INVESTIGATION INTO THE EFFECTS OF PROBIOTIC, PREBIOTIC AND SYNBIOTIC FEED SUPPLEMENTS ON GUT MICROBIOTA, IMMUNE FUNCTION AND PERFORMANCE OF BROILER CHICKENS

By

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Abstract

The aim of this project was to evaluate the effects of probiotics, prebiotics and synbiotics on the gut ecosystem, immune function and growth parameters of broiler. The first study screened naturally occurring *Campylobacter* levels in four local sites and revealed the NTU broiler research unit and the NTU animal unit laying hens were *Campylobacter* free, but a small holding with laying hens was positive and the commercial broiler farm was negative until thinning, after which it was positive. The second study investigated possible delivery routes of a novel strain of *Lactobacillus johnsonii* (FI9785) into broiler chicken gut and concluded feed was the optimum method for delivery. A third study compared the effect *L. Johnsonii* FI9785 supplied via feed to control and showed no significant difference in the CFU of caecal *Campylobacter,* no significant ($p \leq 0.05$) effects on growth performance and serum uric acid concentration over 4 weeks. However, mucin layer thickness in the jejunum was significantly ($P \leq 0.05$) increased. Concentration of IgA in the serum blood of probiotic treated birds was also increased but IgM and IgG were not significantly altered.

Study 4 involved isolation and in vitro screening of candidate probiotic isolates of lactic acid bacteria and a prebiotic from Jerusalem artichoke plant (JA). All tests confirmed the isolates had the characteristics of lactic acid bacteria and have an inhibition activity toward *Campylobacter*. All isolates belonged to the genus of *Lactobacillus* and all retained viability during freezing and drying and the poultry gastrointestinal environment, indicating all were potential probiotic agents. Assessment of JA inulin levels indicated the plant to be a potentially good prebiotic source with these isolates. Study 5 investigated *in vivo* effects of the *Lactobacillus* isolates (probiotic), JA powder (prebiotic), synbiotic (mix of pre and probiotic). Caecal content were negative for *Campylobacter* throughout but at day 7, abundance of *Firmicutes* phyla were higher ($p \leq 0.05$) than control for all of supplements treatments and abundance of *Faecalibacterium* genus numerically increased in all treatments but significantly ($p \leq 0.05$) only in 5% prebiotic and probiotic supplemented diets. At day 42, abundance of genus of *Erysipelotrichaceae* decreased in all treatments. Assessment of growth performance showed JA had no effects but probiotic and synbiotic supplementation caused a degradation in the body weight and increased feed intake. Supplements downregulated the cytokine expression IFNγ, IL-10 and IL-6 in the ileum tissue but showed no effect in the bursa tissue.

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Dedicate

Finally, and most of all, I would like to dedicate this dissertation to my parents for all their love, encouragement, and great support. It is the best thing in my life to be a part of their family.

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List of abbreviations

BF	Bursa of fabricius
CCDA	Cefoperazone Deoxycholate agar
cDNA	complementary DNA
CFU	Colony forming unit
DNA	deoxyribonucleic acid
DP	Degree polymerisation
EPEF	European Production Efficiency Factor
FCR	Feed conversion ratio
FI	Feed intake
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIT	Gastrointestinal tract
IL-10	Interleukin 10
IL-6	Interleukin
INF-y	Interferon gamma
JA	Jerusalem artichoke
LAB	Lactic acid bacteria
MOS	Mannanoligosaccharide
mRNA	Messenger RNA
MRS	De Man, Rogosa and Sharpe agar
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RT-	Reverse transcription
qPCR	Quantitative Polymerase chain reaction
rDNA	Ribosomal DNA
RNA	ribonucleic acid)
rRNA	Ribosomal RNA
SCFA	Short chain fatty acid
SE	Standard error

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Chapter 1

Literature Review

1.1 Introduction

The poultry industry has grown rapidly since Second World War and the volume of poultry products continues to increase (FAO, 2009). The Food and Agriculture Organisation of the United Nations reported that about 23 billion broiler chickens were produced worldwide in 2016 (FAO, 2018). Therefore broiler chickens are raised in high stocking densities and new strains are genetically selected for very fast growth. Over past 50 years, the growth rate of broiler have increased by over 300% due to intense genetic selection (Knowles, et al. 2008). However selection for fast growth has some side effects such as limited disease resistance, poor skeletal integrity and heart failure. In addition, intensive rearing of broiler chicken has raised a particular issue with disease. Diseases are now considered by many to be the most important obstacle for poultry sector itself and for public health (van Asselt, et al. 2018).

Antibiotics have been used widely for prevention and treatment of infectious disease in farm animals alongside their utilization for human medication for many decades (Edens 2003) to improve the performance of broiler chicken (Allen and Stanton 2014). The numerous disease challenges impacting on the poultry industry have prompted the sector to routinely use antibiotics for the prevention and treatment of disease, as well as for their growth promoting effects. Heat production from individual birds combined with environmental heat presents a major additional challenge to meat poultry production in hot countries such as Iraq. This stressor increases the vulnerability of birds to infectious disease, enhancing gut health of meat chickens in hot countries is a priority commercial poultry production. There is now a growing interest in non-EU countries to follow Europe in reducing in-feed antibiotics due to concerns over antibiotic resistance (Lea, 2013).

Use of in-feed antibiotics led to improved feed conversion efficiency and reduced pathological load associated with poultry production. The greatest problem with antibiotics for poultry as well as for human is antibiotic–resistant bacteria (Nhung, Chansiripornchai and Carrique-Mas 2017). In broiler chickens this has made controlling disease hard because there are many antibiotics previously used in the poultry production which now fail to treat many disease cases. In addition, this presents a major risk for humans as some antibiotics considered important for human health have lost

their efficacy. These reasons together prompted the EU to ban using antibiotics as growth promoters and trying to reduce its therapeutic use on the poultry farms as well as encouraging other, non-EU countries to reduce their use of antibiotics as growth promoters (European Commission, 2005). However, banning or decreasing using antibiotics in poultry farms results in increased mortality rate, feed intake and decrease body weight and growth rate which means increased cost of production and an increased probability of contaminating poultry products intended for human consumption.

The gastrointestinal tract (GIT) is one of the most important system of organs in poultry – not only for nutrition but also as a route for disease entry and for its other, indirect effects on bird performance. (Huyghebaert, Ducatelle and Immerseel 2011) stated that the quality and quantity of GIT microflora and morphological structure of the inner lining (mucosal layer and epithelial cells) have a strong correlation with livestock performance and feed efficiency. Enteric pathogens of animals constitute a direct source for food contamination therefore, poultry production is considered as one of the most important sources for human infection (Santini, et al. 2010). One of the main causes for these illnesses is the contamination of poultry meat by *Campylobacter*, which is reported as an organism that is very easily spread among the birds especially in high population densities such as those associated with intensive poultry production (Santini, et al. 2010). Currently, there is a growing interest to use alternatives to antibiotics in poultry farms to improve the health of these birds and to produce fewer contaminated products.

1.2 Digestive system of broiler chickens

The gastrointestinal tract (GIT) of poultry, specifically broiler chickens, compromises of the oesophagus which continues down past the crop, proventriculus, and gizzard, then continues through the small intestine (duodenum, jejunum, ileum), includes the caeca and ends at the colon and cloaca (Pan and Yu 2014).

1.2.1 Crop & Oesophagus

The majority of bird species have a crop, the main role is as a transient store for consumed food (Svihus 2014a). The crop is a necessity for birds as the feed storage capacity of gizzard and proventriculus is limited (Jackson and Duke 1995). In broiler

chickens, the crop may store between 5 to 10 g of feed but there is no secretion of enzymes or absorption of nutrients in this region of the gastrointestinal tract (Svihus 2014a).

