniprot.org)

Entry	Protein names	Gene ontology (biological process)	Gene ontology (cellular component)	Gene ontology (cellular location)	Gene ontology (molecular function)
A0A412VK70	TonB-dependent receptor		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
A0A1M6M8D7	C4-dicarboxylate transporter, DctM subunit		integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]	integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]	
A0A174K9C2	Cell surface protein				
I9TUB5	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
E5UWV3	DUF3869 domain-containing protein				
R9HHV2	Biopolymer transporter ExbB	protein transport [GO:0015031]	integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]	integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]; protein transport [GO:0015031]	
I9A6Z2	DUF5017 domain-containing protein				
S6CPS1	TonB dependent receptor		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	

I9IW03	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
I9R4F4	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
I9U4W6	OmpA-like domain-containing protein		membrane [GO:0016020]	membrane [GO:0016020]	
R9H3W5	Uncharacterized protein				
S3Y2M8	Uncharacterized protein	protein secretion by the type VI secretion system [GO:0033103]	type VI protein secretion system complex [GO:0033104]	type VI protein secretion system complex [GO:0033104]; protein secretion by the type VI secretion system [GO:0033103]	
I9QUZ5	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
R9GYT7	Uncharacterized protein				
A0A1Y4TDW8	Purine nucleoside phosphorylase DeoD-type (EC 2.4.2.1)	nucleoside metabolic process [GO:0009116]		purine-nucleoside phosphorylase activity [GO:0004731]; nucleoside metabolic process [GO:0009116]	purine-nucleoside phosphorylase activity [GO:0004731]
R9HT89	SusE domain-containing protein		outer membrane [GO:0019867]	outer membrane [GO:0019867]; starch binding [GO:2001070]	starch binding [GO:2001070]
R9HTP3	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	

A0A416W9T5	DUF4136 domain-containing protein				
U6RCI3	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
A0A1Y4HYH7	Uncharacterized protein				
A0A1Y4I144	Uncharacterized protein				
A0A415QBP2	SusC/RagA family TonB-linked outer membrane protein		cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	
A0A1Y4TDT7	Preprotein translocase subunit YajC		integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]	integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]	
A0A1Y4TE45	BMP family ABC transporter substrate-binding protein		plasma membrane [GO:0005886]	plasma membrane [GO:0005886]	
R6QI05	Single-stranded DNA-binding protein (SSB)	DNA replication [GO:0006260]		single-stranded DNA binding [GO:0003697]; DNA replication [GO:0006260]	single-stranded DNA binding [GO:0003697]
A0A1Y4T5W8	Alkaline phosphatase			phosphatase activity [GO:0016791]	phosphatase activity [GO:0016791]
A0A1Y4T6I2	Pyruvate, phosphate dikinase (EC 2.7.9.1)	pyruvate metabolic process [GO:0006090]		ATP binding [GO:0005524]; kinase activity [GO:0016301]; metal ion binding [GO:0046872]; pyruvate, phosphate dikinase activity	ATP binding [GO:0005524]; kinase activity [GO:0016301];

			[GO:0050242]; pyruvate metabolic process [GO:0006090]	metal ion binding [GO:0046872]; pyruvate, phosphate dikinase activity [GO:0050242]
U6RC22	SusC/RagA family TonB-linked outer membrane protein	cell outer membrane [GO:0009279]	cell outer membrane [GO:0009279]	

5.5. Discussion

Xylans are abundantly present in the cereal based diets of poultry and are fermented by the commensal bacteria in the caeca yet addition of supplemental xylanases or addition of small amounts of XOS in poultry diets is an increasingly popular practice. Addition of xylanases or XOS has been shown to improve production performance and gut health parameters of broilers in many studies (Aachary and Prapulla, 2011b; Pourabedin and Zhao, 2015). However, the currently suggested mechanism of action of XOS being quantitatively fermented into SCFA does not explain the increases in their concentration given the miniscule amounts of XOS added to poultry diets. Hence, the stimbiotic mechanism of XOS has been advanced which essentially proposes that the presence of XOS in the caeca serves as a signal to the xylanolytic bacteria to produce their own xylan degrading enzymes in higher quantities (Bedford, 2019b). Effectively, this leads to better digestion of the dietary fibre and increased energy efficiency of the diets. However, this mechanism has not been studied *in vivo*. In this study, a proteomic approach was applied to evaluate the range of proteins produced by the entire caecal microbiota in response to XOS supplementation in the diets. It was found that the Sus-like proteins from *Bacteroides spps* were significantly higher in the XOS group compared to the control. In addition, both the TonB dependent receptor and the biopolymer transport protein ExbB was also found to be higher the same *spps*. Sus-C like proteins are members of the TonB receptor family involved in the transport of oligosaccharides across the outer membrane into the periplasmic space with help of energy derived from proton motive force and the TonB-ExbBD complex (Martens *et al.*, 2009).

Sus-C is a highly conserved protein of the sus-like system of the Gram-negative Bacteroidetes. The sus-like system is a cluster of cell envelope-associated proteins that confer the ability to *Bacteroides* (a genus of Bacateroidetes) to metabolize a single glycan or a group of related glycans (Shipman, Berleman and Salyers, 2000; Martens, Chiang and Gordon, 2008; Dodd *et al.*, 2010; Sonnenburg *et al.*, 2010). The sus-like system is located within the series of augmented and permuted gene clusters

termed polysaccharide utilization loci (PULs) in the Gram-negative bacteria Bacteroides. The Sus (starch utilization system) was discovered in *Bacteroides thetaiotaomicron*, a prominent human gut Bacteroidete by Slayers et al through their work on starch degradation by the bacterium (1977a; 1977b) (Figure 5.6). Subsequent research in microbial genome sequencing uncovered the derivatives of this prototypic system or "Sus-like systems" are particularly well represented in the genomes of *B. thetaiotaomicron* and many other Bacteroidetes. An important characteristic of the Sus-like systems is the synchronized action of a number of gene products involved in substrate binding and degradation (Martens et al., 2009).

Each Sus-like system contains at least one pair of outer membrane proteins homologous to SusC and SusD, which are essential for the import and degradation of starch in the prototypic system, Sus (Shipman, Berleman and Salyers, 2000). SusC-like proteins are predicted TonB-dependent receptors that span the outer membrane and transport oligosaccharides in an energy-dependent manner. SusD-like proteins are outer membrane lipoproteins that are oriented towards the external environment; they bind directly to specific glycans and contribute to the capture and delivery of oligosaccharides to the SusC transporter (Koropatkin *et al.*, 2008; Sonnenburg *et al.*, 2010). SusC- and SusD-like proteins work in a coordinated manner with other outer membrane glycan binding proteins and polysaccharide degrading enzymes (glycoside hydrolases, polysaccharide lyases, and carbohydrate

esterases), which are grouped into sequence-based families in the Carbohydrate Active Enzymes (CAZy) database (Cantarel *et al.*, 2009).

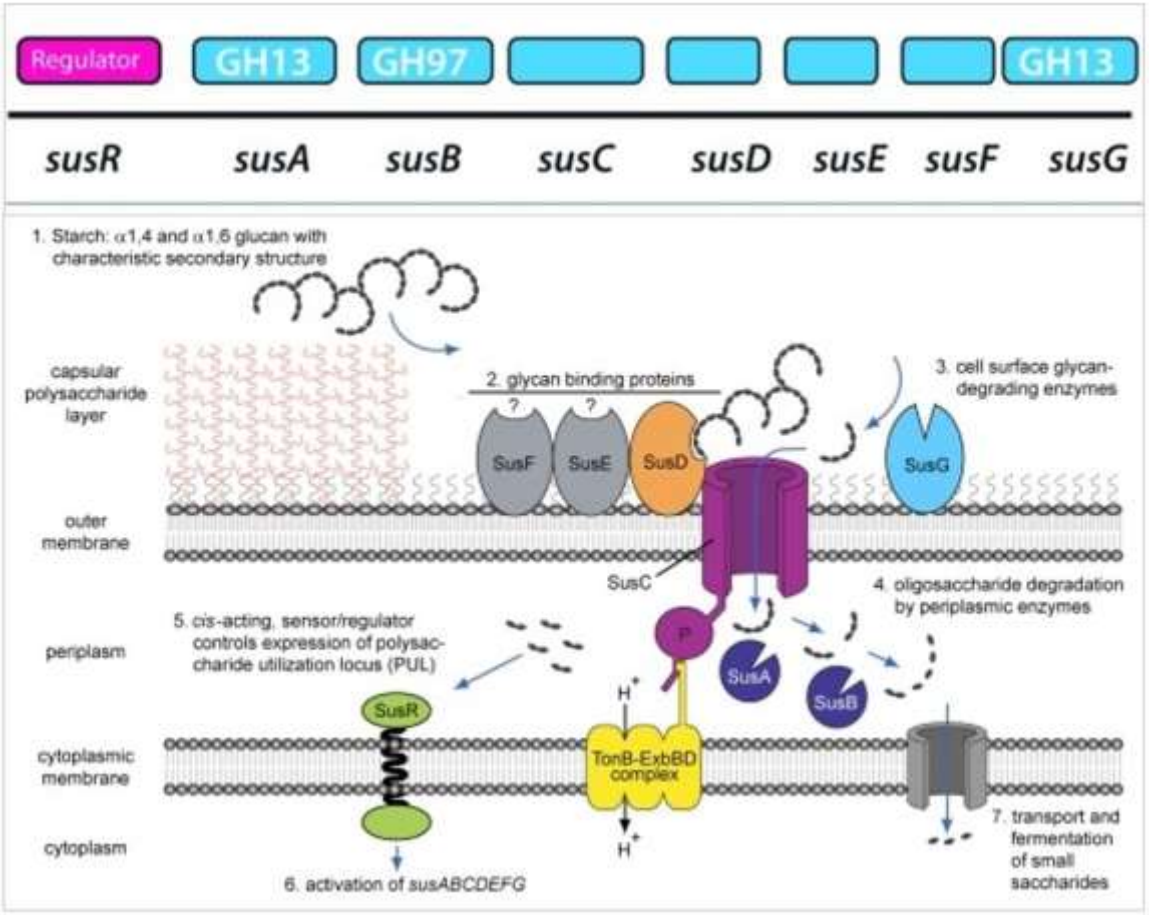


Figure 5.6: shows the order of genes in the eight-gene sus cluster that is responsible for starch utilization in *B. thetaiotaomicron* and the functional model of glycan processing (Adapted from Flint et al., 2012; Martens et al., 2009) '*Reproduced under a CC-BY license*'

A sus-like xylan degrading machinery was reported by Dodd *et al* (2010) in *Prevotella bryanatii* B_14, a Bacteroidete that has been frequently isolated from rumen microbiome. The authors reported the discovery of an invariant 6 gene cluster flanked by either biochemically categorised or predicted glycoside hydrolases and carbohydrate esterases in *P. bryantii* B_14. This gene cluster (figure 5.7) was found to be critical to xylan utilisation in this bacterium and more importantly was highly conserved in other xylanolytic *Prevotella* and *Bacteroides spp*. derived from the bovine rumen and the human colonic microbiomes which suggests "a conserved mechanism for xylan utilization by xylanolytic Bacteroidetes" (Dodd *et al.*, 2010).