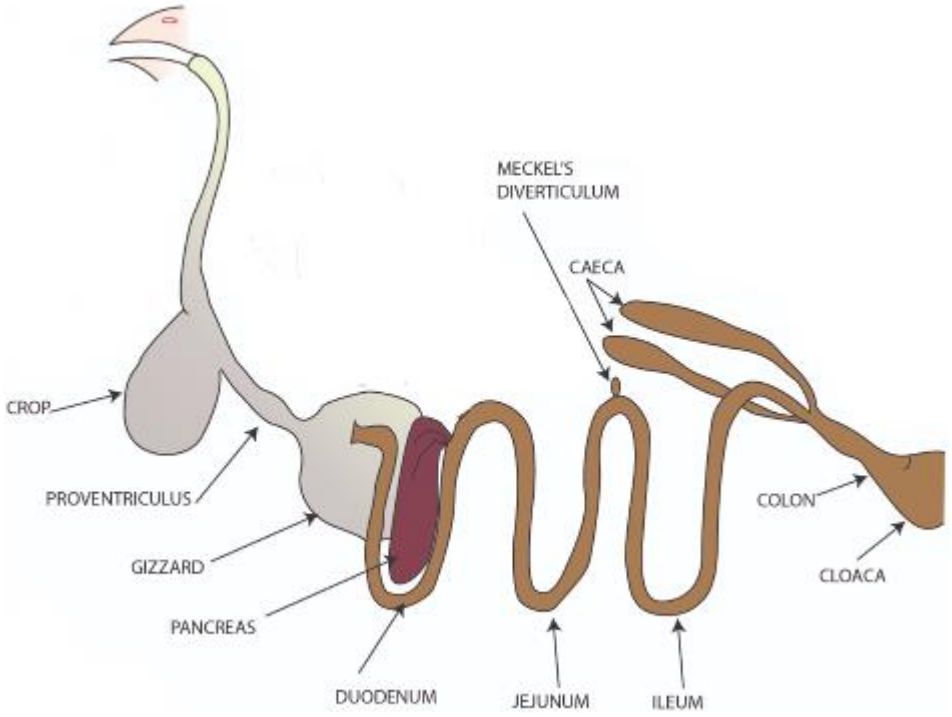

Figure 1:1 Broiler chickens gastrointestinal tract Poultry Hub, (2018)

1.2.2 Proventriculus and Gizzard

The proventriculus and gizzard are the stomach compartments of birds. The proventriculus is a mixing organ where feed and enzymes are mixed before entering the gizzard. Hydrochloric acid and pepsinogen are secreted by the proventriculus and then mixed with contents in the gizzard (Svihus 2014a). The main function of gizzard is grinding feed material, as the bird does not have teeth.

1.2.3 Small Intestine

The small intestine in broiler chickens consists of three sections: duodenum, jejunum and ileum, located between the gizzard and caeca. The duodenum is the first part of the small intestine in which pancreatic and bile ducts release enzymes and bile salts to neutralise the acidic contents from the gizzard and continue the process of digestion (Duke 1986). The first section of the small intestine is referred to as the duodenum, and forms adherent loop around the pancreas. The second section, the jejunum, ends at the

yolk sack residue (Meckel's diverticulum) and has a key role, as large proportion nutrients digested and absorbed here (Wu, et al. 2013). The last segment of the small intestine is the ileum which ends at the ileo-caeco-colonic junction (Nkukwana, et al. 2015). The function of this final section is mainly nutrient absorption and it has been recently proposed that there is a significant role of the ileum in digestion and absorption of starch in broiler chickens. (Svihus 2014b) Svihus (2014b) observed that total starch digestion may increase from 91 to 99% from the beginning to the end of the ileum respectively.

1.2.4 Caeca

Caeca are formed as two paired, blind-ended pouches located at the junction of the ileum and colon (McLelland 1989). In most avian species, the caeca are the unique features of the digestive tract and various sizes and forms are associated with different species (Clench 2015). The functions associated with the caeca are breakdown of fibre and storage of undigested material in addition to absorption of electrolytes and water, which give the caeca some importance in the gut (Svihus 2014a). Depending on the bird species, caecal material is generally retained 3-4 times longer than faecal material (Duke 1986). Caeca of broiler chickens have been observed to undergo morphological change as a result of different dietary components, such as increased fibre content or fermentable content of food (Jozefiak, et al. 2011, Rehman, et al. 2007). These morphological changes in the caeca as a result of shifts in diet indicate that the function of the caeca may include fermentation of dietary compounds (Svihus, Choct and Classen 2013). In the caeca of birds fermentation occurs selectively for some feed stuffs such as fibre (carbohydrate) as each bird contains a unique microbiota (Waite and Taylor, 2014).

1.2.5 Colon and cloaca

The large intestine of birds is relatively short and the avian colon is located between the caeca and the cloaca. In birds, its main function is water and electrolyte reabsorption - unlike the fermentative role of the colon in mammals as fermentation in the avian digestive tract predominantly occurs in the caeca (Lei, et al. 2012). The cloaca has no digestive function, but serves as the exit cavity for the digestive and urogenital systems.

1.3 Microbiota in the gastrointestinal tract

The gut microbiota is a topic that been widely studied because of its impact on health and performance (Roto, Rubinelli and Ricke 2015, Apajalahti and Vienola 2016). The gut microbiota is home to one of the biggest bacterial populations on earth, its level ranging from 10^8 to 10^{14}cfu/g of digesta (Apajalahti, Kettunen and Graham 2004a, Gill, et al. 2006). It have been found that microorganisms that comprise the microbiota of gut directly impact the health of the host, which can provide protection against the damage that may occur to the epithelial layer, and they can promote the development of a healthy immune system (Brisbin, Gong and Sharif 2008b, Hoffmann, et al. 2009). In addition commensal bacteria, in the animal gut can aid in digestion and absorption of nutrients as well as contribute to the enhancement of nutrient utilization (Delzenne and Cani 2011). Meanwhile there is a second group of harmful bacteria, which may be involved in infection, intestinal putrefaction and toxin production (Jeurissen, et al. 2002). Research has suggested that better growth and fewer health issues in poultry could achieve if early development of a mature and diverse microbiota (Munyaka, Khafipour and Ghia 2014). This is in part due to healthy competition among microorganisms. The gut microbiota generally refers to the intestinal regions and most studies focus on the duodenum, jejunum, ileum and caeca (Roto, et al. 2015). Caeca have been given most attention and their contents (digesta) exhibit the most diverse bacterial communities, which in turn, indicate its potential for impact on host health (Pan and Yu 2014). Microbiota in the gut plays an important role in the health of the GIT through several different mechanisms. A primary example is competitive exclusion of pathogenic bacteria by different mechanisms such as reducing available attachment sites on the epithelium, increasing mucin production and reducing pH and competition for nutrients. Other protective effects of microbiota are via selective stimulation of the immune system; production of compounds, like antimicrobial compounds such as bacteriocin and production of short-chain fatty acids (SCFA) (Kogut 2013). Different components of the GI tract vary in their biochemical properties such as oxygen content and pH, which can pose a selective pressure on the microbial community. The oesophagus, crop and cloaca are considered semi-oxic environments, facilitating communities of aerobes, micro-aerobes and facultative anaerobes. The sections of the GI tract located between the crop and cloaca are dominated by obligate or facultative

anaerobes, including members of the *Firmicutes* and *Proteobacteria* (Hird, et al. 2015, Waite and Taylor 2015). (Wei, Morrison and Yu 2013) stated that *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the largest phyla which accounted for >90% of all the sequences. (Pan and Yu 2014) stated that there are about 13 phyla of bacteria, however, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* accounted more than 90% of the intestinal bacteria of broiler chickens. (Amit-Romach, Sklan and Uni 2004) stated that the different sections of the small intestine possess similar microbiota and are dominated by Lactobacilli and Clostridia at the genera level.

The proventriculus is usually acidic which likely poses the first screening of microorganisms entering the digestive tract with feed (Beasley, et al. 2015) and likely biases the resident microbiota towards acidophiles. Stomach acidity varies among bird notably having most acidic stomachs, suggesting a possible role of diet in shaping acidity (Roggenbuck, et al. 2014). The importance of the caeca comes from the fact that it is considered to as act as a reservoir of microbiota in the broiler chickens, the diversity of which is generated in the caeca offering an important section to study pathogens such as Campylobacter (Thibodeau, et al. 2015, Yan, et al. 2017). Also fermentation in the avian digestive tract predominantly occurs in the caeca (Lei, et al. 2012). However microbial communities of the caeca are distinct from the rest of the GI tract (Sohail, et al. 2015). Table 1.1 gives insight into typical taxa and genera of microbiota associated with each region of the gastrointestinal tract but the immense influence of external factors on colonisation means that each situation will vary from this example.

Table 1.1 Surveyed bacteria along the gastrointestinal tract of broiler chickens.

Gut site and CFU	pH	Taxa	Genus
Crop (10^8-10^9)	4-6	*Firmicutes*	*Lactobacillus*
		Actinobacteria	*Bifidobacteria*
		Proteobacteria	*Enterobacter*
Gizzard (10^7-10^8)	2-5	*Firmicutes*	*Lactobacillus, Enterococcus*
Small intestine (10^8-10^9)	6-7.5	*Firmicutes*	*Lactobacillus, Clostridium, Ruminococcus*
		Proteobacteria	*Escherichia, Enterococcus*
Caeca(10^10-10^11	5-5.7	*Firmicutes (44-56%)*	*Faecalibacterium, Pseudobutyrivibrio, Subdoligranulum, Acetanaerobacterium, Lactobacillus, Clostridium, Megamonas, Sporbacter, Peptococcus, Ruminococcus Campylobacter (Hermans, et al. 2011)*
		Fungi	*Candida*
		Bacteroidetes (23-46%)	*Bacteroides*
		Proteobacteria (1-16%)	*Escherichia, Bilophila*
		Archaea (0.81%)	*Methanobrevibacter(woesei,thaueri), Methanobacterium, Methanosphaera, Methanothermus, Methanothermobacter, Methanopyrus, Methanococcus*
Large intestine	7	*Proteobacteria*	*Escherichia , other*

Data adapted from (Yeoman, et al. 2012).

The concept of host factors affecting microbial diversity offers the opportunity to use established and healthy microbiomes to generate a working GIT microbial profile. However, this may prove to be quite challenging as it has been found that broiler chickens interacting together in the same conditions, receiving the same feed, and of the same age and sex still display uniquely dominant bacterial communities (van der Wielen, et al. 2002). The quantity and profile of microflora in the GIT are very important as there is a dynamic balance between the beneficial and pathogenic bacteria in gut. When this balance is altered through any type of physiological or environmental stress, the disruption can lead to disease (Thursby and Juge 2017, Sugiharto 2016). This

disruption to the GIT is reflected by an overall reduction in bird health and gut function, resulting in deteriorating production performance (Gaggia, Mattarelli and Biavati 2010).

1.4 Culture-free methods to study the gut microbiota

Traditional methods for assessing gut microbiota involve culturing diluted intestinal samples on selective media under specific incubation conditions. This technique has a number of limitations. Firstly the selective media limits the microbes cultured to particular species or genera, so offers no insight into the range of microbiota. Also most gut microbiota are anaerobic and require a very low concentration of oxygen to survive, so any air exposure during plating will damage or kill some species, so they are not represented at enumeration (Walker et al., 2014). Intestinal microbiota that are identified from culture-based methods may be incomplete and inaccurate because only 10 to 60% of the total intestinal tract bacteria are culturable (Gong 2007). Several studies have used 16s rDNA clone libraries to investigate the distribution of microorganisms in different regions of the gastrointestinal tract (Wang, et al. 2004). These have confirmed earlier information from cultural studies, indicating a major shift between the stomach, small intestine and large intestine in non-ruminant mammals and man, with the more dense and complex anaerobically dominated communities occurring in the large intestine (Russell and Rychlik 2001). These studies have expanded the knowledge about the gut microbiota and has found that only about 10% of the identified caecal bacterial 16S rDNA sequences represent previously known bacterial species, and the remaining sequences belong to new species or even new genera (Apajalahti, Kettunen and Graham 2004b). Representatives of the same groups of bacteria, described in the cultivation studies, were found using molecular methods, although the species were found in different abundance among the cloned sequences (Bjerrum, et al. 2006). Culture-independent methods have revealed that there is a highly diverse bacterial community in the caeca, which mainly comprise Gram-positive bacteria (Zhu, et al. 2002, Gong 2007). Subsequently this method using molecular and sequencing technique are recognized as able to provide a more comprehensive representation of the microbiome (Zhu, et al. 2002, Lan, et al. 2002).

1.5 Microbial development of the broiler chickens

. In the commercial production of poultry, chicks are hatched away from their parents, which will affect the development of gut microbiota (Stanley, et al. 2013a). In addition they are hatched from disinfected eggs in very clean hatchery (Methner, et al. 1997). The gastrointestinal (GI) tract of poultry come in contact with exogenous microorganisms after hatch immediately which then becomes a warm home for a complex microbiome with majority anaerobic bacteria (Pan and Yu 2014). Then as chicks growing the diversity of the microbiome will become varied until it reaches a relatively stable. Consequently the initial colonization of the GIT by non-pathogenic microbiota in newly hatched chicks will be strongly affected by the microorganism that present in the hatchery or the environment of housing (Schokker, et al. 2015). Therefore using competitive exclusion products that contain complex microbiota from healthy adult hens to colonise young chicks can therefore prevent the infection with pathogens (Norris and Ngambi 2006, Havenstein, Ferket and Qureshi 2003).

The initial gut colonizers are the facultative anaerobes and soon, within a week after hatching, *Firmicutes* representatives begin to appear, then finally, representatives of *Bacteroidetes* become part of the intestinal tract microbiota (Videnska, et al. 2014). However, the gastrointestinal tract of poultry may contain more than 650 microbial species (Apajalahti, et al. 2004a). A recent evaluation of the ecology of the microflora of the broiler chickens intestine using 16s rRNA confirms that *Lactobacillus* is the predominant genus in young birds, while in older broiler chickens Bifidobacteria are dominant (Amit-Romach, et al. 2004).

A balanced intestinal microbial population is generally considered to be the chief characteristic of a healthy and well-functioning gastrointestinal tract. Chicks establish a protective microflora within the first couple of days after hatching which then develops with age (Gabriel *et al*., 2006). Within one day after hatching, the ileum and caeca that were previously sterile contain 10^8 and 10^9 bacteria per gram of content respectively (Apajalahti, et al. 2004a) then after 3 days this will increase to 10^9 and 10^{11} per gram of content respectively. Afterward the numbers will remain relatively stable until 30 days of age (Gabriel, et al. 2006). Coliforms and Enterococci were found to be the most dominate microbial in the gut of the chicks initially. *Lactobacillus* bacteria colonise

broiler chickens gut slowly, but finally, they become the most dominant species in the upper part of the GI tract (Apajalahti, et al. 2004a). The broiler chickens gizzard microbiota is highly similar to crop microbiota (Sekelja, et al. 2012): lactobacilli comprising 43% of the gizzard microbiota in the domestic broiler chickens GI tract (Gong 2007). Lactobacilli are expected in the gizzard because these bacteria tolerate acidic environments, and also produce acids (Amit-Romach, et al. 2004).

In the caeca the bacterial population is more diverse, especially with the slow turnover of the digesta (1 to 2 times a day) which can result in the development of more and different types of bacteria. However although there is an incredibly diverse range of microorganisms in the gut microbiota of poultry, the most abundant are primarily anaerobic (Pan and Yu 2014) probably because there is low to zero oxygen levels available in the lumen (Sun and O'Riordan 2013).

1.6 Protective role of the gastrointestinal microbiota in broiler chickens

Intestinal bacteria play an important role in host health which comes from different effects on; nutrition, infection, morphology and immunity. In addition the microbiota contributes to vitamin and amino acid production (Apajalahti, 2005). Moreover, broiler chickens gut microbiota can act as a reservoir of pathogenic or antibiotic resistant bacteria which can be transferred to other microorganisms including pathogens, which in turn can spread to humans by consuming their products (Zhou, Wang and Lin 2012). The most studied broiler chickens microbiota are from caeca as a sampling site because of the importance of it in health, production and the wellbeing of broiler chickens (Stanley, et al. 2015). In the avian host, caeca generally have a more important role preventing infectious disease than the mammalian caecum, where the preimarly role is digestion for energy. It is considered to be a multi-purpose organ that is vital to the birds physiology; as there is a very dense microbial community which makes the caeca to be considered as a powerhouse for fermentation (Clench 2015) resulting in the production of energy metabolites that can aid birds to achieve the requirements of energy (Lei, et al. 2012). (McBride and Kelly 1990) reported that about 23% of whole body energy consumption is utilized by the GIT and liver. Also it was reported that the microbiota present in the broiler chickens intestinal tract significantly increased the metabolizable

energy associated with broiler chickens feed, indicating that the gut microbiota are responsible for the additional dietary energy that is utilized (Hegde, Rolls and Coates 1982).

The indigenous microflora are considered to be a key component in protecting the gut from pathogen invasion. The GIT of the mature bird is much more resistant to pathogen colonization compared with newly hatched neonates whose GIT is sterile and highly susceptible (Nurmi and Rantala, 1973; Mead, 1998). Therefore to maintain the intestinal microflora balance in animals it is important to prevent diseases by controlling the overgrowth of potential pathogenic bacteria. The control of infections through a non-antibiotic approach is urgently required. The natural bacterial flora (e.g. probiotic bacteria) represents a promising alternative therapy.

The protective influence of maternal transfer of enteric microflora is known for various warm-blooded species, including humans. Unfortunately, in many poultry operations, transfer of microflora from the hen to her offspring no longer occurs, because chicks are raised separately from parent flocks. The concept of accelerating development of the normal enteric microflora, thereby increasing the resistance of young poultry to infection, was first described by (Nurmi and Rantala 1973). These researchers collected microflora from the alimentary tract of mature broiler chickens and used it to inoculate newly hatched chicks, thereby reducing considerably *Salmonella* colonization. This strategy has been called 'competitive exclusion', 'the Nurmi effect' or 'probiotic supplementation' and, subsequently, numerous studies have demonstrated reductions in *Salmonella* colonization of poultry using mixed, undefined enteric cultures. In an early example, (Schoeni and Doyle 1992) isolated caecum-colonizing bacteria that produced anti-*Campylobacter* metabolites from *C. jejuni*-free hens and demonstrated that these isolates could protect chicks against a subsequent challenge with C. *jejuni*. In other studies, bacterial strains isolated from washed caeca were shown to possess hydrophobic properties and their use improved the efficacy of competitive exclusion cultures in broiler chickens (Stavriac and D'aoust 1993). A competitive exclusion culture was developed from the microflora occurring in the same niche as that occupied by *Campylobacter*, using scrapings of intestinal mucosa ((Stern, 2008; Stern, 1994). (Koenen, et al. 2004) developed a method for in vitro selection of lactic acid bacteria

with immuno-modulating properties in broiler chickens. The mechanisms that gut microbiota can protect the host from pathogenic are listed in table 1.2.