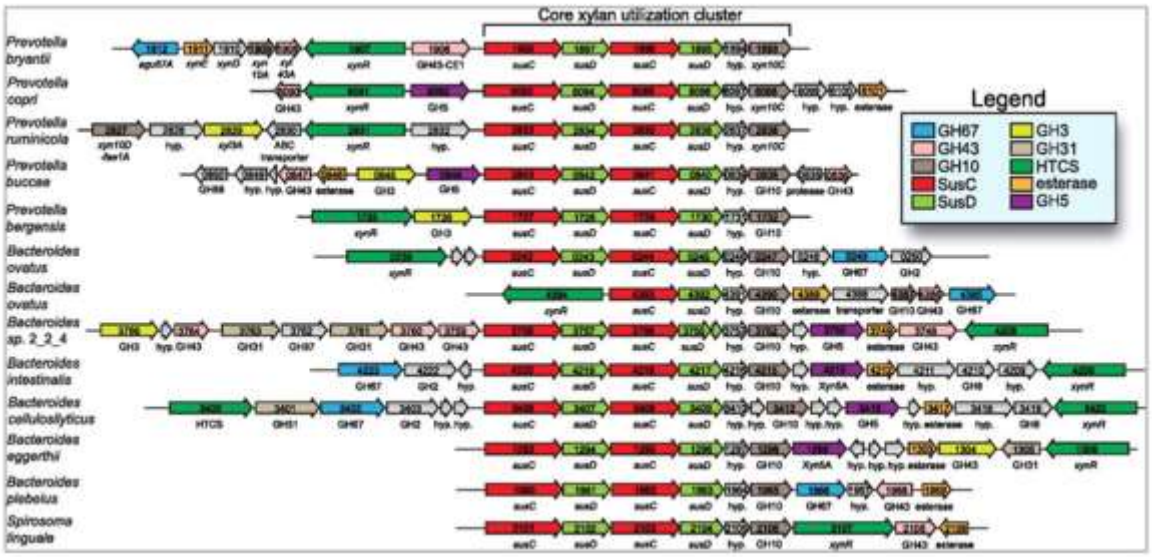


Figure 5.7: shows that the core xylan utilization system is conserved among certain species within the phylum Bacteroidetes (Adapeted from Dodd et al., 2010). *'Reproduced under a CC-BY license'*

In addition, *sus*C gene is a part of the core xylan utilization system which is conserved among certain species within the phylum Bacteroidetes (Dodd *et al.*, 2010). The presence of two xylan PULs, large xylan PUL (PUL-xylL) and the small xylan PUL (PUL-xylS) in *Bacteroides ovatus* was described by Rogowski et al (2015), both of which encode SusC-like proteins. Mendis et al. (2018) used three different *susC* transcripts as a proxy for expression PUL-xylL and PUL-xylS in *B. ovatus* growing on xylan substrates differing in their degree of polymerization and degree of substitution. The increased expression of SusC-like proteins in the present study may therefore indicate the upregulation of the entire *sus-like* system which includes glycoside hydrolases (GH) responsible for breakdown of oligosaccharides encoded by either one or both xylan-PULs which supports the proposed stimbiotic mechanism of XOS.

Future work to support this study would involve isolation of several different *Bacteroides* from the chicken caeca and growing them on media containing XOS used in this study as the sole carbon source. The levels of the *sus*C transcript from these bacteria can then be determined using RT-PCR that would give an indication of the expression of the cognate PULs. Furthermore, the activity of xylosidases released in the growth media could be quantified spectrophotometrically by continuous monitoring of xylose released using a D-xylose detection kit.

5.6. Conclusions

Shot-gun proteomics for the first time indicated that supplementation of XOS in broiler diets stimulated the Gram negative Bacteroides to upregulate their xylan degrading (sus-like) system via upregulation of *SusC*- like and other membrane proteins involved in transport of complex polysaccharides across the outer membrane. However further evidence from *in vitro* studies using pure cultures of *Bacteroides* from the chicken caecum is required to warrant these findings.

Chapter 6: General discussions, conclusions and recommendations

6.1. Introduction

This chapter had been divided into three sections to discuss the potential of XOS as a feed ingredient to promote gut health of broiler chickens. Firstly, the key findings from the two broiler trials are reviewed and contrasted in the context of current global poultry production practices. Secondly the impact of the findings on the industry and global food security are discussed. Subsequently, key areas for future research and development are outlined and recommendations based on this work are given.

Chicken meat and eggs are an affordable source of animal protein for people all over the world regardless of their economic status. Poultry has an important role to play in the future food security considering its production rate, affordability, acceptability, and low environmental impact. However, the size of the sector means that small alterations in production methods have profound global effects on a volume basis. This means advancements in the poultry sector must be achieved through sustainable means. One of the sustainable development objectives of the poultry industry is to produce chickens without prophylactic use of antibiotics. Since the ban of in-feed antibiotics in the EU in 2006 and consumer pressure for antibiotic free chicken in the US and Canada, research in the field of natural alternatives to antibiotics has gained momentum. Probiotics, prebiotics, synbiotics, yeast cell wall oligosaccharides, essentials oils and plant polyphenols have been widely explored in monogastric nutrition as alternatives to in-feed antibiotics. These alternatives aim to maximise performance by optimising the gut health of the animal.

XOS as prebiotics in poultry feed have gained significant attention over the past few years. Compared to other commonly used prebiotics like mannan-oligosaccharides, galacto-oligosaccharides and fructo-oligosaccharides, the parent molecule of XOS, xylan, is abundantly present in the cereal-based diets of poultry. Two approaches for increasing XOS in the GIT have been investigated. One, creating XOS *in situ* in the GIT via addition of xylanases in the diet (Bedford, 2000). These xylanases breakdown the xylan in the cell wall of cereal grains to create XOS or arabinoxylooligosaccharides (AXOS) in the GIT, thereby increasing the nutritive value of feed. Second, addition of small amounts XOS in the feed,

derived from human inedible agricultural residues. Addition of XOS or xylanase have similar effects in terms of increasing the concentration of potentially prebiotic oligosaccharides in the GIT of broilers (Craig *et al.*, 2020). XOS has shown to improve performance of birds in many trials but there is more conclusive evidence from *in vitro* and *in vivo* studies regarding benefits of XOS in beneficially modulating the gut microbiota by enhancing the abundance of lactate producing *Lactobacillus* and the SCFA producers of Lachnospiraceae and Ruminoccaceae families. Furthermore, XOS was shown to ameliorate the detrimental effects of pathogens in challenge studies (Eeckhaut *et al.*, 2008; Keerqin *et al.*, 2017; Pourabedin *et al.*, 2017). Thus the use of XOS serves as an insurance against dysbacteriosis, a term commonly used for the poor performance and inflammatory response associated with colonisation of the intestinal tract with undesirable bacteria in the post-antibiotic era (Teirlynck *et al.*, 2011). This thesis includes two broiler trials one with birds raised under optimal conditions and the other under challenging environmental conditions to examine the effect of XOS on performance and gut health parameters. Furthermore, effects of XOS on the carbohydrate degrading capacity of the caecal microbiota was examined to assess its role as a stimbiotic.

6.2. Key findings, contrast and critique of investigations based on broiler trials

Two broiler trials were conducted with male Ross308 birds, one at the PRU at Nottingham Trent University under controlled environmental conditions and high biosecurity and the other on a farm barn with very limited temperature control and unhygienic conditions. In the PRU trial, the birds were fed a wheat-maize-soyabean meal diet supplemented with or without 0.1 g/kg XOS or 0.1 g/kg xylanase or their combination in three feeding phases. Three hundred and eighty four birds were divided in 4 treatment groups with 12 replicate pens; 8 birds per pen and 96 birds per treatment. Body weights and feed intake measured weekly. In the farm trial the diet was based on a maize and soya bean meal supplemented with the same dose of XOS (0.1 g/kg). Birds were divided in 4 pens of 2 replicate treatments and each pen consisted of either 190 or 225 birds. Sixty birds per pen were wing

tagged for weekly measurements body weights only. Due to the trial design, it was not possible to measure feed intake in this trial.

In the PRU trial there were no significant effects of XOS, xylanase or their combination on performance, composition of caecal microbiota or relative abundance of bacterial groups, caecal SCFA and the expression of genes considered as biomarkers of gut integrity. This is contradictory to many studies where XOS was shown to significantly improve either growth performance (De Maesschalck et al., 2015; Zhenping et al., 2012) or increase the abundance of the beneficial lactate or SCFA producers or increased concentrations of SCFA in caeca (Craig *et al*., 2019; De Maesschalck *et al*., 2015; Ding *et al*., 2018; Ribeiro *et al*., 2018). The lack of any significant effect in the PRU trial (chapter 3) can be distilled down to two main reasons, one, the birds were already performing at their maximum potential as they were raised under optimal conditions and fed a nutritionally complete diet and two, the low dose of XOS, 0.1 g/kg used in this study. As discussed in Chapter 3 Section 3.5.1, higher doses of XOS were used in studies where XOS had demonstrated significant improvements in performance and other measured parameters (Pourabedin *et al*., 2015a)

In the trial conducted on a farm barn, XOS significantly improved bird weights up to 28 days of age. Unlike the PRU trial, where Bacteroidetes were completely absent from amongst the caecal microbiota, the relative abundance of Bacteroidetes in this trial was 17 – 30%. As discussed in section 3.5.2 of chapter 3, it is evident from published studies that the absence of Bacteroidetes is not uncommon in broiler trials (Kers *et al*, 2018). Secondly the difference in the environment may have also contributed to the absence of Bacteroidetes. A trial undertaken as a part of another PhD project at the NTU, poultry research unit also showed complete lack of the phylum Bacteroidetes (Desbruslais, 2020). This adds weight to the finding from this study. Moreover because of the high variability in microbiome studies and their results it is not possible to single out a reason for the absence of Bacteroidetes. Families Lachnospiraceae and Erysipelotrichaceae were significantly increased in birds fed the XOS supplemented diet. The expression of genes of proteins considered as biomarkers of gut

integrity were either significantly (*MUC*2 and OCLN) or numerically (*sIgA* and CLDN5) improved, expression of pro-inflammatory cytokine IL-1β was reduced and finally acetate and propionate were significantly higher while butyrate was numerically higher in the XOS supplemented diet. These findings indicate that XOS improved performance and gut health parameters in birds raised under challenging environmental conditions.

When comparing the two trials an important point to note is the use of individual chickens within a pen on the farm trial (chapter 4) as opposed to the use of pen as an experimental unit in the poultry research unit trial (chapter 3); the former introduces more variability in the data and may not accurately represent the average pen weight. An individual bird can vary from other birds in the pen in its microenvironment, eating behaviour, feed intake, exposure to microflora in the environment and its immunological response to the microflora, all of which can impact the performance of the bird which in this case was assessed through the weight gain. The use of pen as an experimental unit, on the other hand, allows one to get an accurate average weight of the birds in the pen thus accounting for variations between birds. Secondly it is not possible to measure the feed intake of an individual bird in a large pen hence the FCR could not be calculated in the farm trial. An FCR rather than weight gain or bird weight is a more reliable indicator of performance as it calculates how efficiently the weight gain occurred. Considering that feed amounts to more than 60% of the production costs, the efficiency of feed conversion may be more valuable to broiler farmers than their weight gain.

In addition to the above there were several other differences between the two trials that could have contributed to the different response to XOS observed.

1) **Environment** – as summarised in above paragraphs and discussed in detail in section 4.5, the effects of feed additives are more obvious in birds raised under challenging environmental conditions. Hence significant improvements in performance and gut health parameters could be seen in birds fed the XOS supplemented diet in the farm trial.

2) **Cereal type** – It is known that wheat and barley contain a higher non starch polysaccharide (NSP) content compared to maize. Therefore feeding wheat or barley to broiler chickens may negatively affect performance due to increased intestinal viscosity and/or nutrient encapsulation (Bedford and Partridge, 2010). This may be one of the reasons for the lower performance of the birds fed the XOS supplemented diet in the PRU trial as the main cereal ingredient of the diet was wheat followed by maize. The fact that the diets containing xylanases reported better performance of birds in the same trial supports the idea that the viscosity effect of NSP may be contributing to the poor performance of diets that lacked the enzyme. On the contrary, the birds in the farm trial were fed diet containing only maize as the cereal ingredient. Therefore, in this trial the birds may not have encountered the nutritional obstacle of a viscous diet and leading to better performance.

3) **Feed form** – in trial the PRU trial a mash diet was fed to the bird in all three phases whilst in the farm trial, a crumb starter and pelleted grower and finisher was provided. The physical form of feed (mash and pellet) is a crucial factor in the weight gain of broilers. Mash is a form of a complete feed that is finely ground and mixed such that ingredients cannot be easily separated out; thus each mouthful provides a well-balanced diet. Mash diet gives greater unification of growth, less mortality and is more economical (Jafarnejad *et al.*, 2010). However, mash feed is not so palatable and does not retain its nutritive value so well compared to ungrounded feed (Jahan, Asaduzzaman and Sarkar, 2006). Pellets on the other hand are small feed particles agglomerated by means of mechanical pressure, moisture and heat. Offering feed to poultry in pellet form enhances the economics of production by improving feed conversion efficiency and growth performance. These improvements are attributed to decreased feed wastage, higher nutrient density, no selective feeding, decreased time and energy spent for eating, decreased ingredient segregation, destruction of pathogenic organisms, reducing thermolabile toxins, thermal modification of starch and protein and improved palatability (Jensen, 2000; Peisker, 2006; Amerah *et al.*, 2007). In the PRU trial the

cumulative weight gain of birds fed a diet supplemented with XOS only was significantly lower compared to the control birds. On the other hand in the farm trial although there was no significant difference in weight gain of the two groups for the entire trial period but the XOS fed birds had a significantly higher weight gain up to 28 days of age.

Thus the difference in response to XOS in the two trials may be due to one or a combination of above the factors.

This thesis also includes a study which for the first time provided evidence supporting the stimbiotic mechanism of XOS. Shot-gun proteomics, identified proteins, part of the carbohydrate degrading machinery of bacteria that were up-regulated in response to XOS.

Some weaknesses in the analysis were identified at a stage when either time or resources did not permit the reanalysis of the samples.