Table 1.2. Defence mechanisms of the avian gastrointestinal tract.

Mechanism	Mode of action
Physical barriers	
Mucin	Mucin secretion and type affect microflora
pH	Low pH of upper GIT inhibits growth of some enteric bacteria
Nutrient competition	Bacteria must compete with the GIT for nutrients
Peristalsis	Movement of digesta and mucin prevents bacterial adherence
Oxygen tension	The anaerobic environment of the GIT and inhibits some microbes
Gut microflora	
Competition for adhesion	Bacteria compete for adhesion sites
Nutrient competition	Bacteria compete for nutrients
Bacteriocins	Antimicrobial compounds produced by other bacteria to inhibit competitors
Bacteriophages	Viruses that replicate within and lyse specific bacteria
Short-chain fatty acids	Antimicrobial compounds that can inhibit the growth of some bacteria
Competitive exclusion	
Mature microflora	Microflora from healthy adults and protects neonates
Mucosal scrapings	Microflora collected from mucosal scrapings that reduce *Campylobacter*
Immuno-modulation	Probiotic bacteria that stimulate an immune response
Bactericidal compounds	Caecal bacteria that secrete metabolites bactericidal to *C. jejuni*
In vitro competition	Enteric bacteria that outcompete pathogens in vitro
Mucosal immunity	
Immune surveillance	M cells and phagocytes constantly monitor the GIT for pathogens
Defences	Antimicrobial peptides expressed in the villus crypts
Secretory IgA	Secreted by B cells to bind to bacteria and prevent bacterial attachment
Mucin secretion	Regulated by pattern-recognition receptors; flow and type affect microflora

Adapted from (Perry 2006)

1.7 Diversity in gut microbial communities

Microbiota characterization has been studied to investigate the changes in broiler chickens microbiota within the gut caused by many factors. It has been documented that broiler chickens microbiota responds to changes in feed (Siragusa, et al. 2008, Jozefiak, et al. 2011), litter composition (Cressman, et al. 2010), antibiotics (Lin, et al. 2013) and probiotic addition to feed (Lee, et al. 2011, Nakphaichit, et al. 2011), disease (Stanley, et al. 2012, Juricova, et al. 2013) and stress (Lan, Sakamoto and Benno 2004, Burkholder, et al. 2008).

The gastrointestinal tract of poultry essentially is coated in a dense layer of commensal bacteria. In general, the crop and the caeca contain the most complex microbial communities. Meanwhile, there is less colonization in the rest of GIT because of the unfavourable environment. In the duodenum for instance there are numerous enzymes and antimicrobial compounds present in high level, such as bile salts, in addition there is the rapidly changing environment due to reflux from the jejunum to the gizzard (Gabriel, et al. 2006). Going down the GIT, the ileum and caeca will become more favourable environments as they contain fewer enzymes and antimicrobial compounds; therefore concentrations of commensal bacteria will increase, which will be around 10^9 and 10^{11} cfu/g, respectively (Thompson, et al. 2012). (Stanley, et al. 2013b) found that the microbiota in the broiler chickens individually of each single bird of three trials which were similar in feed and all conditions. The authors identified that there was a variation from batch to batch across the three trials and in addition they found that the variations were large within each trial. Hence, it seems individual bird to bird variation is normal in the gut microbiota of broiler chickens.

1.8 Human pathogenic bacteria in the avian gastrointestinal tract

Understanding the strategies by which zoonotic bacteria survive and adapt in the avian gut is important, as a major mode of carcass contamination occurs during processing, when edible meat is exposed to intestinal contents. *Campylobacter* and *Salmonella* are the most prevalent pathogens derived from poultry that infect humans through foodborne illness (CDC, 2004). Also *Salmonella* is still one of the most prevalent food safety risks and has always been associated with poultry products (de Oliveira, et al. 2014). Other foodborne pathogens, including *Listeria monocytogenes* and *Clostridium*

perfringens, can also colonize the avian gut and are potentially pathogenic to humans. Enteric *E. coli* isolates from avian species tend to be non-pathogenic to humans; however, there is some evidence that broiler chickens can be colonized by *E. coli* 0157:H7, a highly pathogenic organism (Ferens and Hovde 2011).

1.9 Concept of probiotics

The previous descriptions of intestinal microbiota focus on healthy situations where the balance of microbiota is tipped towards a high percentage of beneficial bacterial. However, modern poultry production often involves a variety of challenges that invoke deviation from this situation, so there is opportunity for interventions to re-establish a high percentage of beneficial bacterial through the use of probiotic supplements. The relative meaning of probiotic is “for life” which is originally derived from Greek language that is currently used to name the bacteria which associated with beneficial effects in animals and humans. There are many definitions that have been proposed for the term probiotics such as Fuller (1989) “a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance”. However according to the currently defined by FAO/WHO, probiotics were defined as “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (Fijan 2014).

(Stanley, et al. 2013b) has reported that in the broiler chickens, colonisation of the gut is thought to start immediately after hatch, which means that the hatching environment can affect the microbial profile significantly. In the commercial poultry production, it is argued that the timeframe for maturity of broiler chickens has been significantly reduced, in which it appears that gut microbiota can stabilise within three days after hatching (Apajalahti, et al. 2004a) then it remain reasonably constant until day 30 of age (Lu, et al. 2003a). However in the poultry industry, there are some factors that can affect the gut profile of birds. Firstly chicks will hatch away from their parents and secondly the strict hygiene implemented in the commercial hatcheries or/with washing or fumigation of the eggs prior to hatching (Varmuzova, et al. 2016a) will reduce the bacterial load in these environments and mean that the spread of bacteria is limited (Donaldson, et al. 2017). Therefore, chicks that are hatched in hatcheries will be exposed to a diverse range of bacteria from the surrounding environment rather than from their

parent. These environments include hatchery, transport, and the farm (Stanley, et al. 2013b). (O'Dea, et al. 2006) reported that exposure to pathogenic bacteria can be avoided by exposing chicks to beneficial bacteria like probiotic bacteria. (Methner, et al. 1997) reported that inoculation of newly hatched broiler chickens with gut microbiota of donor hens can prevent against colonization of *Salmonella* spp.

1.9.1 Probiotic Microorganisms

Species of a wide range of different genera of microorganisms (*Lactobacillus, Bifidobacterium*, *Bacillus* , *Saccharomyces, Aspergillus, Candida*, *Lactococcus*, *Streptococcus, Enterococcus, Bacillus* and *E. coli)*, as well as undefined mixed cultures which have a beneficial effect on performance of broiler chickens through different mechanisms have been added to broiler chickens diets (Lutful Kabir 2009) (Patterson and Burkholder 2003). Lactic acid bacteria (LAB) are Gram-positive bacteria that are natural inhabitants of the gastrointestinal (GI) tracts of mammals, including humans. They include *Lactobacilli*, *Lactococci*, *Enterococci*, *Streptococci*, *Leuconostoc* and *Pediococci* (Pessione 2012) in addition to *Bifidobacterium* (Sule, et al. 2014). Also there are variety of genera and species thereof that have been used as probiotic organisms in humans or animals. Probiotic species used in broiler chickens diets usually belong to *Lactobacillus, Streptococcus*, *Bifidobacterium, Bacillus*, *Enterococcus*, *Aspergillus*, *Saccharomyces* and *Candida* (Lutful Kabir 2009). The most common species currently being used as probiotic, isolated from the intestinal tract are *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus salivarius, Lactobacillus plantarum, Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis, Bifidobacterium spp.* and *Escherichia coli* (Fuller 1989). Organisms from the *Lactobacillus* genus are Gram-positive, facultatively anaerobic, catalase-negative, non-spore-forming rod-shaped bacteria. This genus is composed of over 170 species (Goldstein, Tyrrell and Citron 2015a) and can ferment carbohydrates and produce lactic acid as a major end-product (Cortón, et al. 2000). Species of *Lactobacillus* such as *Lb salivarius*, *Lb reuteri* and *Lb fermentum* are common species in the broiler chickens gut and they have been used previously as probiotic organisms to improve the health and performance of broiler (Olnood, Beski, Choct, et al. 2015, Shokryazdan, et al. 2017). (Santini, et al. 2010) selected *Lactobacillus* isolates from different sources and they

found that some of these isolates were able to inhibit the growth of *Campylobacter*. Also (Ghareeb, et al. 2012) found that *Lb*. *salivarius* and *Lb*. *reuteri*, *Enterococcus faecium* and *Pediococcus acidilactici* inhibited the growth of *Campylobacter jejuni*.

1.9.2 Characteristics and properties of probiotics

Probiotics to be active as supplement should have some specified criteria and characteristics : (1) non-pathogenic bacteria (Hardy, et al. 2013), and (2) resistant to gastric pH and processing/storage, to allow them to persist in the intestinal tract, (3) they are able to produce inhibitory compounds and (4) compounds that modulate immune responses (Patterson and Burkholder 2003). Lactobacilli and bifidobacteria are well-documented examples of beneficial bacteria and the most common use as probiotic, they are beneficial and indigenous to the human and broiler chickens GIT (Olnood, Beski, Iji, et al. 2015, Walter 2008). Lactobacilli belong to big group collectively referred to as lactic acid bacteria, which metabolize carbohydrates and produce lactic acid as the primary end product (Sun, et al. 2015). Bifidobacteria are often associated with lactic acid bacteria for their production of lactic acid, however, they are phylogenetically distinct. Bifidobacteria are Gram-positive, and heterofermentative (Pokusaeva, Fitzgerald and van Sinderen 2011). Bifidobacteria also digest oligosaccharides to use it as carbon and energy sources, they produce lactic acid, acetic acid, ethanol, and formic acid (Van der Meulen, Avonts and De Vuyst 2004). In addition, they can prevent pathogenic bacteria by competing for nutrients as they are capable of internalizing simple sugars remaining in the environment, (Roto, et al. 2015). Lactobacilli and bifidobacteria are both known to be members of the intestinal microbiota in animals and humans; their presence is important to maintain the gut microbiota (Hemarajata and Versalovic 2013).

1.10 Modes of action of probiotics

Gut microbiota plays an important role in the health of the GIT through many different mechanisms as previously summarised in table1:2. A primary example is competitive exclusion of pathogenic bacteria by different methods such as reducing available attachment sites on the epithelium, increasing mucin production, reducing pH and competition for nutrients. Other protective effects of microbiota are through selective stimulation of the immune system; production of compounds like antimicrobial

compounds such as bacteriocins and production of short-chain fatty acids (SCFA) (Clavijo and Florez 2018). Key features of probiotic bacteria are the capability of manipulating the gut microbiota to host advantage via three main mechanisms as detailed below:

1.10.1 Competitive exclusion

In the lower intestines, the complex lining with bacteria can work as a barrier to prevent pathogenic bacteria from colonization. Infection will occur without these barriers in place: the lining of the intestines when the bacteria settle first will make other microorganisms to compete for space and nutrients in order to survive and colonize in the intestine (Hardin 1960, Gabriel, et al. 2006, Lawley and Walker 2013). Therefore infection by pathogens can be prevented by establishing the early foundation of a mature GIT microbiota, as the beneficial bacteria will compete with the pathogenic bacteria (*Salmonella*) for space and nutrients (Gleeson, Stavric and Blanchfield 1989, Crhanova, et al. 2011). Naturally, rapid colonization of members from the parents gut microbiome will occur when chicks hatched in the presence of maternal faecal contents (Lutful Kabir 2009). Researchers stated that native microflora can compete with pathogenic microbes for essential nutrients which can be a limiting factor in colonization of the gut by invading pathogens (Lan, et al. 2005, Woo and Ahn 2013). For example, for almost all species of bacteria, iron is essential as it is an important component of many proteins. Therefore, iron acquisition during infection of a human host is a challenge that must be surmounted by every successful pathogenic microorganism. Iron is essential for bacterial and fungal physiological processes such as DNA replication, transcription, metabolism, and energy generation via respiration (Ratledge and Dover 2000, Caza and Kronstad 2013). (Deriu, et al. 2013) found that probiotic of *E. coli* (Nissle) outcompetes *Salmonella* for iron in the inflamed gut and reduced *S*. Typhimurium colonization in mouse models.

1.10.2 Antimicrobial substances

Probiotic organisms can either by themselves produce or indirectly induce the host cells to produce peptides that interfere with pathogens and prevent invasion of epithelium cells such as anti-bacterial protein defences, cathelicidins and bacterial/permeability-inducing protein (BPI) which display an antimicrobial activity against wide variety of microorganisms (Kelsall 2008). Probiotics have shown the capability to inhibit the growth of pathogens due to production of a variety of antimicrobial factors such as defences, bacteriocin, hydrogen peroxide, nitric oxide, and short chain fatty acid (SCFA) which will reduce the pH of the lumen (Henningsson, Björck and Nyman 2001). Some are able to produce H_2O_2 which is one important mode of action of LAB to be used as probiotic as an antimicrobial agent against pathogenic bacteria (Servin 2004).

Another substance produced by organisms such as *Lactobacillus* and *Bifidobacterium* spp. are SCFA that are organic acids resulting from fermentation of indigestible carbohydrates in the GIT (Liu, Gibson and Walton 2016). The predominant SCFA present in the GIT are acetic, propionic and butyric acids (Rios-Covian, et al. 2016). SCFA increase from undetectable levels in the caeca of day-old broilers to the highest concentrations at 15 days of age (van Der Wielen, et al. 2000). SCFA have been reported to inhibit growth or reduce levels of *S.* Enteritidis*, S.* Typhimurium*, S.* Pullorum*, E. coli, C. jejuni* and *C. coli* (van Der Wielen, et al. 2000, Chaveerach, et al. 2004). Although not fully understood, the antibacterial mechanisms include bacteriostatic and bactericidal properties, depending on the physiological status of the bacteria and the physico-chemical characteristics of the external environment (Jones and Ricke 2003). SCFA that are not dissociated can diffuse across bacterial lipid membranes and decrease the intracellular pH which then can cause cellular damage or death of those microbes that are sensitive to such conditions (van der Wielen, et al. 2002). Furthermore, other mechanism that SCFA can also depress bacterial growth, since additional energy is required to return the internal pH of the cells to homeostatic levels (Van Immerseel, et al. 2004).

Al-Tarazi and Alshawabkeh (2003) reported that the administration of formic and propionic acids via feed reduced S. pullorum related mortality in broilers by 58% and caecal colonization by 75%. While the direct, oral administration of SCFA has had limited success, utilizing probiotics and/or prebiotics to increase SCFA is more effective. The

effectiveness of butyric acid, the optimal acid for preventing bacterial invasion/colonization (Van Immerseel, et al. 2004), was increased by supplementing the diet with fructo-oligosaccharide and *Bifidobacterium* spp. (Blay et al., 1999; Kaplan and Hutkins, 2000). Corrier et al. (1990) fed dietary lactose to broiler chickens in combination with a competitive exclusion preparation and demonstrated a significant increase in lactic acid, a decrease in caecal pH and a 3.5–4.0 log reduction in *S*. Typhimurium. The combined treatment also increased concentrations of acetic, propionic and butyric acids in the caeca of these birds. (Donalson, et al. 2008) treated chicks with a competitive exclusion preparation and found that concentrations of propionic acid were increased and that treated birds had greater protection from caecal colonization by S. *Typhimurium*. Also a contributing factor to the inhibition of pathogen growth was the drop in cytoplasmic pH which occurred when SCFA were present (Mani-López, García and López-Malo 2012). In addition, pH is another physico-chemical factor that provide an unfavourable for bacterial pathogens segments of the gastrointestinal tract (Beasley, et al. 2015).

1.10.3 Bacteriocins

Bacteriocins are peptides or proteins that are produced by bacteria which can kill or inhibit the growth of other bacteria (Cleveland et al., 2001). Bacteriocins have activity against a number of pathogenic, Gram-negative bacteria (Mota-Meira et al., 2000; Arques et al., 2004) and are one of the proposed mechanisms of action of competitive exclusion preparations (Nurmi and Rantala, 1973; Mead, 2000; Patterson and Burkholder, 2003). The administration of bacteriocins isolated from *L*. *salivarius* and *Paenibacillus polymyxa* reduced *Campylobacter* colonization to undetectable levels in the caeca of broiler chickens and turkeys (Stern et al., 2005; Cole et al., 2006), whereas 10^6 cfu/g of *Campylobacter* were detected in the caeca of control birds.