- In case of Trial 1 the treatment groups were not blocked. This was a major oversight. The trial could be improved by blocking to minimize the effect of geographical location of pens within the room thereby making the data more sensitive
- Following extraction of caeca from the birds, they were snap frozen in dry ice and stored at -20^{o}C. However, a review published in 2018 after the completion of both trials suggests that rapid freezing of samples to -80^{o}C is the best practice for sample storage for 16S sequencing (Pollock *et al.*, 2018).
- Two different versions of the Illumina Miseq kit were ordered in error. These two versions differed in their read lengths, Oligo26 was sequenced using version 3, with read length of 2x 300 base pairs while Oligo13 was sequenced using version 2 with read lengths of 2x 250 base pairs. As long reads allow for more stringent trimming of the reads based on quality, and still provide sufficient overlap between forward and reverse reads to generate a complete V3-V4 sequence for further analysis.

- Use of two reference genes as opposed to one in qPCR experiments could have increased the resolution and accuracy of results (Kozera and Rapacz, 2013). A single reference gene, GAPDH was selected for three reasons, (1) it is the most commonly used reference in qPCR studies and referred to as the "classical" housekeeping gene (Kozera and Rapacz, 2013) (2) it was used in published studies investigating the effect of feed additives in broilers and was the only reference gene used (Palamidi and Mountzouris, 2018; Paraskeuas and Mountzouris, 2019; Yuan et al., 2018) and was also used in a previous PhD study conducted at the PRU (Al-Sudani, 2018), finally (3) use of a single reference gene is accepTable, if it was previously tested in similar experimental conditions and properly validated (Thellin *et al.*, 1999).
- The RNA integrity was assessed using the semi-quantitative agarose gel electrophoresis method which is a less accurate as compared to the quantitative Agilent Bioanalyzer which assesses the quality of RNA, produces an electropherogram and gives an RNA integrity number (RIN). Electropherograms can give a clear visual indication of any degraded RNA in the sample. RIN values range from 1 to 10 and values above 6.5 are considered as good quality RNA for qPCR studies. The costs of consumables required for the bioanalyzer and its non-availability at Brackenhurst campus were the main reasons for assessing RNA integrity using electrophoresis only.
- The expression of immune markers of gut health such as IL-1β, IL-10, IFN-γ and LITAF are best studied in the Bursa of Fabricius or caecal tonsils which are organs of the immune system. However, tissue samples of these organs were not collected at the time of sampling the birds.
- As the concentrations of SCFA was measured in the caeca, it would have been extremely useful to examine the expression of FFAR2, the receptor for SCFA, in the caecal tissue. Although whole caeca were sampled from birds for sequencing and quantification of

SCFA but for gene expression studies samples need to be fixed in RNAlater at the time of sampling which was not done for caecal tissue

- The protocol used for quantification of SCFA using GCMS did allow the quantification of lactate. It would have been beneficial to trial and validate different methods for quantification of SCFA and lactate. Lactate is converted to butyrate by lactate-utilizing-butyrate-producing gut bacteria (De Maesschalck *et al.*, 2015). Measurement of lactate could thus serve as an additional parameter of modulation of gut microbiota by XOS. However due to time constraints this was not achievable.

6.3. Potential impact of project on industry

In the studies undertaken as a part of this project, XOS derived from corn cobs was used as a prebiotic in boiler diets with beneficial effects on performance and gut health parameters under conditions similar to commercial units. This demonstrate that prebiotic XOS are an effective way of converting agricultural waste into value added supplements for animal feed industry. This approach is particularly important in countries with an abundance of agro-industrial residues. Secondly, both XOS and xylanase have similar effects on performance and gut health of broilers, hence the choice of using one or both of these supplements needs to be considered against the cost of procurement and financial returns obtained which may vary from one production unit to another. Third, dietary supplementation of XOS as an alternative for antibiotic growth promoters may alleviate detrimental effects of suboptimal growing conditions that are commonly observed on commercial poultry units such as large variations in temperature and wet and dirty litter on broiler performance and gut health. Finally, the effectiveness of prebiotics also depends on other factors including, dose, composition of the basal diet, and environmental condition, showing variable effects on poultry species, so that it is necessary to determine conditions under which prebiotics are effective ensuring their effective use.

6.4. Potential impact of project on research and future research directions

This project for the first time investigated the effects of XOS on performance and gut health parameters of birds raised under suboptimal conditions. The results indicated the 0.1 g/kg of XOS was successful in mitigating the effects of challenging environmental conditions on performance and gut health parameters. Another study as a part of this project suggests that in birds growing to their maximum potential under controlled research conditions the same dose of XOS may not improve on performance or gut health. This was also the first study to provided evidence using a proteomics approach to the proposed stimbiotic mechanism of XOS.

1) Considerable research remains on optimising the dose of XOS along with the type of cereal grain in the basal diet needed to achieve the best performance outcome, studying the effect of XOS in birds following pathogen challenge and optimising a method of administration. For example, it would have been beneficial to include 0.5, 1.0 and 2.0 g/kg of XOS in this study to determine if higher doses of XOS were needed to improve performance of birds growing under optimal environmental and nutritional conditions.
2) According to published studies, there are conflicting results on effect of XOS on performance. However, these studies differ in the composition of their basal diet (Courtin et al; 2008; De Maesschalck et al., 2015; Pourabedin et al., 2015; Ribeiro et al., 2018; Samanta et al., 2016). Designing a trial to include, maize-soybean meal (SBM), wheat-SBM and wheat-maize-SBM diets with or without XOS will help to determine if there is XOS x cereal-type interaction. Data from the results of the trials conducted as a part of this project also hints that there may a diet x cereal type interaction as the diet used the first trial was a wheat-maize-SBM diet while in the second trial the diet used was maize-SBM based.
3) It would also have been interesting to note the effect of XOS in mitigating the effects of pathogens in a challenge study. Since the ban on the use in feed antibiotics once well-controlled poultry diseases, such as necrotic enteritis, have re-emerged to cause tremendous

production losses (M'Sadeq *et al.*, 2015). Only two challenge studies published to date that have assessed the role of XOS in ameliorating the detrimental effects of *Salmonella enteritidis or Clostridium perfringens* infection (Keerqin *et al.*, 2017; Pourabedin *et al.*, 2017). However, such a study cannot be undertaken at Nottingham Trent University due to the lack of Home Office License permitting the deliberate infection of birds with pathogens.

4) Considering that prebiotics are added in miniscule amounts, an interesting avenue would be to explore *in ovo* as a method of administering of XOS. Chicken feed is made in large batches and small amounts of XOS added to rather large volumes of feed may not guarantee its consumption by every bird and perhaps could be the reason for the conflicting results of XOS on bird performance. *In ovo* technology involves mechanically delivery of substances directly into the incubating egg. This technique was developed for vaccination against viral diseases but has been employed for delivery of prebiotics, probiotics, synbiotics, hormones, vitamins and peptides (Siwek *et al.*, 2018). The applications of *in ovo* technology and its potential as a highly effective method for early programming of microbiota and development of immune system has been highlighted in several review papers (Kadam *et al.*, 2013; Roto, Kwon and Ricke, 2016; Siwek *et al.*, 2018; Taha-Abdelaziz *et al.*, 2018; Jha *et al.*, 2019). Two independent studies have demonstrated that prebiotics administered *in ovo* increased the number of *Bifidobacteria* in newly hatched chicks (Villaluenga *et al.*, 2004; Tako *et al.*, 2014). The advantages of delivering XOS *in ovo* would ensure uniform delivery to every bird and early stimulation of beneficial microbiota rather than random colonizers from the environment post hatch. The *in ovo* administration of XOS could be followed by feeding a XOS supplemented diet to ensure the continuity of its beneficial effects.

5) Another potential application of XOS that has not yet been studied *in vivo* is its antioxidant capacity. The intestinal epithelium is prone to oxidative damage by reactive oxygen species (ROS) produced as a result of aerobic metabolism. Oxidative damage can also be induced by luminal oxidants such as mycotoxins in feed (Kouadio *et al.*, 2005; Osselaere *et al.*, 2013;

Antonissen *et al.*, 2015) and fats or lipids in aged grains (Ringseis, Piwek and Eder, 2007; Varady, Eder and Ringseis, 2011; Liang *et al.*, 2015). Heat stress may also cause oxidative damage to the intestine (Yu *et al.*, 2013; Liu *et al.*, 2017). On the other hand, cold stress can provoke physiological responses and enhance energy consumption and ROS production (Blagojević, 2007; Blagojevic, Grubor-Lajsic and Spasic, 2011; Fu *et al.*, 2013). The animals defence to oxidative damage includes antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidise) and antioxidants substances which maintain the redox balance, important for intestinal health and functions of visceral organs such as kidney, liver and heart (Nordberg and Arnér, 2001; Nain *et al.*, 2008; Wang, Xiao and Zhan, 2018). Oxidative stress occurs when the generation of ROS exceeds the scavenging capacity of the antioxidant system (Burton and Jauniaux, 2011; Sack *et al.*, 2017). To date, only one published study has demonstrated the *in vitro* antioxidant activity of XOS alone and in combination with probiotic *L. plantarum* (Yu *et al.*, 2015). In this study by Yu et al., XOS, *L. plantarum*, and their combination were assayed by free radical scavenging activity and metal chelating ability compared to the control, ascorbic acid. The antioxidant properties of XOS, *L. plantarum*, and their combination were found to be better than the control activity with the combination of XOS and *L. plantarum* performing better that the two alone. Given the results from this study the *in vivo* antioxidant capacity of XOS would be worth investigating.

6) The histomorphological examination of the intestinal tissue is an economical and speedy method that gives an indication of the intestinal health of the animal. Measurements of villus height, crypt depth and goblet cell count of birds fed diet with and without XOS can give a prompt idea of the role of XOS as a gut health promoter. Longer villi, shorter crypts and increased goblet cell counts have been reported in studies in chicken or pigs fed pre or probiotic supplemented diets to elucidate the beneficial effects of these feed additives (De Maesschalck *et al.*, 2015; Yin *et al.*, 2019; Pourabedin *et al.*, 2014)

7) In order to further investigate the stimbiotic effect of XOS, Bacteroides from chicken caecum can be isolated and grown on medium containing XOS as the sole carbon source but differing in their DP and substitution. Following growth, the expression of genes responsible for carbohydrate degradation can be assessed using qPCR. In addition, knockout mutants of PUL-XylL and PUL-XylS can be constructed to understand the role of their genes in carbohydrate metabolism.

6.5. Recommendations based on practical applications of these findings

1) The market for prebiotics in food and feed in growing rapidly (FAO, 2007). Considering the abundance of XOS in the plant kingdom and its benefits as a prebiotic in human and animal health, there is tremendous scope for the feed industry to exploit other agricultural wastes such as sugarcane bagasse, coconut and palm kernels, nut shells and even marine algae for production of XOS.

2) For underperforming old farms where substantial financial investments are needed to improve infrastructure, XOS may serve as an economical option to improve bird performance.

3) As newly hatched broilers chicks in hatcheries have no maternal contact they are exposed to a wide variety of bacteria from environmental sources. Therefore, inclusion of XOS in post hatch diets or starter diets is highly beneficial for early colonization of the gut by Lactobacilli and *Bifidobacteria*.

In conclusion, these recommendations show that there is a clear role for XOS in the antibiotic-free production of meat poultry and their efficacy will be further enhanced by increased understanding of optimum conditions for their application.