1.10.4 Cost of beneficial bacteria

Beneficial bacteria in the gut have many advantages to the host, however, these bacteria need nutrients to grow and be active, which will draw from the nutrient reservoir in the gut. Therefore they will compete with host the on the nutrients in the gut as this bacteria may use these nutrients such as simple carbohydrate and mineral (Wasielewski, Alcock and Aktipis 2016). (Fak and Backhed 2012) reported that a strain of *Lactobacillus reuteri*

(ATCC PTA 4659) caused a loss of weight when administrated to human. Although the commensal bacteria has benefit to the host by competing with pathogenic bacteria, an overgrowth of these bacteria can be detrimental to the host by excessive uptake of nutrients making them limited to the host (Zaidel and Lin 2003). The presence of the gut microbiota increases the cost of energy by altering the rate of energy-consuming reactions (Muramatsu, Nakajima and Okumura 1994). For example, the amount of dietary energy spent to maintain gut will increase as a result of stimulation and renewal of epithelial lining from pathogen attachment (Yang, IJI, and Choct 2009). Therefore, through a variety of mechanisms, commensal bacterial can divert nutrients from the diet of host as energy sources, so those nutrients are unavailable to the host for growth and maintenance.

1.11 Probiotic effects on poultry

1.11.1 Probiotic effects on performance

Overall, probiotic supplements have been shown to have inconsistent effects on performance in poultry. (Haghighi, et al. 2006) found that oral gavage of chicks at day 1 with probiotics (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis)* resulted in improved IgG and IgA levels in probiotic treatments. (De Cesare, et al. 2017) found that using *Lactobacillus acidophilus* D2/CSL (CECT 4529) in the chicks the body weight was improved at day 15 of age.

(Panda, et al. 2000) did not find any significant difference in the BWG of broiler chickens that were given feed containing probiotics *L*. *acidophilus* and *Streptococcus faecium* compared with control. (Awad, et al. 2009) found that adding supplement of probiotic had no effect on the body weight at day 35 along with body weight gain, meanwhile symbiotic supplementation affected these parameters significantly ($p \leq 0.05$). (Sarangi, et al. 2016) observed that using prebiotic, probiotic and synbiotic in feed of broiler chickens did not affect the body weight, feed intake and FCR up to day 42 of age. (Salehimanesh, Mohammadi and Roostaei-Ali Mehr 2016) reported that using the additives of prebiotic, probiotic and synbiotic in the broiler rations had not been significantly BW and gain.

(Mountzouris, et al. 2010) found that using the probiotic PoultryStar ME (Biomin GmbH, Herzogenburg Austria) that was comprised of 5-bacterial species *Lactobacillus*

reuteri DSM 16350, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* DSM 16284, *Pediococcus acidilactici* DSM 16210 and *Lactobacillus salivarius* DSM 16351) in the diet of broiler, improved the body weight, body weight gain and feed conversion rate (FCR) of these broiler chickens. Also it was demonstrated that using probiotic supplementation of Bactocell (*Pediococcus acidilactici* 10^9 CFU/g) improved the body weight and daily weight gain and feed conversion ratio of broiler chicks at late ages (3–6 weeks and at the end of age 6 weeks) significantly (Alkhalf, Alhaj and Al-Homidan 2010). (De Cesare, et al. 2017) investigated the effects of the probiotic dietary supplementation of *Lactobacillus acidophilus* D2/CSL (CECT 4529) on productive performance, and found that this supplementation improved the FCR of the broiler. Another study conducted by (Li, et al. 2014) studied the effects of *Bacillus subtilis*, *Rhodopseudomonas palustris*, *Candida utilis* and *Lactobacillus acidophilus* in broiler chickens feed on broiler growth performance. They found that this probiotic treatment improved the growth of broilers significantly as the body weight was bigger and FCR was lower at 42 days of age in the probiotic treatments. Also broiler chickens diet inoculated with LactoFeed which consists of 2.5×10^{10} CFU/kg of each *Lactobacillus acidophilus*, *Lactobacillus casei, Bifidobacterium thermophilum* and *Enterococous faecium* improved the body weight and FCR at day 42 of broiler (Zarei, Lavvaf and Motamedi Motlagh 2018). However other studies found that there was no significant effects on the performance of broiler chickens. (Eckert, et al. 2010) stated that administering a *Lactobacillus*-based probiotic either by drinking water or feed improved the body weight significantly compared with control treatment from 15 days of age until 48 days. Eckert et al., (2010) also reported that *Lactobacillus* via drinking water can improve the body weight and feed conversion of broiler chickens within commercial environments. (Mountzouris, et al. 2007) conducted an experiment of adding a mixture of different species of probiotic which contained 2 *Lactobacillus* strains, 1 *Bifidobacterium* strain, 1 *Enterococcus* strain, and 1 *Pediococcus* strain in broiler diets for 6 weeks. They added probiotic either with feed or water which were compared with control (basal diet). Then they studied the effects of these treatment on performance of the broiler. They found that overall body weight, feed intake, and feed conversion ratio, were not affected by the probiotic supplement. (Olnood, et al. 2015), studied the effects of adding of four *Lactobacillus* strains (*Lactobacillus johnsonii, Lactobacillus*

crispatus, *Lactobacillus salivarius* and an unidentified *Lactobacillus* sp.). Compared with control on performance of broiler chickens, they found that the probiotic supplement had no significant effect on weight gain, feed intake and feed conversion rate (FCR) during the 6-week of age.

1.11.2 Probiotic effects on mucin in broiler chickens

Mucin dynamics can also be altered by dietary supplements. Fernandez et al. (2000) fed broiler chickens a diet containing xylanase, which reduces *C. jejuni* colonization by decreasing the viscosity of caecal mucous, altering gut transit time and possibly by 'flushing' *C. jejuni* from the GIT. (Forte, et al. 2018) found that using *Lactobacillus acidophilus* in the broiler chickens feed as probiotic improved the thickness of mucin of the ileum. (Deplancke and Gaskins 2001) stated that there is a symbiotic relationship between commensal bacterial colonization and the host and also these bacteria typically enhanced the secretion of mucus. (Smirnov, Sklan and Uni 2004) Smirnov et al. (2004) observed that goblet cell density was greater in the ileum and jejunum and mucin glycoprotein levels were lower in the duodenum of chicks fed antibiotic growth promoters. In the same study, use of probiotics increased the goblet cell cup in the lower intestines of chicks. In vitro studies utilizing *L. plantarum* 299v demonstrated the ability of probiotics to inhibit enteropathogenic organisms by inducing the expression of intestinal mucin genes ((Mackenzie, et al. 2010). (Tsirtsikos, et al. 2012) found that using probiotic (PoultryStar ME, Biomin GmbH, Austria) containing *Lactobacillus reuteri, Lactobacillus salivarius*, *Enterococcus faecium*, *Bifidobacterium animalis* and *Pediococcus acidilactici* increased the thickness of the mucus layer in the duodenum of broilers.

1.11.3 Probiotics and gut microbiota

(Swiatkiewicz, Koreleski and Arczewska-Wloek 2011) studied the effect on the performance of diet supplementation with selected prebiotics (control (none); inulin, 70 g/kg; oligofructose, 70 g/kg) in a 6-week experiment of broiler They found that at 21 or 42 d of age, there was an effect of inulin or oligofructose on performance of broilers compared with control which has no supplements. Also (Olnood, et al. 2015), studied the effects of adding of four *Lactobacillus* strains: *Lactobacillus johnsonii, Lactobacillus crispatus*, *Lactobacillus salivarius* and an unidentified *Lactobacillus* sp. on the gut

microbial profile and production performance of broiler chicken. It was found that probiotic supplements tended to increase the number of total anaerobic bacteria in the ileum and caeca, and the number of LAB and lactobacilli in the caeca.

(Dibaji, et al. 2014) investigated the efficacy of different levels (0.075% to 0.0375%) of a synbiotic (Biomin Imbo, consisting of: *Enterococcus faecium* and fructo-oligosaccharides) compared with control basal diet (synbiotic free) over a 42-d feeding period, and then measured the microbial population in the caeca. They found that the addition of different levels of synbiotic increased the numbers of lactobacilli in the caecal contents of broiler chickens. At the same time this supplement reduced *Escherichia coli* and total coliform populations in the intestines of broiler chickens. (Mountzouris, et al. 2007) conducted an experiment of adding a mixture of different species of probiotic which contained 2 *Lactobacillus* strains, 1 *Bifidobacterium* strain, 1 *Enterococcus* strain, and 1 *Pediococcus* strain in broiler diets for 6 weeks. They added probiotic either with feed or water which were compared with control (basal diet) and diets containing antibiotic (Avilamycin). Then they studied the effects of these additions on the caecal microbial ecology. Their results indicated that in the caecal microflora composition concentrations of bacteria belonging to *Bifidobacterium* spp., *Lactobacillus* spp., and Gram-positive cocci were significantly higher in probiotic treatments in water or feed compared with the control and antibiotic treatments. (Fukata, et al. 1999) found that addition of *Lactobacillu*s to broiler chickens feed did not bring about differences in lactobacilli or *Bifidobacterium* in broiler chickens caeca at day 7 or day 21. Hong Park, (2016) stated that when adding prebiotic-based Mannanoligosaccharide (MOS) the abundance of the *Faecalibacterium* genus was increased in the treatment compared with the control. *Faecalibacterium* is also known as one of the butyrate-producing genera (Wang, Lilburn and Yu 2016, Egshatyan, et al. 2016, Pryde, et al. 2002). Butyrate has been shown to have anti-inflammatory activity (Van Immerseel, et al. 2010, Celasco, et al. 2014). Also Ramirez-Farias, et al. (2009) and (Wang, et al. 2017) when they used prebiotic and probiotic respectively. *Blautia* is a genus belong to the phylum *Firmicutes* which has been traditionally believed to carry genes related to polysaccharide metabolism which is thought to enhance the efficiency of energy harvesting by the host (Kasai, et al. 2015). During this metabolism, acetate is also produced (Kettle, et al. 2015, Turroni, et al. 2016), Most of the bacteria within the

Firmicutes phylum are considered to be butyrate producers in the gut microbiota of broiler chickens (Varmuzova, et al. 2016b), which correlates with the health of the host and has been shown to improve intestinal defence and protect the host against lethal infection (Fukuda, et al. 2011).