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Appendix

Appendix A: Trial 1 (Oligo26)- Ingredients of Basal diet

Starter – 50kg batch

Ingredients	Quantity Added (kg)
Wheat	17.5
Corn	11.95
Soya bean meal	17.18
Soya oil	1.38
Salt	0.15
Limestone	0.05
Dicalcium Phos 18%P	1.13
Sodium Bicarbonate	0.05
Lysine - HCl	0.115
DL- Methionine	0.155
Threonine	0.065
Vitmin mineral premix	0.25
Quantum Blue 5G	0.05

Grower – 130 kg batch

Ingredients	Quantity Added (kg)
Wheat	22.75
Corn	17.27
Soya bean meal	20.18
Soya oil	2.5
Salt	0.2
Limestone	0.03
Dicalcium Phos 18%P	1.3
Sodium Bicarbonate	0.065
Lysine HCl	0.117
DL- Methionine	0.1755
Threonine	0.0585
Vitamin mineral premix	0.325
Quantum Blue 5G	0.0065

Finisher – 80 kg batch

Ingredients	**Quantity Added (kg)**
Wheat	28
Corn	23.34
Soya bean meal	22.04
Soya oil	3.96
Salt	0.248
Limestone	0.024
Dicalcium Phos 18%P	1.47
Sodium Bicarbonate	0.08
Lysine HCl	0.144
DL- Methionine	0.208
Threonine	0.06
Vitamin mineral premix	0.4
Quantum Blue 5G	0.008

Appendix B: Concentration of extracted RNA and purity ratios

Trial 1 Oligo26

Sample/Pen No	Tissue weight (g)	conc (ng/ul)	260/280	260/230
1	0.0254	378.04	2.098	1.399
2	0.0271	159.12	2.081	0.706
3	0.0298	96.68	1.951	1.531
4	0.032	285.6	2.076	1.85
5	0.0322	172.08	2.05	2.089
6	0.0312	81.64	1.995	0.264
7	0.0268	111.68	2.064	1.356
8	0.341	87.4	2.05	0.449
9	0.0294	174.68	2.073	0.612
10	0.0407	214.2	2.06	1.25
11	0.0394	406.88	2.094	2.276
12	0.0368	232.04	2.067	0.788
blank		0.6	16	0.007
			25/05/2020	
13	0.0277	380.88	2.07	2.403
14	0.0335	308.76	2.072	2.423
15	0.0245	296.12	2.066	2.397
16	0.0281	316.32	2.074	2.443
17	0.0273	388.84	2.08	2.321
18	0.0311	368	2.069	2.419
19	0.0322	283.2	2.054	2.437
20	0.028	187.68	2.042	2.509
21	0.0315	297.68	2.053	2.47
22	0.0316	174.12	2.04	2.448
23	0.0313	325.76	2.059	2.372
24	0.0288	229.68	2.06	2.432
blank		0	2.25	0.708
			01/06/2020	
25	0.0306	526.08	2.086	2.42
26	0.0287	308.68	2.053	2.427
27	0.0274	211.44	2.077	2.338
28	0.0265	271	2.071	2.424
29	0.034	298.24	2.069	2.32
30	0.0278	216.88	2.064	2.389
31	0.0332	323.72	2.08	2.242
32	0.0325	321.48	2.064	2.383
33	0.0375	288.36	2.065	2.353
34	0.0308	310.24	2.071	2.386

	35	0.0298	363.48	2.084	2.227
	36	0.0305	336.52	2.078	2.386
blk			1.32	2.063	0.273
			08/06/2020		
	37	0.0334	212.52	2.019	2.308
	38	0.0361	359.28	2.096	2.402
	39	0.0337	261.44	2.086	2.427
	40	0.0351	193.44	2.088	2.437
	41	0.0324	215.92	2.089	2.399
	42	0.0293	187.88	2.081	2.441
	43	0.0337	244.68	2.085	2.441
	44	0.0275	206.28	2.083	2.396
	45	0.0282	234.08	2.094	2.266
	46	0.039	242.52	2.086	2.456
	47	0.0314	177.32	2.077	2.358
	48	0.0343	239.28	2.084	2.451
blk		0			

Trial 2 – Oligo13

Bird number	Tissue Weight (g)	conc	260/280	260/230
1	0.0348	273.48	2.068	1.933
2	0.0323	146.08	2.06	2.127
3	0.0323	253.32	2.06	2.389
4	0.0322	242.72	2.057	2.396
5	0.0374	305.84	2.069	2.072
6	0.0294	259.12	2.072	2.009
7	0.0371	229.8	2.05	2.31
8	0.0326	301.88	2.062	2.396
9	0.0273	226.8	2.061	1.985
10	0.035	258.28	2.058	2.391
11	0.0333	259.76	2.056	2.325
12	0.0313	362.52	2.071	2.393
13	0.0274	330	2.076	2.419
14	0.0356	195.2	2.056	2.384
15	0.305	214.24	2.058	2.37
16	0.0263	194.16	2.046	2.389
17	0.0292	176.04	2.045	2.271
18	0.0262	265.4	2.05	2.416
19	0.0346	274.32	2.071	2.108
20	0.0287	285.32	2.062	2.406
blank		0.68	5.667	0.013

Appendix C: Number of outlier observations removed due to excessive feed spillage in Trial 1 (Oligo26)

	D0 BW	D8 BW	D0-8 BWG	D0-8 FI	D0-8 FCR	D14 BW	D8-14 BWG	D8-14 FI	D8-14 FCR	D22 BW	D14-22 BWG	D14-22 FI	D14-22 FCR	D28 BW	D22-28 BWG	D22-28 FI	D22-28 FCR	D35 BW	D28-35 BWG	D28-35 FI	D28-35 FCR	D0-35 BWG	D0-35 FI	D0-35 FCR
CON	0	0	0	3	3	0	0	1	1	0	0	0	0	0	1	3	3	0	1	1	1	1	1	1
XOS	0	0	0	1	1	0	1	2	2	0	1	1	2	0	0	3	3	0	0	0	0	0	0	0
XYL	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
XOS+XYL	0	0	0	1	1	0	0	1	1	0	0	1	1	0	0	1	1	0	1	1	1	1	1	1
Total	0	0	1	5	6	0	1	5	5	0	1	2	3	0	1	7	7	0	2	2	2	2	2	2

Appendix D: List of all proteins (382) quantifiable from all samples along with gene names and organism.

Protein accession was used to generate this table from Uniprot.org using “retrieve ID/mapping” function. Note - some proteins have been deleted from the database since the date of the proteome used for analysis (Oct 2019).

Entry	Entry name	Protein names	Gene names	Organism
A0A174MXP4	A0A174MXP4_BACUN	Collagen triple helix repeat (20 copies)	ERS852510_04211	Bacteroides uniformis
A0A174K9C2	A0A174K9C2_PHOVU	Cell surface protein	ERS852457_03365	Phocaeicola vulgatus (Bacteroides vulgatus)
A0A1U7LBG6	A0A1U7LBG6_9BACT	DUF4988 domain-containing protein	BHV81_08890	Butyricimonas synergistica
R9HRK8	R9HRK8_BACUN	SusC/RagA family TonB-linked outer membrane protein	C801_03513	Bacteroides uniformis dnLKV2
A0A174P3E2	A0A174P3E2_BACUN	Outer membrane receptor proteins, mostly Fe transport	ERS417307_03860	Bacteroides uniformis
A0A3A5WNH7	A0A3A5WNH7_9BACE	Uncharacterized protein	DWZ67_10585	Bacteroides sp. AF34-31BH
R9HQK9	R9HQK9_BACUN	SusC/RagA family TonB-linked outer membrane protein	C801_03189	Bacteroides uniformis dnLKV2
R9HTP3	R9HTP3_BACUN	SusC/RagA family TonB-linked outer membrane protein	C801_03011	Bacteroides uniformis dnLKV2
A0A373ITS7	A0A373ITS7_9BACE	Uncharacterized protein (Fragment)	DW175_10675	Bacteroides sp. AM16-15
A0A3E4G8C8	A0A3E4G8C8_9BACE	TonB-dependent receptor	DXD78_09395	Bacteroides sp. D20

U6RAN7	U6RAN7_9BACT	Uncharacterized protein	HMPREF1534_03679	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A3A5PEZ3	A0A3A5PEZ3_9BACE	Cell envelope biogenesis protein OmpA	DW039_05875	Bacteroides sp. AF39-16AC
U6RAE4	U6RAE4_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1534_02829	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A3A5WYB5	A0A3A5WYB5_9BACE	DUF4988 domain-containing protein	DWZ03_09355	Bacteroides sp. AF29-11
R7EA11	R7EA11_9BACE	TonB_dep_Rec domain-containing protein	BN594_01687	Bacteroides uniformis CAG:3
R9HUW0	R9HUW0_BACUN	SusC/RagA family TonB-linked outer membrane protein	C801_02294	Bacteroides uniformis dnLKV2
U6RC22	U6RC22_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1534_02487	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A174W2A6	A0A174W2A6_PHOVU	Cell surface protein	ERS852556_03039	Phocaeicola vulgatus (Bacteroides vulgatus)
A0A1U7LBQ5	A0A1U7LBQ5_9BACT	Uncharacterized protein	BHV81_08880	Butyricimonas synergistica
A0A1Y4QN01	A0A1Y4QN01_9FIRM	Uncharacterized protein	B5E91_01780	[Clostridium] spiroforme
A0A143XH05	A0A143XH05_9BACT	BACON domain-containing protein	BN3659_00474	Alistipes sp. CHKCI003
A0A174CDM4	A0A174CDM4_BACUN	TonB-dependent receptor plug	ERS417307_00988	Bacteroides uniformis
A0A173Z0I2	A0A173Z0I2_BACUN	TonB-dependent receptor	ERS417307_00481	Bacteroides uniformis
A0A143XJC8	A0A143XJC8_9BACT	TonB dependent receptor	BN3659_00999	Alistipes sp. CHKCI003
A0A143XWR7	A0A143XWR7_9FIRM	Clostridial hydrophobic W	NDGK_02190	Clostridiales bacterium CHKCI001
A0A4U6MYJ2	A0A4U6MYJ2_CAMJU	Deleted.		

A0A373J7U4	A0A373J7U4_9BACE	TonB-dependent receptor	DW175_02670	Bacteroides sp. AM16-15
E5UWV3	E5UWV3_9BACE	DUF3869 domain-containing protein	HMPREF9011_03176	Bacteroides sp. 3_1_40A
R9HUD1	R9HUD1_BACUN	DUF5115 domain-containing protein	C801_02032	Bacteroides uniformis dnLKV2
R5RPR4	R5RPR4_9FIRM	Uncharacterized protein	BN747_00441	Firmicutes bacterium CAG:646
S6CPS1	S6CPS1_9BACT	TonB dependent receptor		uncultured bacterium
S3Y2M8	S3Y2M8_BACSE	Uncharacterized protein	HMPREF1181_03300	Bacteroides stercoris CC31F
I8ZTB6	I8ZTB6_BACUN	Deleted.		
R9HT89	R9HT89_BACUN	SusE domain-containing protein	C801_03009	Bacteroides uniformis dnLKV2
R7EAN8	R7EAN8_9BACE	MotA_ExbB domain-containing protein	BN594_01610	Bacteroides uniformis CAG:3
A0A318D0I4	A0A318D0I4_9GAMM	Uncharacterized protein	DL796_12175	Kangiella spongicola
A0A143XJV0	A0A143XJV0_9BACT	Uncharacterized protein	BN3659_00782	Alistipes sp. CHKCI003
A0A414WCZ2	A0A414WCZ2_BACUN	SusC/RagA family TonB-linked outer membrane protein (TonB-dependent receptor)	DW216_10390 EAJ11_17760 GAP44_12425	Bacteroides uniformis
R7EK70	R7EK70_9BACE	TPR_REGION domain-containing protein	BN594_00431	Bacteroides uniformis CAG:3
A0A143XSZ0	A0A143XSZ0_9BACT	Uncharacterized protein	BN3659_01737	Alistipes sp. CHKCI003
A0A143XX16	A0A143XX16_9BACT	Uncharacterized protein	BN3659_02234	Alistipes sp. CHKCI003
U6RBK7	U6RBK7_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1534_02486	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
E5UXY0	E5UXY0_9BACE	TonB-dependent receptor plug domain-containing protein	HMPREF9011_03553	Bacteroides sp. 3_1_40A

I9QUZ5	I9QUZ5_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1065_03948	Bacteroides dorei CL03T12C01
A0A1Y3RNH9	A0A1Y3RNH9_9FIRM	Uncharacterized protein	B5G40_07675	Flavonifractor sp. An9
R9HHV2	R9HHV2_PHOVU	Biopolymer transporter ExbB	C800_02022	Bacteroides vulgatus dnLKV7
R8VUS9	R8VUS9_9CLOT	AP_endonuc_2 domain-containing protein	HMPREF1526_02342	Butyricicoccus pullicaecorum 1.2
R9HVE8	R9HVE8_BACUN	Uncharacterized protein	C801_02503	Bacteroides uniformis dnLKV2
R9I9W8	R9I9W8_BACUN	Uncharacterized protein	C801_01120	Bacteroides uniformis dnLKV2
I9U4W6	I9U4W6_PHOVU	OmpA-like domain-containing protein	HMPREF1058_02553	Bacteroides vulgatus CL09T03C04
A0A4Q5EFC5	A0A4Q5EFC5_BACUN	TonB-dependent receptor	EAJ11_00735 GAP44_02285	Bacteroides uniformis
U6RQ26	U6RQ26_9BACT	Glutamate dehydrogenase	HMPREF1534_00144	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
R9H3W5	R9H3W5_PHOVU	Uncharacterized protein	C800_03708	Bacteroides vulgatus dnLKV7
G1V333	G1V333_9DELT	OmpA-like domain-containing protein	HMPREF0178_01930	Bilophila sp. 4_1_30
U6RQG1	U6RQG1_9BACT	SusC/RagA family TonB-linked outer membrane protein (Fragment)	HMPREF1534_00001	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A143XT19	A0A143XT19_9BACT	Uncharacterized protein	BN3659_01733	Alistipes sp. CHKCI003
R6TE04	R6TE04_9STAP	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	BN609_01033	Staphylococcus sp. CAG:324
A0A1Y4QIE2	A0A1Y4QIE2_9FIRM	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	B5E91_06845	[Clostridium] spiroforme

R9HSR7	R9HSR7_BACUN	Xylose isomerase (EC 5.3.1.5)	xylA C801_02830	Bacteroides uniformis dnLKV2
A0A173ZX38	A0A173ZX38_BACUN	Putative exported lipoprotein	ERS417307_00608	Bacteroides uniformis
A0A1Y4T7X8	A0A1Y4T7X8_9FIRM	Bifunctional metallophosphatase/5'-nucleotidase	B5E66_06695	Faecalibacterium sp. An121
E7H3L4	E7H3L4_9BURK	Autotransporter domain-containing protein	HMPREF9464_01302	Sutterella wadsworthensis 3_1_45B
A0A143XN94	A0A143XN94_9BACT	Uncharacterized protein	BN3659_01170	Alistipes sp. CHKCI003
A0A1Q6UBC4	A0A1Q6UBC4_9PROT	Peptidoglycan-associated protein	pal BHW58_06720	Azospirillum sp. 51_20
A0A143XHX1	A0A143XHX1_9BACT	Uncharacterized protein	BN3659_00623	Alistipes sp. CHKCI003
A0A143XPR0	A0A143XPR0_9BACT	Glutamate decarboxylase (EC 4.1.1.15)	gadB BN3659_01336	Alistipes sp. CHKCI003
A0A1Y4MFM1	A0A1Y4MFM1_9FIRM	D-galactose-binding periplasmic protein (D-galactose/ D-glucose-binding protein)	B5F11_17715	Anaerotruncus colihominis
R9HS63	R9HS63_BACUN	Uncharacterized protein	C801_03511	Bacteroides uniformis dnLKV2
A0A1Y4SYT3	A0A1Y4SYT3_9FIRM	PTS glucose transporter subunit IIBC	B5E66_11770	Faecalibacterium sp. An121
A0A1M6QCW3	A0A1M6QCW3_9FIRM	S-layer homology domain-containing protein	SAMN02745138_01294	Anaerotignum lactatifermentans DSM 14214
R7AK20	R7AK20_9CLOT	Glutamate dehydrogenase	BN684_01032	Clostridium sp. CAG:505
F4TB93	F4TB93_ECOLX	Outer membrane porin protein NmpC	ECJG_04942	Escherichia coli M718
A0A1Y3UIY1	A0A1Y3UIY1_9FIRM	Sodium:alanine symporter family protein	B5G26_02700	Anaerotignum lactatifermentans

A0A174CSL2	A0A174CSL2_BACUN	TonB-dependent receptor (TonB-dependent receptor plug)	DW988_18515 ERS417307_01054 GAQ27_04980 GAQ49_03195	Bacteroides uniformis
A0A1Y3Y8G9	A0A1Y3Y8G9_9FIRM	Pyruvate:ferredoxin (Flavodoxin) oxidoreductase	B5G00_04160	Blautia sp. An46
A0A143XQ85	A0A143XQ85_9BACT	Outer membrane protein Omp28	BN3659_01734	Alistipes sp. CHKCI003
E5YBD1	E5YBD1_BILW3	Uncharacterized protein	HMPREF0179_03504	Bilophila wadsworthia (strain 3_1_6)
E3GH71	E3GH71_9FIRM	Ethanolamine utilization protein eutL (Microcompartment protein PduB)	ELI_4084 SAMN04487888_11042	Eubacterium callanderi
A0A3E4W3Y6	A0A3E4W3Y6_9BACE	SusF/SusE family outer membrane protein	DXD78_09385	Bacteroides sp. D20
A0A373UTK6	A0A373UTK6_9BACT	Deleted.		
A0A1Y3U9M2	A0A1Y3U9M2_9FIRM	Phenyllactate dehydratase	B5G26_00105	Anaerotignum lactatifermentans
A0A1Y4S958	A0A1Y4S958_9FIRM	PTS acetylgalactosamine transporter subunit IID	B5E77_10935	Lachnoclostridium sp. An131
A0A414JRA3	A0A414JRA3_BACUN	SusE domain-containing protein	DW729_07360	Bacteroides uniformis
A0A3A5X5B6	A0A3A5X5B6_9BACE	Uncharacterized protein	DWZ03_18575	Bacteroides sp. AF29-11
R9I1P7	R9I1P7_BACUN	Uncharacterized protein	C801_01122	Bacteroides uniformis dnLKV2
A0A143XRF8	A0A143XRF8_9BACT	Outer membrane protein Omp28	BN3659_01736	Alistipes sp. CHKCI003
A0A1Y4QN11	A0A1Y4QN11_9FIRM	Uncharacterized protein	B5E91_01830	[Clostridium] spiroforme
A0A143XGZ8	A0A143XGZ8_9BACT	Cna protein B-type domain protein	BN3659_00471	Alistipes sp. CHKCI003

A0A1Y4TC37	A0A1Y4TC37_9FIRM	Uronate isomerase (EC 5.3.1.12) (Glucuronate isomerase) (Uronic isomerase)	uxaC B5E66_04180	Faecalibacterium sp. An121
A0A1Y4RF00	A0A1Y4RF00_9FIRM	Uncharacterized protein (Fragment)	B5E82_14730	Lachnoclostridium sp. An138
A0A143XSI0	A0A143XSI0_9BACT	Fibronectin type III domain protein	BN3659_01738	Alistipes sp. CHKCI003
A0A1Y4T5W8	A0A1Y4T5W8_9FIRM	Alkaline phosphatase	B5E67_07760	Faecalibacterium sp. An122
F3AAB2	F3AAB2_9FIRM	CO-methylating acetyl-CoA synthase (EC 2.3.1.169)	HMPREF0992_00007	Lachnospiraceae bacterium 6_1_63FAA
U6RTS6	U6RTS6_9BACT	TonB-dependent receptor	HMPREF1534_00108	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
R9I3N3	R9I3N3_BACUN	Multifunctional fusion protein [Includes: Protein translocase subunit SecD; Protein-export membrane protein SecF]	secD secF C801_00660	Bacteroides uniformis dnLKV2
A0A396QST6	A0A396QST6_9FIRM	Elongation factor Tu (EF-Tu)	tuf DXA96_08245	Lachnospiraceae bacterium OF09-33XD
A0A2V2PN55	A0A2V2PN55_ENTFC	Deleted.		
U6RBY9	U6RBY9_9BACT	SusE domain-containing protein	HMPREF1534_02827	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
R9I424	R9I424_BACUN	Alpha-D-xyloside xylohydrolase	C801_00142	Bacteroides uniformis dnLKV2
S3CFF4	S3CFF4_9BURK	Porin_4 domain-containing protein	HMPREF1476_01281	Sutterella wadsworthensis HGA0223
A0A1Y4UMH0	A0A1Y4UMH0_9FIRM	ABC transporter substrate-binding protein	B5E56_09450	Flavonifractor sp. An112

A0A174P284	A0A174P284_BACUN	LamG domain-containing protein	ERS417307_03863	Bacteroides uniformis
A0A412Q7W3	A0A412Q7W3_PHOVU	Uncharacterized protein	DWX04_21775	Phocaeicola vulgatus (Bacteroides vulgatus)
A0A1Y4TF80	A0A1Y4TF80_9FIRM	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.-)	B5E66_00095	Faecalibacterium sp. An121
R6PPB7	R6PPB7_9FIRM	Uncharacterized protein	BN668_01752	Firmicutes bacterium CAG:466
A0A3E4Q3B7	A0A3E4Q3B7_BACUN	DUF3869 domain-containing protein	DW795_19485 DXC91_08820	Bacteroides uniformis
A0A143XQ81	A0A143XQ81_9BACT	Peptidase family S41	BN3659_01739	Alistipes sp. CHKCI003
A0A143XRV7	A0A143XRV7_9BACT	Outer membrane protein Omp28	BN3659_01735	Alistipes sp. CHKCI003
A0A1Y4THU8	A0A1Y4THU8_9FIRM	Urocanate reductase (EC 1.3.99.33)	B5E66_00300	Faecalibacterium sp. An121
A0A1Y4KR11	A0A1Y4KR11_9FIRM	Uncharacterized protein	B5F19_16735	Pseudoflavonifractor sp. An184
A0A1Y4TGS3	A0A1Y4TGS3_9FIRM	Sugar ABC transporter ATP-binding protein	B5E66_02470	Faecalibacterium sp. An121
A0A1C6CE34	A0A1C6CE34_9CLOT	Glutamate dehydrogenase	gdhA SAMEA3545390_01411	uncultured Butyricicoccus sp.
A0A143XVN3	A0A143XVN3_9BACT	BACON domain-containing protein	BN3659_02080	Alistipes sp. CHKCI003
I9IW03	I9IW03_PHOVU	SusC/RagA family TonB-linked outer membrane protein	HMPREF1058_02268	Bacteroides vulgatus CL09T03C04
R9I383	R9I383_BACUN	Phosphoenolpyruvate carboxykinase (ATP) (PCK) (PEP carboxykinase) (PEPCK) (EC 4.1.1.49)	pckA C801_00635	Bacteroides uniformis dnLKV2

A0A4Q5EFA4	A0A4Q5EFA4_BACUN	RagB/SusD family nutrient uptake outer membrane protein	EAJ11_00740 GAP44_02280	Bacteroides uniformis
R8W0F3	R8W0F3_9CLOT	Propanediol utilization protein PduA	HMPREF1526_01212	Butyricicoccus pullicaecorum 1.2
A0A2V2PJ49	A0A2V2PJ49_ENTFC	Deleted.		
A0A1Y4U1W5	A0A1Y4U1W5_9FIRM	Putative K(+)-stimulated pyrophosphate-energized sodium pump (EC 7.2.3.-) (Membrane-bound sodium-translocating pyrophosphatase) (Pyrophosphate-energized inorganic pyrophosphatase) (Na(+)-PPase)	hppA B5E62_05360	Lachnoclostridium sp. An118
U6RCI3	U6RCI3_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1534_02679	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A3N5IG31	A0A3N5IG31_9PLAN	TAF domain-containing protein	EHM77_06745	Planctomycetaceae bacterium
A0A354M5F0	A0A354M5F0_9BACT	GGGtGRT protein (Fragment)	DDY73_12145	Coprobacter fastidiosus
R9IAT4	R9IAT4_BACUN	Uncharacterized protein	C801_00425	Bacteroides uniformis dnLKV2
G1V312	G1V312_9DELT	Peptidoglycan-associated protein	pal HMPREF0178_01909	Bilophila sp. 4_1_30
C0DBY2	C0DBY2_9FIRM	Rubrerythrin (Fragment)	CLOSTASPAR_06789	[Clostridium] asparagiforme DSM 15981
A0A1Y4WEE1	A0A1Y4WEE1_9FIRM	3-hydroxybutyryl-CoA dehydrogenase	B5E42_14860	Flavonifractor sp. An10

R6QG46	R6QG46_9FIRM	PTS system mannose/fructose/sorbose family IID protein	BN583_00941	Anaerostipes sp. CAG:276
A0A370CMH6	A0A370CMH6_9MYCO	Keratin_2_tail domain-containing protein (Fragment)	DVS77_34450	Mycolicibacterium moriokaense
A0A1Y4TE45	A0A1Y4TE45_9FIRM	BMP family ABC transporter substrate-binding protein	B5E66_02510	Faecalibacterium sp. An121
A0A1Y3YNJ7	A0A1Y3YNJ7_9FIRM	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	B5F98_05355	Pseudoflavonifractor sp. An44
U6RLW7	U6RLW7_9BACT	Uncharacterized protein	HMPREF1534_00843	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
U6REY3	U6REY3_9BACE	Uncharacterized protein	HMPREF1214_04318	Bacteroides sp. HPS0048
A0A1Y4TFC5	A0A1Y4TFC5_9FIRM	ABC transporter substrate-binding protein	B5E66_00810	Faecalibacterium sp. An121
A0A1Y4SDS3	A0A1Y4SDS3_9FIRM	LacI family transcriptional regulator	B5E77_04355	Lachnoclostridium sp. An131
U6RA64	U6RA64_9BACT	Glycosyl hydrolase family 109 protein 1	HMPREF1534_03270	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A1Y4T1Z7	A0A1Y4T1Z7_9FIRM	Glycogen synthase (EC 2.4.1.21) (Starch [bacterial glycogen] synthase)	glgA B5E66_11710	Faecalibacterium sp. An121
U3NGG1	U3NGG1_9BACT	DNA gyrase subunit A (EC 5.6.2.2)	gyrA	uncultured bacterium EB4
R9HV20	R9HV20_BACUN	Uncharacterized protein	C801_02295	Bacteroides uniformis dnLKV2