(Krumbeck, et al. 2015) found that when using a prebiotic (galactooligosaccharides) in humans they observed an increase in the *Blautia* genus. van Zanten, et al. (2014) found that the addition of a synbiotic to human food did not increase the abundance of *Blautia*, but actually brought about a decrease compared with the non-treated control. The genus *Erysipelotrichaceae* was decreased in the caecal content at day 42. The importance of these bacteria is in inflammation which is related to disorders of the gastrointestinal tract in humans (Chen, et al. 2012, Dinh, et al. 2015). Neveling, et al. (2017) added probiotic strains that were isolated from broiler chickens which consisted of *L. crispatus*, *L. gallinarum*, *L. johnsonii*, *L. salivarius*, *Enterococcus faecalis* and *Bacillus amyloliquefaciens* to the broiler chickens diet and found degradation decrease in the abundance of this *Erysipelotrichaceae* genus, while Tanner, et al. (2014) found that using FOS in swine feed increased the abundance of *Erysipelotrichaceae.* Meanwhile at day 42 abundance of *Erysipelotrichaceae* was decreased in the treatments of supplements compared with control so it may be concluded that these supplements modified the gut microflora in a mildly positive manner, as this genus is used as indictor for inflammation (Palm, et al. 2014) and these supplements caused a degradation in this bacteria.

1.11.4 Probiotic efficacy and *in vitro* pathogen inhibition

(Kizerwetter-Swida and Binek 2005) isolated 16 different strains of *Lactobacillus* from the broiler chickens gut and examined them for their potentially probiotic properties to inhibit the growth of enteropathogenic bacteria (*Salmonella* Enteritidis*, Escherichia coli and Clostridium perfringens*) by using the supernatants from *Lactobacillus.* Their results demonstrated that some isolates have an in vitro antagonistic effect against enteropathogenic bacteria especially in controlling necrotic enteritis caused by C. *perfringens*. Shokryazdan, et al. (2014) used 9 strains of *Lactobacillus* isolated from human milk, infant faeces, and fermented grapes and dates and examined them for their antimicrobial activity toward twelve pathogenic human strains including *Candida albicans* (ATCC 44831), *Enterococcus faecium* (ATCC

51558), *Staphylococcus epidermidis* (ATCC 12228), *Propionibacterium acnes* (ATCC 6919), *E. coli* (ATCC 29181), *Shigella sonnei* (ATCC 25931), *Helicobacter pylori* (ATCC 43579), *Enterobacter cloacae*, *Vibrio parahaemolyticus*, *Listeria monocytogenes, Klebsiella pneumoniae* (K36) and *Staphylococcus aureus* (S244). They found that all the isolated *Lactobacillus* strains, except *L. acidophilus* HM1, exhibited strong inhibition on the growth of *Staphylococcus epidermidis* (ATCC12228), *Enterobacter cloacae*, and *Listeria monocytogenes* , and the three *L. casei* strains (BF1, BF2, and BF3) showed strong activities against *Helicobacter pylori* and good inhibition against *Staphylococcus aureus*. Moreover they found that inhibitory effects of the *Lactobacillus* strains were due to their organic acid production.

In an attempt to colonize newly hatched chicks with a mature and healthy microbiome that will discourage pathogenic bacteria from colonizing, chicks have been experimentally inoculated with competitive exclusion culture mixtures (Nurmi and Rantala 1973, Nisbet 2002). Introduction of the competitive exclusion cultures has proven to be effective in protecting young animals from enteric pathogens and several reviews have been written on various aspects of this research (Callaway, et al. 2008). Several investigators have attempted to exploit and improve the competitive exclusion phenomenon by mimicking properties of efficacious bacteria, using defined cultures or by measuring beneficial effects within the GIT. For example, Schoeni and Doyle (1992) isolated caecum-colonizing bacteria that produced anti-*Campylobacter* metabolites from *C. jejuni*-free hens and demonstrated that these isolates could protect chicks against a subsequent challenge with *C. jejuni*.

1.12 The immune system

The first level of defence from exogenous pathogens that colonize host cells and tissues is the gastrointestinal tract, it is also the largest organ with immune properties (Surai 2013). Modern strains of chicken and particularly broilers have developed very fast growth but these developments impact on health of these birds as there are negative relationship between body weight and immunity. For example there is a relationship between the acute phase of immune response and feed intake and productivity, and the consumption of nutrients will increase up to 10% to maintain growth and development (Klasing 2007a).

The immune function of the gut comes from different features. The most essential is the gut-associated lymphoid tissue (GALT). GALT includes; lymphoid aggregate located within lamina propria, Meckel's diverticulum, payer's patches and caecal tonsils. These structures of lymphoid tissue are distributed throughout the gut which is considered the intestinal arm of immunity (Peralta, et al. 2017).

1.12.1 Generation of intestinal immune response

As gut microbiota are in close contact with cells of the gut-associated immune system, T- or B-cell-mediated immune responses can be modulated by the commensal bacteria or their structural components, either locally or systemically as a result of interactions between host cells and these bacteria (Macpherson, et al. 2000). Gut microbiota or its products, may play a role in the development of immune response. It has been found that the broiler chickens GALT will reach its functional maturity by week 2 of age, and involves cells of the immune system, such as T and B cells, natural killer (NK) and macrophages 4 and 18 (Haghighi, et al. 2005). Possibly, the immune response can be generated in the foregut as well, though primary responses most likely start in the hindgut, bursal duct, bursal sac, and spleen.

1.12.2 Mucins and gut immunity

The intestinal epithelium has a range of mechanisms to protect the body against the invasion of intestinal pathogens. The first factor is the mucin layer, which include two layers: an inner and outer layer. The outer layer can be colonised by microorganisms as this layer is loose and exposed to the bacteria that enter to the gut, while the inner layer prevents the bacteria from adhering to the epithelium (Hansson and Johansson 2010). Therefore mucus represents the first line of defence to foreign pathogenic bacteria (Brisbin, Gong and Sharif 2008a). Mucin is synthesized and secreted by goblet cells that cover the epithelium of the intestinal tract (Smirnov, et al. 2004), forming a gel that adheres to the mucosal surface (Forstner et al., 1995). Gastrointestinal mucin acts as the luminal barrier and a primary line of defence against invading pathogens (Moncada, Kammanadiminti and Chadee 2003). This mucous layer in the gut may prevent bacteria to pass to epithelial cells through mucous which is first layer that the bacteria need it in order to adhere to and invade, to make infection (Ribet and Cossart 2015). The mucosal layer of the GIT covers the epithelial surface and acts as an interface between the external and internal environment. Its function is as a medium for protection, lubrication and transport between the epithelial cells and lumen (Perry 2006).

1.12.3 Gut-associated lymphoid tissue (GALT)

There is a correlation between functional maturity of the intestine and complete development of a local immune system. The induction and development of responses of immunity occur mainly in GALT and in the spleen as broiler chickens do not have lymph nodes like mammals, GALT is considered to be one of the fundamental immunological phenomena which including in birds the immune response or reflex to antigens (Klipper, Sklan and Friedman 2001). Lymphoid follicles in caecal appendices are quite frequent around this area especially in caecal tonsils (Surai 2013). Meanwhile there are no lymphoid follicles in the colon of birds, which instead occur in the terminal part that opens into the cloacal bursa which is involved in primary and secondary immune responses, and in the bursal duct mucosa and submucosa contain many lymphoid follicles (Casteleyn, et al. 2010). The Bursa of Fabricius is considered to be an important immune organ with functions of a peripheral lymph node and is a source of differentiation of B-lymphocytes in the birds (Parra, Takizawa and Sunyer 2013).