A0A1Y4AB18	A0A1Y4AB18_9FIRM	BMP family ABC transporter substrate-binding protein	B5F94_05215	Flavonifractor sp. An4
A0A1Y3ST11	A0A1Y3ST11_9FIRM	Transcriptional regulator	B5G33_13960	Blautia sp. An81
A0A1Y4TDW8	A0A1Y4TDW8_9FIRM	Purine nucleoside phosphorylase DeoD-type (EC 2.4.2.1)	B5E66_02520	Faecalibacterium sp. An121
R9H325	R9H325_PHOVU	SusC/RagA family TonB-linked outer membrane protein	C800_03755	Bacteroides vulgatus dnLKV7
S3ZLM1	S3ZLM1_BACSE	Succinate dehydrogenase flavoprotein subunit	HMPREF1181_00307	Bacteroides stercoris CC31F
A0A1Y4RRE1	A0A1Y4RRE1_9FIRM	Uncharacterized protein	B5E84_06440	Lachnoclostridium sp. An14
I9A6Z2	I9A6Z2_PHOVU	DUF5017 domain-containing protein	HMPREF1058_00306	Bacteroides vulgatus CL09T03C04
A0A1Y4S971	A0A1Y4S971_9FIRM	PTS acetylgalactosamine transporter subunit IIC	B5E77_10940	Lachnoclostridium sp. An131
S3BCB3	S3BCB3_9BURK	Porin_4 domain-containing protein	HMPREF1476_01398	Sutterella wadsworthensis HGA0223
A0A328HLU7	A0A328HLU7_9ENTR	30S ribosomal protein S5 (Fragment)	rpsE DOZ52_28585	Enterobacter hormaechei
A0A1Y4T5C9	A0A1Y4T5C9_9FIRM	Glucose-1-phosphate adenylyltransferase subunit GlgD	B5E67_08840	Faecalibacterium sp. An122
A0A1Y4K6D7	A0A1Y4K6D7_9ACTN	ABC transporter substrate-binding protein	B5F23_03095	Olsenella sp. An188
A0A1Q6IUB2	A0A1Q6IUB2_9BACT	Deleted.		
R6Y0B0	R6Y0B0_9FIRM	Rubrerythrin	BN815_01162	Firmicutes bacterium CAG:94
A0A1Y4L8M2	A0A1Y4L8M2_9FIRM	Uncharacterized protein	B5F19_12710	Pseudoflavonifractor sp. An184
R5ZBK8	R5ZBK8_9LACO	S-layer protein	BN764_00067	Lactobacillus amylovorus CAG:719

R9I1M4	R9I1M4_BACUN	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.-)	C801_01089	Bacteroides uniformis dnLKV2
A0A1Y4T4Q2	A0A1Y4T4Q2_9FIRM	Sugar ABC transporter permease	B5E66_09415	Faecalibacterium sp. An121
W6PHV6	W6PHV6_9BACE	OMP_b-brl domain-containing protein	BN890_51260	Bacteroides xylanisolvens SD CC 1b
R6YJM2	R6YJM2_9FIRM	Uncharacterized protein	BN815_00945	Firmicutes bacterium CAG:94
A0A2V2PLT1	A0A2V2PLT1_ENTFC	Deleted.		
A0A1Y4FA84	A0A1Y4FA84_9FIRM	Uncharacterized protein	B5F53_02490	Blautia sp. An249
A0A143XES4	A0A143XES4_9BACT	Peptidase C10 family protein	BN3659_00442	Alistipes sp. CHKCI003
R9I3V3	R9I3V3_BACUN	SusC/RagA family TonB-linked outer membrane protein	C801_00822	Bacteroides uniformis dnLKV2
E5Y381	E5Y381_BILW3	Ethanolamine utilization protein EutM	HMPREF0179_00642	Bilophila wadsworthia (strain 3_1_6)
A0A1Y3ZND6	A0A1Y3ZND6_9FIRM	Uncharacterized protein	B5F94_13515	Flavonifractor sp. An4
A0A412VK70	A0A412VK70_PHOVU	TonB-dependent receptor	DWW27_14115	Phocaeicola vulgatus (Bacteroides vulgatus)
B1BZF9	B1BZF9_9FIRM	PTS system, mannose/fructose/sorbose family, IID component	CLOSPI_00336 CLOSPI_02206	[Clostridium] spiroforme DSM 1552
C0CUH8	C0CUH8_9FIRM	Putative potassium/sodium efflux P-type ATPase, fungal-type	CLOSTASPAR_00670	[Clostridium] asparagiforme DSM 15981
E5VA13	E5VA13_9BACE	TonB-dependent Receptor Plug domain-containing protein (TonB-dependent receptor)	DXD65_08040 HMPREF1007_01596	Bacteroides sp. 4_1_36

A0A2V2PM25	A0A2V2PM25_ENTFC	Deleted.		
A0A174CRV4	A0A174CRV4_BACUN	DUF5017 domain-containing protein	DW795_11300 DW988_20090 EP145_00805 ERS417307_01052 GAQ27_04990 GAQ49_03205	Bacteroides uniformis
W1F721	W1F721_ECOLX	Outer membrane protein A (Outer membrane porin A)	ompA	Escherichia coli ISC7
R9HPC2	R9HPC2_BACUN	Ferritin (EC 1.16.3.2)	C801_03802	Bacteroides uniformis dnLKV2
R9HRV8	R9HRV8_BACUN	Biopolymer transporter ExbB	C801_03630	Bacteroides uniformis dnLKV2
R8VZN7	R8VZN7_9CLOT	Uncharacterized protein	HMPREF1526_00928	Butyricicoccus pullicaecorum 1.2
A0A3C1BVU6	A0A3C1BVU6_9FIRM	ABC transporter substrate-binding protein	DCO69_00935 DFH97_00265	Clostridiales bacterium
A0A3E4XGS2	A0A3E4XGS2_BACUN	SusD/RagB family nutrient-binding outer membrane lipoprotein	DXC07_15195	Bacteroides uniformis
A0A143XIL5	A0A143XIL5_9BACT	Outer membrane protein Omp28	BN3659_00781	Alistipes sp. CHKCI003
A0A4T9Y902	A0A4T9Y902_9FIRM	Deleted.		
A0A412PRL1	A0A412PRL1_9FIRM	Deleted.		
A0A2U2ELR2	A0A2U2ELR2_9ACTN	Type I glyceraldehyde-3-phosphate dehydrogenase (Fragment)	C1C94_033895	Streptomyces sp. SMS_SU21
A0A350XV71	A0A350XV71_9FIRM	Glutaconyl-CoA decarboxylase subunit beta (EC 7.2.4.5)	DCY15_01425	Ruminococcaceae bacterium
A0A2A4AQP3	A0A2A4AQP3_9PSED	Uncharacterized protein	CO192_17590	Pseudomonas pelagia

A0A4Q4ICB7	A0A4Q4ICB7_9BACL	Tubulin domain-containing protein (Fragment)	EWI07_14530	Sporolactobacillus sp. THM7-4
A0A1Y4WDG3	A0A1Y4WDG3_9FIRM	Sodium:proton antiporter	B5E42_14920	Flavonifractor sp. An10
U6RC26	U6RC26_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1534_03094	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A1Y4T2W3	A0A1Y4T2W3_9FIRM	Uncharacterized protein	B5E66_10295	Faecalibacterium sp. An121
A0A414WEJ4	A0A414WEJ4_BACUN	TonB-dependent receptor	DW216_06300 EAJ11_07930 GAP44_06670	Bacteroides uniformis
R9HUT7	R9HUT7_BACUN	LTD domain-containing protein	C801_02269	Bacteroides uniformis dnLKV2
R6VK10	R6VK10_9FIRM	Glycogen synthase (EC 2.4.1.21) (Starch [bacterial glycogen] synthase)	glgA BN546_01891	Firmicutes bacterium CAG:227
R6TPQ8	R6TPQ8_9STAP	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	BN609_00821	Staphylococcus sp. CAG:324
R9GYT7	R9GYT7_PHOVU	Uncharacterized protein	C800_04312	Bacteroides vulgatus dnLKV7
A0A1Y4IR87	A0A1Y4IR87_9FIRM	Glutaconyl-CoA decarboxylase subunit beta (EC 7.2.4.5)	B5F29_08345	Lachnoclostridium sp. An196
F0R247	F0R247_PHOSB	Na(+)-translocating NADH-quinone reductase subunit B (Na(+)-NQR subunit B) (Na(+)-translocating NQR subunit B) (EC 7.2.1.1) (NQR complex subunit B) (NQR-1 subunit B)	nqrB Bacsa_0786	Phocaeicola salanitronis (strain DSM 18170 / JCM 13657 / BL78) (Bacteroides salanitronis)

A0A1Y4KRZ7	A0A1Y4KRZ7_9FIRM	Uncharacterized protein (Fragment)	B5F19_16760	Pseudoflavonifractor sp. An184
U6RSE7	U6RSE7_9BACT	Uncharacterized protein	HMPREF1534_00002	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
G9RX93	G9RX93_9FIRM	6-carboxy-5,6,7,8-tetrahydropterin synthase (EC 4.1.2.50) (Queuosine biosynthesis protein QueD)	HMPREF1032_00822	Subdoligranulum sp. 4_3_54A2FAA
A0A351WLT9	A0A351WLT9_9BACT	Glutamate dehydrogenase	DCZ95_15140	Verrucomicrobia bacterium
B1BZ10	B1BZ10_9FIRM	PTS system mannose/fructose/sorbose family IID component	CLOSPI_00167	[Clostridium] spiroforme DSM 1552
A0A143XJN3	A0A143XJN3_9BACT	Colicin uptake protein TolQ	BN3659_00849	Alistipes sp. CHKCI003
R7EHL7	R7EHL7_9BACE	DUF1080 domain-containing protein	BN594_02319	Bacteroides uniformis CAG:3
A0A3A5PCL4	A0A3A5PCL4_9BACE	Uncharacterized protein	DW039_02875	Bacteroides sp. AF39-16AC
A0A373IR95	A0A373IR95_9BACE	Uncharacterized protein	DW175_11765	Bacteroides sp. AM16-15
A0A143XUN4	A0A143XUN4_9BACT	Uncharacterized protein	BN3659_01943	Alistipes sp. CHKCI003
A0A1Y4UK18	A0A1Y4UK18_9FIRM	Peptide ABC transporter	B5E56_11230	Flavonifractor sp. An112
A0A1Y4IM87	A0A1Y4IM87_9FIRM	ABC transporter permease	B5F29_02845	Lachnoclostridium sp. An196
A0A1Y4T4P2	A0A1Y4T4P2_9FIRM	ABC transporter substrate-binding protein	B5E66_09425	Faecalibacterium sp. An121
R8W5Z8	R8W5Z8_9CLOT	Fe-ADH domain-containing protein	HMPREF1526_00643	Butyricicoccus pullicaecorum 1.2
M2Q308	M2Q308_9FIRM	RNA polymerase sigma factor SigA	sigA HMPREF9943_01190	Eggerthia catenaformis OT 569 = DSM 20559
A0A1Y4SCL1	A0A1Y4SCL1_9FIRM	Sugar ABC transporter permease	B5E77_06345	Lachnoclostridium sp. An131

A0A4Z4VFF6	A0A4Z4VFF6_VIBPH	Deleted.		
A0A099IC77	A0A099IC77_CLOIN	PTS acetylgalactosamine transporter subunit IID	CIAN88_02135	Clostridium innocuum
U6RS84	U6RS84_9BACT	MotA_ExbB domain-containing protein	HMPREF1534_00096	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
R6TA85	R6TA85_9STAP	Uncharacterized protein	BN609_00572	Staphylococcus sp. CAG:324
A0A1Y3U9Q4	A0A1Y3U9Q4_9FIRM	Phenyllactate dehydratase	B5G26_00110	Anaerotignum lactatifermentans
A0A415QBP2	A0A415QBP2_9BACT	SusC/RagA family TonB-linked outer membrane protein	DWZ68_17035 DXA50_06385	Butyricimonas virosa
A0A1Y4LXW9	A0A1Y4LXW9_9CLOT	Cu_amine_oxidN1 domain-containing protein	B5F15_03725	Butyricicoccus pullicaecorum
A0A1Y3WKQ9	A0A1Y3WKQ9_9FIRM	Phosphate ABC transporter phosphate-binding protein	B5G12_06560	Faecalibacterium sp. An58
A0A4S2AWX0	A0A4S2AWX0_9BACE	Glutamate dehydrogenase	E5355_10330	Bacteroides sp. NM69_E16B
A0A1U7LA50	A0A1U7LA50_9BACT	SusC/RagA family protein	BHV81_11135	Butyricimonas synergistica
A0A143XTJ1	A0A143XTJ1_9BACT	Uncharacterized protein	BN3659_01805	Alistipes sp. CHKCI003
U6RDM4	U6RDM4_9BACT	F5/8 type C domain-containing protein	HMPREF1534_03096	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A1Y4TC00	A0A1Y4TC00_9FIRM	Sugar ABC transporter substrate-binding protein	B5E66_06405	Faecalibacterium sp. An121
U6RBJ0	U6RBJ0_9BACT	Uncharacterized protein	HMPREF1534_03175	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
R9HMV4	R9HMV4_PHOVU	Uncharacterized protein	C800_00784	Bacteroides vulgatus dnLKV7
A0A2V2PGU5	A0A2V2PGU5_ENTFC	Deleted.		
R9HWT3	R9HWT3_BACUN	Uncharacterized protein	C801_01483	Bacteroides uniformis dnLKV2

A0A1Y4EML9	A0A1Y4EML9_9FIRM	Fn3_like domain-containing protein (Fragment)	B5F54_14520	Anaeromassilibacillus sp. An250
W4PKA8	W4PKA8_9BACE	Glutamate dehydrogenase	JCM6294_3300	Bacteroides pyogenes DSM 20611 = JCM 6294
A0A3G1KSB8	A0A3G1KSB8_9FIRM	Ethanolamine utilization protein EutM	DCMF_11620	Peptococcaceae bacterium DCMF
D1PIH8	D1PIH8_9FIRM	Rubrerythrin	SUBVAR_04143	Subdoligranulum variabile DSM 15176
I9TUB5	I9TUB5_PHOVU	SusC/RagA family TonB-linked outer membrane protein	HMPREF1058_03865 HMPREF1058_03867	Bacteroides vulgatus CL09T03C04
A0A417TVY5	A0A417TVY5_9CLOT	4-hydroxybutyryl-CoA dehydratase	DXB50_04095	Butyricicoccus sp. OM04-18BH
R9I4P4	R9I4P4_BACUN	SusC/RagA family TonB-linked outer membrane protein	C801_01567	Bacteroides uniformis dnLKV2
R9I3L2	R9I3L2_BACUN	Nitroreductase domain-containing protein	C801_00634	Bacteroides uniformis dnLKV2
A0A1Y4HYH7	A0A1Y4HYH7_9FIRM	Uncharacterized protein	B5F35_05925	Anaeromassilibacillus sp. An200
A0A1Y4TEB0	A0A1Y4TEB0_9FIRM	PTS N-acetylgalactosamine transporter subunit IID	B5E66_02110	Faecalibacterium sp. An121
C7MP28	C7MP28_CRYCD	Pyruvate:ferredoxin (Flavodoxin) oxidoreductase, homodimeric	Ccur_09770	Cryptobacterium curtum (strain ATCC 700683 / DSM 15641 / 12-3)
V6HZZ4	V6HZZ4_9LEPT	ATP synthase subunit alpha (EC 7.1.2.2) (ATP synthase F1 sector subunit alpha) (F-ATPase subunit alpha)	atpA LEP1GSC062_3324	Leptospira alexanderi serovar Manhao 3 str. L 60

B1BZ34	B1BZ34_9FIRM	Phosphate-binding protein	pstS CLOSPI_00191	[Clostridium] spiroforme DSM 1552
U6REJ9	U6REJ9_9BACT	Outer membrane autotransporter barrel domain-containing protein	HMPREF1534_02645	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A1M6XAE3	A0A1M6XAE3_9FIRM	Copper amine oxidase N-terminal domain-containing protein	SAMN02745138_02752	Anaerotignum lactatifermentans DSM 14214
I9R4F4	I9R4F4_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1064_01249	Bacteroides dorei CL02T12C06
R9I7M9	R9I7M9_9BACT	Xylose isomerase (EC 5.3.1.5)	xylA C802_02021 E5339_17635	Phocaeicola sartorii
K2B5H6	K2B5H6_9BACT	Tr-type G domain-containing protein (Fragment)	ACD_47C00338G0002	uncultured bacterium
R6BVI8	R6BVI8_9CLOT	Uncharacterized protein	BN513_00647	Clostridium sp. CAG:169
A0A2A7QU21	A0A2A7QU21_9LACO	S-layer protein	CP357_09365	Lactobacillus sp. UMNPBX6
D1PJ75	D1PJ75_9FIRM	Acyl-CoA dehydrogenase, C-terminal domain protein	SUBVAR_04395	Subdoligranulum variabile DSM 15176
T0TC41	T0TC41_9STRE	Glyceraldehyde-3-phosphate dehydrogenase	HSISB1_1622	Streptococcus sp. HSISB1
A0A2U1C1L1	A0A2U1C1L1_9FIRM	Triosephosphate isomerase (TIM) (TPI) (EC 5.3.1.1) (Triose-phosphate isomerase)	tpiA C7373_105219	Intestinimonas butyriciproducens
R5DWJ3	R5DWJ3_9FIRM	Uncharacterized protein	BN798_00014	Eubacterium sp. CAG:86
A0A1Y4KVL3	A0A1Y4KVL3_9FIRM	Uncharacterized protein (Fragment)	B5F19_16730	Pseudoflavonifractor sp. An184
R9HW23	R9HW23_BACUN	Preprotein translocase, YajC subunit	C801_01405	Bacteroides uniformis dnLKV2

A0A1Y4VDD7	A0A1Y4VDD7_9FIRM	Ethanolamine utilization protein EutM	B5E53_06600	Eubacterium sp. An11
A0A1Y4TC98	A0A1Y4TC98_9FIRM	ATPase	B5E66_05615	Faecalibacterium sp. An121
B1BZ09	B1BZ09_9FIRM	PTS system sorbose-specific iic component	CLOSPI_00166	[Clostridium] spiroforme DSM 1552
A0A4Z4VFP1	A0A4Z4VFP1_VIBPH	Deleted.		
R8VSL1	R8VSL1_9CLOT	Uncharacterized protein	HMPREF1526_02735	Butyricicoccus pullicaecorum 1.2
A0A416W9T5	A0A416W9T5_9BACT	DUF4136 domain-containing protein	DWW52_08120	Odoribacter sp. AF15-53
W1X860	W1X860_ECOLX	Outer membrane protein W	Q609_ECAC01329G0005	Escherichia coli DORA_A_5_14_21
R9I1G0	R9I1G0_BACUN	Uncharacterized protein	C801_01098	Bacteroides uniformis dnLKV2
R9HJN9	R9HJN9_PHOVU	MFS domain-containing protein	C800_01599	Bacteroides vulgatus dnLKV7
R6QI05	R6QI05_9FIRM	Single-stranded DNA-binding protein (SSB)	BN792_00519	Faecalibacterium sp. CAG:82
K6BTP7	K6BTP7_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF0999_01403	Parabacteroides sp. D25
D1Y6G5	D1Y6G5_9BACT	Rubrerythrin	HMPREF7215_1824	Pyramidobacter piscolens W5455
D1PNS9	D1PNS9_9FIRM	Formate acetyltransferase (EC 2.3.1.54) (Pyruvate formate-lyase)	pflB SUBVAR_06000	Subdoligranulum variabile DSM 15176
A0A3P3QUS9	A0A3P3QUS9_9FIRM	ABC transporter substrate-binding protein	EHV10_14105	Lachnoanaerobaculum gingivalis
A0A318D3C3	A0A318D3C3_9GAMM	L-lactate dehydrogenase (EC 1.1.1.27)	DL796_12005	Kangiella spongicola
A0A1Y4UUY5	A0A1Y4UUY5_9FIRM	Nitrate ABC transporter permease	B5E56_06330	Flavonifractor sp. An112

A0A1Y4TCA1	A0A1Y4TCA1_9FIRM	3-hydroxybutyryl-CoA dehydrogenase	B5E67_00260	Faecalibacterium sp. An122
A0A1Y4MC39	A0A1Y4MC39_9FIRM	GRAM_POS_ANCHORING domain-containing protein	B5F11_19495	Anaerotruncus colihominis
A0A1Y3W770	A0A1Y3W770_9FIRM	Uncharacterized protein	B5G12_11865	Faecalibacterium sp. An58
A0A143XJV3	A0A143XJV3_9BACT	TPR_REGION domain-containing protein	BN3659_00932	Alistipes sp. CHKCI003
A0A0S8L0V0	A0A0S8L0V0_9PROT	50S ribosomal protein L5	rplE AMJ72_06915	Acidithiobacillales bacterium SM1_46
R9I1K3	R9I1K3_9BACT	Uncharacterized protein	C802_03534	Phocaeicola sartorii
A0A0V8QCL0	A0A0V8QCL0_9FIRM	GGGtGRT protein	ASU35_02615	Acetivibrio ethanolgignens
A0A1Y4TG31	A0A1Y4TG31_9FIRM	Uncharacterized protein	B5E66_02690	Faecalibacterium sp. An121
R6DEK6	R6DEK6_9CLOT	Uncharacterized protein	BN558_01125	Clostridium sp. CAG:242
A0A1Y3YEV6	A0A1Y3YEV6_9FIRM	Uronate isomerase (EC 5.3.1.12) (Glucuronate isomerase) (Uronic isomerase)	uxaC B5G00_01585	Blautia sp. An46
R6PB87	R6PB87_9FIRM	Carbon monoxide dehydrogenase (EC 1.2.7.4)	BN627_01065	Lachnospiraceae bacterium CAG:364
A0A318CZF4	A0A318CZF4_9GAMM	Chaperone protein HtpG (Heat shock protein HtpG) (High temperature protein G)	htpG DL796_11965	Kangiella spongicola
R7EE73	R7EE73_9BACE	AP_endonuc_2 domain-containing protein	BN594_02318	Bacteroides uniformis CAG:3
A0A4Q5DYA9	A0A4Q5DYA9_BACUN	Uncharacterized protein	EAJ11_20415 GAP44_20490	Bacteroides uniformis
A0A417TE71	A0A417TE71_9FIRM	Thioredoxin reductase (EC 1.8.1.9)	trxB DXB59_08440	Ruminococcus sp. OM05-10BH
R9I3W8	R9I3W8_BACUN	Uncharacterized protein	C801_02575	Bacteroides uniformis dnLKV2

A0A1Q6UFY4	A0A1Q6UFY4_9PROT	Uncharacterized protein	BHW58_01030	Azospirillum sp. 51_20
A0A1Y4TEL9	A0A1Y4TEL9_9FIRM	D-galactose-binding periplasmic protein (D-galactose/ D-glucose-binding protein)	B5E66_02085	Faecalibacterium sp. An121
R7BCP6	R7BCP6_9CLOT	Uncharacterized protein	BN684_00413	Clostridium sp. CAG:505
S3Z8W3	S3Z8W3_BACSE	Uncharacterized protein	HMPREF1181_03301	Bacteroides stercoris CC31F
A0A1C7PNF9	A0A1C7PNF9_9GAMM	Deleted.		
U7UES6	U7UES6_9FIRM	Glutamate dehydrogenase	gdh HMPREF1250_1128	Megasphaera sp. BV3C16-1
A0A1Y4T6G1	A0A1Y4T6G1_9FIRM	Uncharacterized protein	B5E66_10160	Faecalibacterium sp. An121
K5Z332	K5Z332_9BACT	Uncharacterized protein	HMPREF1077_01874	Parabacteroides johnsonii CL02T12C29
H6VTN5	H6VTN5_9LACO	Surface layer protein SlpB (Fragment)		Lactobacillus crispatus
R6Y3M9	R6Y3M9_9FIRM	Phosphoenolpyruvate carboxykinase [GTP] (PEP carboxykinase) (PEPCK) (EC 4.1.1.32) (GTP-dependent phosphoenolpyruvate carboxykinase) (GTP-PEPCK)	pckG BN815_01789	Firmicutes bacterium CAG:94
R6NVA0	R6NVA0_9FIRM	PTS system N-acetylglucosamine-specific IIB component Glc family (TC 4.A.1.1.2)/PTS system N-acetylglucosamine-specific IIC component Glc family (TC 4.A.1.1.2)	BN703_01142	Ruminococcus sp. CAG:55
U6RBU2	U6RBU2_9BACT	Uncharacterized protein	HMPREF1534_03304	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223

R9HXF2	R9HXF2_BACUN	Branched-chain-amino-acid transaminase (EC 2.6.1.42)	C801_01887	Bacteroides uniformis dnLKV2
I8ZET9	I8ZET9_BACUN	Deleted.		
V8C507	V8C507_9FIRM	Uncharacterized protein	HMPREF1202_01565	[Ruminococcus] lactaris CC59_002D
A0A1M6MVV3	A0A1M6MVV3_9FIRM	S-layer homology domain-containing protein	SAMN02745138_00691	Anaerotignum lactatifermentans DSM 14214
A0A415QF00	A0A415QF00_9BACT	Porin	DWZ68_14370	Butyricimonas virosa
A0A143XYK9	A0A143XYK9_9BACT	Thiol-disulfide oxidoreductase ResA	resA_9 BN3659_02387	Alistipes sp. CHKCI003
R6Y8A3	R6Y8A3_9FIRM	Repeat protein	BN815_01400	Firmicutes bacterium CAG:94
A0A1Y4QDW9	A0A1Y4QDW9_9FIRM	Reverse rubrerythrin	B5E92_14620	Erysipelatoclostridium sp. An15
A0A410QF56	A0A410QF56_9FIRM	Formate--tetrahydrofolate ligase (EC 6.3.4.3) (Formyltetrahydrofolate synthetase) (FHS) (FTHFS)	fhs EQM13_13225	Tissierellia sp. JN-28
U6RBV6	U6RBV6_9BACT	Protein translocase subunit SecY	secY HMPREF1534_02598	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
R6GC52	R6GC52_9BACE	Pyruvate ferredoxin/flavodoxin oxidoreductase	BN744_00279	Bacteroides sp. CAG:633
U6R8T6	U6R8T6_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1534_03990	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A1Y4I0F3	A0A1Y4I0F3_9FIRM	30S ribosomal protein S2	rpsB B5F35_02035	Anaeromassilibacillus sp. An200
R7EI53	R7EI53_9BACE	Uncharacterized protein	BN594_02861	Bacteroides uniformis CAG:3
A0A1Y4TH05	A0A1Y4TH05_9FIRM	Uncharacterized protein	B5E66_02695	Faecalibacterium sp. An121

U6RRD1	U6RRD1_9BACT	Sodium ion-translocating decarboxylase, beta subunit	HMPREF1534_00565	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A1Y4TDT7	A0A1Y4TDT7_9FIRM	Preprotein translocase subunit YajC	B5E66_03225	Faecalibacterium sp. An121
S2ZDM4	S2ZDM4_9FIRM	50S ribosomal protein L22	rplV HMPREF1216_02385	Coprococcus sp. HPP0048
A0A416ZCG6	A0A416ZCG6_9FIRM	Sodium:alanine symporter family protein	DWV52_03335	Ruminococcaceae bacterium AF10-16
R9HW10	R9HW10_BACUN	Uncharacterized protein	C801_01390	Bacteroides uniformis dnLKV2
A0A1Q6UFM7	A0A1Q6UFM7_9PROT	Autotransporter domain-containing protein	BHW58_01695	Azospirillum sp. 51_20
W9AFS9	W9AFS9_ECOLX	Major outer membrane lipoprotein Lpp (Braun lipoprotein) (Murein lipoprotein) (BLP)	too many to list	Escherichia coli O25b:H4-ST131
A0A1Y4L476	A0A1Y4L476_9FIRM	ATPase	B5F19_14410	Pseudoflavonifractor sp. An184
C0C223	C0C223_9FIRM	Rubredoxin	CLOHYLEM_06194	[Clostridium] hylemonae DSM 15053
R9L6P5	R9L6P5_9BACL	Multiple sugar transport system permease	C812_03478	Paenibacillus barengoltzii G22
A0A2D9LBZ4	A0A2D9LBZ4_9ALTE	Succinate dehydrogenase flavoprotein subunit (EC 1.3.5.1)	CMG81_07925	Marinobacter sp.
I9U8L0	I9U8L0_BACUN	Deleted.		
R7ELB1	R7ELB1_9BACE	Mannan endo-1,4-beta-mannosidase (EC 3.2.1.78)	BN594_00627	Bacteroides uniformis CAG:3
A0A328HLR1	A0A328HLR1_9ENTR	30S ribosomal protein S15 (Fragment)	DOZ52_28910	Enterobacter hormaechei
A0A1Y4QGV0	A0A1Y4QGV0_9FIRM	F5/8 type C domain-containing protein	B5E91_10210	[Clostridium] spiroforme

W9E405	W9E405_PELUQ	ATP synthase subunit beta (EC 7.1.2.2) (ATP synthase F1 sector subunit beta) (F-ATPase subunit beta)	atpD Pelub83DRAFT_1028	Candidatus Pelagibacter ubique HIMB083
E5UNJ2	E5UNJ2_9BACE	Cell surface protein	HMPREF9011_00261	Bacteroides sp. 3_1_40A
A0A1M6M8D7	A0A1M6M8D7_9FIRM	C4-dicarboxylate transporter, DctM subunit	SAMN02745138_00522	Anaerotignum lactatifermentans DSM 14214
U6RQY9	U6RQY9_9BACE	Uronate isomerase (EC 5.3.1.12) (Glucuronate isomerase) (Uronic isomerase)	uxaC HMPREF1214_02300	Bacteroides sp. HPS0048
A0A2V2PBB5	A0A2V2PBB5_ENTFC	Deleted.		
A0A1Y3UAA6	A0A1Y3UAA6_9FIRM	Branched-chain amino acid transport system carrier protein	B5G26_01215	Anaerotignum lactatifermentans
A0A1Y4T6I2	A0A1Y4T6I2_9FIRM	Pyruvate, phosphate dikinase (EC 2.7.9.1)	B5E66_07600	Faecalibacterium sp. An121
R6TSD9	R6TSD9_9FIRM	Uncharacterized protein	BN626_01947	Eubacterium rectale CAG:36
A0A1C6HKY6	A0A1C6HKY6_9FIRM	NADH peroxidase (EC 1.11.1.1)	rbr3A SAMEA3545394_02830	uncultured Flavonifractor sp.
U6REI6	U6REI6_9BACT	SusD_RagB domain-containing protein	HMPREF1534_02828	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
X8HWM6	X8HWM6_9ACTN	Uncharacterized protein	HMPREF1503_0463	Olsenella uli MSTE5
I9QFZ3	I9QFZ3_9BACT	SusC/RagA family TonB-linked outer membrane protein	HMPREF1064_04101	Bacteroides dorei CL02T12C06
A0A1Y3U546	A0A1Y3U546_9FIRM	Acyl-CoA dehydrogenase	B5G26_06285	Anaerotignum lactatifermentans
R7B7R4	R7B7R4_9CLOT	D-isomer specific 2-hydroxyacid	BN684_00660	Clostridium sp. CAG:505

		dehydrogenase NAD-binding		
A0A328HLM3	A0A328HLM3_9ENTR	30S ribosomal protein S8 (Fragment)	DOZ52_29140	Enterobacter hormaechei
A0A7D9N4D0	A0A7D9N4D0_LACJH	PTS fructose transporter subunit IIC	T285_00670	Lactobacillus johnsonii N6.2
R6QKR7	R6QKR7_9CLOT	Uncharacterized protein	BN618_00327	Clostridium nexile CAG:348
A0A2M6WCQ9	A0A2M6WCQ9_9BACT	K(+)-insensitive pyrophosphate-energized proton pump (EC 7.1.3.1) (Membrane-bound proton-translocating pyrophosphatase) (Pyrophosphate-energized inorganic pyrophosphatase) (H(+)-PPase)	hppA COU22_01365	Candidatus Komeilibacteria bacterium CG10_big_fil_rev_8_21_14_0_10_41_13
A0A1Y4TFE6	A0A1Y4TFE6_9FIRM	Peptidase	B5E66_00485	Faecalibacterium sp. An121
A0A316PKN5	A0A316PKN5_9FIRM	Reverse rubrerythrin	DBY17_05610	Ruminococcaceae bacterium
A0A2V2PLD9	A0A2V2PLD9_ENTFC	Deleted.		
G1WFC9	G1WFC9_9ACTN	Uncharacterized protein	HMPREF9452_00042	Collinsella tanakaei YIT 12063
A0A1Y4B0C4	A0A1Y4B0C4_9FIRM	Sugar ABC transporter substrate-binding protein	B5F86_08445	Lachnoclostridium sp. An298
A0A143XIB7	A0A143XIB7_9BACT	Outer membrane protein OprM	oprM_2 BN3659_00877	Alistipes sp. CHKCI003
R9I9S1	R9I9S1_BACUN	Large-conductance mechanosensitive channel	mscL C801_01090	Bacteroides uniformis dnLKV2
U6R7V3	U6R7V3_9BACT	Uncharacterized protein	HMPREF1534_03721	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223

A0A2U1CDP7	A0A2U1CDP7_9FIRM	Nucleoside-binding protein	C7373_10221	Intestinimonas butyriciproducens
A0A1H4D9T1	A0A1H4D9T1_9BACT	Multifunctional fusion protein [Includes: Protein translocase subunit SecD; Protein-export membrane protein SecF]	secF secD SAMN05444145_105206	Alistipes timonensis JC136
R7B772	R7B772_9CLOT	EIICB-Mtl (EC 2.7.1.197) (EIICBA-Mtl) (Mannitol permease IIC component) (Mannitol-specific phosphotransferase enzyme IIB component) (PTS system mannitol-specific EIIB component) (PTS system mannitol-specific EIIC component) (PTS system mannitol-specific EIICB component) (PTS system mannitol-specific EIICBA component)	BN684_00922	Clostridium sp. CAG:505
A0A4S2FU71	A0A4S2FU71_9BACT	Fimbrillin family protein	E5339_02645	Phocaeicola sartorii
A0A3A9BAI6	A0A3A9BAI6_9BACT	Dehydrogenase	D7X10_03540	bacterium D16-63
U6R7S6	U6R7S6_9BACT	Uncharacterized protein	HMPREF1534_03760	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
I9R2H2	I9R2H2_9BACT	DUF4988 domain-containing protein	HMPREF1065_02940	Bacteroides dorei CL03T12C01
U2ACP8	U2ACP8_9CLOT	SLH domain-containing protein (Fragment)	HMPREF0239_04570	Clostridium sp. ATCC BAA-442

R9HVH9	R9HVH9_BACUN	Galactokinase	C801_02367	Bacteroides uniformis dnLKV2
A0A1Q6UBP2	A0A1Q6UBP2_9PROT	Uncharacterized protein	BHW58_05555	Azospirillum sp. 51_20
R5VEJ4	R5VEJ4_9FIRM	Branched-chain amino acid ABC transporter permease protein	BN518_00031	Roseburia sp. CAG:18
R9HZQ8	R9HZQ8_BACUN	Uncharacterized protein	C801_03490	Bacteroides uniformis dnLKV2
R7EFF1	R7EFF1_9BACE	Uncharacterized protein	BN594_02370	Bacteroides uniformis CAG:3
R9I0M1	R9I0M1_BACUN	Uncharacterized protein	C801_01280	Bacteroides uniformis dnLKV2
A0A1Y4I144	A0A1Y4I144_9FIRM	Uncharacterized protein	B5F35_01590	Anaeromassilibacillus sp. An200
A0A1Y4UJN7	A0A1Y4UJN7_9FIRM	BMP family ABC transporter substrate-binding protein	B5E56_10660	Flavonifractor sp. An112
R6YP56	R6YP56_9FIRM	S-layer domain protein	BN815_01607	Firmicutes bacterium CAG:94
A0A2V2PDY0	A0A2V2PDY0_ENTFC	Deleted.		
A0A143XM02	A0A143XM02_9BACT	Putative propionyl-CoA carboxylase beta chain 5 (EC 6.4.1.3)	accD5 BN3659_01347	Alistipes sp. CHKCI003
A0A1Y4IMQ7	A0A1Y4IMQ7_9FIRM	Peptidase	B5F29_03310	Lachnoclostridium sp. An196
A0A1Y4TAY9	A0A1Y4TAY9_9FIRM	Sugar ABC transporter permease	B5E66_06885	Faecalibacterium sp. An121
R6YNS6	R6YNS6_9FIRM	Xylose isomerase domain protein TIM barrel	BN815_00114	Firmicutes bacterium CAG:94
D1PQP0	D1PQP0_9FIRM	ABC transporter, solute-binding protein	SUBVAR_06709	Subdoligranulum variabile DSM 15176
R9HTY2	R9HTY2_BACUN	Uncharacterized protein	C801_02000	Bacteroides uniformis dnLKV2
A0A1Y4JYU9	A0A1Y4JYU9_9ACTN	PTS mannose family transporter subunit IID	B5F23_08525	Olsenella sp. An188
A0A328HLL1	A0A328HLL1_9ENTR	Uncharacterized protein (Fragment)	DOZ52_29190	Enterobacter hormaechei

U6RFP3	U6RFP3_9BACT	Uncharacterized protein	HMPREF1534_02485	Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223
A0A416WEW0	A0A416WEW0_9BACT	PorT family protein	DWW52_02350	Odoribacter sp. AF15-53
R5D0W9	R5D0W9_9FIRM	Bmp domain-containing protein	BN795_00812	Firmicutes bacterium CAG:83