1.12.4 Cytokines

Cytokines are the proteins produced by different cells which act as signals to regulate and activate cells and tissue during inflammatory and immune responses (Wigley and Kaiser 2003). Cytokines play an important role in the immune response, therefore studying cytokines will give more understanding on how probiotic and prebiotics work on the immunity as cytokines are considered as key for the immune response. Furthermore there are interactions between the cells of the gut-associated lymphoid tissue (GALT) which are in contact with the intestinal microbiota, known as cellular cross talk, the function of cytokines depends on the secreting cell. One of the important parameters to investigate the effects of pre- and probiotic on immunity is to measure the level of cytokines. (Brisbin, et al. 2010a) assumed that probiotic bacteria may be able to induce the production of cytokines which in turn regulate both innate and adaptive immune responses. Lactobacilli can induce cytokines type IL-1B, IL-10, IL-12, IL18 and IFN-γ (Brisbin, et al. 2010a).

IL-10 is a cytokine that has an anti-immune and anti-inflammatory activity (Mosser and Zhang 2008). The key role of this cytokine is inhibiting the production and function of pro-inflammatory cytokines, which in turn will regulate the inflammatory responses (Yamana, et al. 2004). It has a crucial role in modulating immune and inflammatory responses during infection with viruses, bacteria, fungi and protozoa (Couper, Blount and Riley 2008). (Cyktor and Turner 2011) indicated that one of the most important roles of IL-10 is to regulate the immunity at the site of infection when it occurs, which means that it will be produced in the case of inflammation or when pathogens enter.

IL-6 is considered to be multifunctional cytokine in both pro-inflammatory and anti-inflammatory role. It is a key cytokine in infection and inflammation and can support the maintenance of immune reactions (Hunter and Jones 2015). IL-6 is an inflammatory cytokine, which provides a protective role during a bacterial infection (Dube, et al. 2004).

Interferon-γ (IFN-γ) is considered to be one of the pro-inflammatory cytokines (Dinarello 2000). It has a pivotal role in host defence, it is considered as a hallmark of innate and adaptive immunity as is produced in response to infection (Mühl and Pfeilschifter 2003). Here, IFN-γ has been chosen as a marker for immune response in inflammation in an

early stage. (Dube, et al. 2004) stated that IFN-γ could be induced and upregulated in the case of inflammation. (P. Kaiser, et al. 2000) found that the level of IFN-γ was increased in the broiler chickens tissues that were infected with *Escherichia coli* or strains of *Salmonella* compared with uninfected tissue. Firstly, the treatments may have had a direct biochemical effect on the immune system, or the treatments may have indirectly affected the gastrointestinal immune system by modulating the intestinal tract microbiome, which in turn produced metabolites that biochemically altered the immune system. The most likely of these two mechanisms is that IFN-γ has been induced in control and upregulated compared with treatments.

(Haghighi, et al. 2008) used treatments of *Salmonella* serovar Typhimurium only and *Salmonella* with a probiotic mixture of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis,* and found that the level of IFN-γ in the caeca of broiler chickens was increased in the first treatment, while in the treatment of *Salmonella* with probiotic the level of this interferon was decreased. (Huang, et al. 2015) observed that when they added the prebiotic inulin to the diet of the broiler, they found that there was a decrease in the level of IFN-γ and IL-6 at day 21 but there was no effect at day 42. These findings also agree with the findings reported by (Janardhana, et al. 2009), who found that there was no difference between control and treatments when they added a prebiotic (fructo-oligosaccharide) to broiler chickens feed. Also, (Brisbin, et al. 2010b) found that *Lactobacillus reuteri* and *Lactobacillus salivarius* did not induce the production of IFN-γ and IL-10 in the caecal tonsil cells of broiler chickens.

(Y. Shang, et al. 2015) found that adding prebiotic (Fructooligosaccharide) to the broiler chickens feed did not induce IL-10 in the ileum tissue compared with control. Meanwhile, these findings do not agree with findings of (Yitbarek, et al. 2015) when they used a synbiotic in broiler chickens feed, as they found that IFN-γ was upregulated in the synbiotic treatments compared with control. (Kareem, et al. 2017) examined the effects of different combinations of inulin and postbiotics (secretions of probiotic) on ileum cytokine expression in the broiler chickens, and found that IFN-γ was upregulated by the addition of the treatments, and IL-6 was downregulated in the tissue of ileum of the broiler. The administration of pre, pro or synbiotics decreased the inflammation, damaged the tissue of the colon, and induced the secretion of IL-10 in this tissue as well, and downregulated the production of IFN-γ (Foye, et al. 2012).

1.13 Methods of probiotic manufacture and delivery

For beneficial activities of probiotic supplementation, there needs to be an appropriate amount of probiotic bacteria which should be alive and remain functional at the site of action (Cook, et al. 2012). It has been stated that a large loss of viability occurs when orally administered bacteria passage through the stomach, because of high acid and presence of bile salt. The loss in viability will lower the efficacy of the supplement (Cook, et al. 2012). It has been shown that *Bifidobacteria* are sensitive with low survival to stresses occurring during production, storage and consumption of these microorganisms. (Doleyres and Lacroix 2005) recommend that probiotics should be present at a minimum level of 6 log colony forming units (CFU)/g in a food product or 7 log CFU/g at the point of delivery (Doleyres and Lacroix 2005, J. Kim, et al. 2016).

There are several factors that can negatively affect the viability of probiotic bacteria during manufacture and/or storage, for example temperature, water activity and other food ingredients. However the main reason for reduced viability is the high temperature during manufacturing processes, this is because of most probiotics have low thermo-resistance (Vesterlund, Salminen and Salminen 2012). Hence, an ideal delivery system is needed which can protect probiotic bacteria from adverse conditions during production and storage and in the acidic gastric environment, that finally make sufficient amount of probiotics available at the site of action (J. Kim, et al. 2016). In terms of delivery, there are several different ways of supplying probiotics to broiler chickens such as, mist spraying, via feed, oral gavage, application to vent lip, and via drinking water (Olnood, et al. 2015) and even delivering probiotic by injection of the egg at the end of incubation (de Oliveira, et al. 2014) and spraying the litter that broiler chickens reared on (Olnood, et al. 2015).

1.14 Prebiotic supplements

Prebiotic materials were defined by (Gibson and Roberfroid 1995), as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health.” This definition was modified to include the requirements for resistance to the acidic gastric environment, gastrointestinal absorption, gastric enzymes, and fermentation by the gastrointestinal microbiota meanwhile stimulate the growth of

beneficial bacteria in the intestine (Roberfroid 2007). Another definition of prebiotic is a ''selectively fermented food ingredient that beneficially affects the host by a selective stimulation of the growth and/or activity of one or a limited number of bacteria in the colon and is not digested by the host digestive enzymes'' (Gibson, et al. 2004). As prebiotics are indigestible by the upper gastrointestinal tract (GIT) they enter the lower GIT where they are considered as a substrate for health-promoting bacteria, such as *Bifidobacteria* and *Lactobacilli*, thus modulating the microbiota in the gut (Gibson and Roberfroid 1995). Oligosaccharides are the main nutritional source, which is reflected in their residence in ecological niches rich in carbohydrate-containing substrates, most commonly plant material, spoiled or fermented foodstuffs, and mucosal membranes of humans and animals (Walter 2008).

1.15 Prebiotic effects in poultry and other animals

1.15.1 Effects on intestinal microbiota

Prebiotics have been studied using different substances to investigate the effects on the gut microbiota. (Konosonoka, et al. 2015) carried out a study to investigate the effects of a combination of the dried Jerusalem artichoke as a prebiotic alongside probiotic bacteria (1 × 10^8 cfu-g-1 *Lactobacillus reuteri* 1 × 10^8 cfu·g-1) fed to broiler chicks from 1 to 42 days old on the presence of bacteria of the family Enterobacteriaceae and lactic bacteria in the ileum part of intestinal tract. Their results established that supplementation of the broiler basic feed with 0.5 % dry powder prebiotic in combination with probiotics improved the level of the favourable lactic acid bacteria in the ileum part of the intestinal tract. (Nabizadeh 2012) conducted an experiment of 3 levels treatments of inulin (0%, 0.5%, or 1%) for 42 days on broiler chicken to evaluate the effectiveness of this prebiotic supplement on the intestinal microbiota of broiler chickens compared with the control group and found that Inulin inclusion had no effect on *Bifidobacteria*, Lactobacilli and *E. coli* counts in ileal contents, but these supplements significantly increased Bifidobacteria counts and decreased *E. coli* counts in caecal contents. Also, Shang and colleagues (2010), found adding inulin to layer hen feed, did not affect lactobacilli but *Bifidobacterium* numbers increased. Samal et al., (2016) found that adding 6% of JA powder into rat feed similarly found improved total count of

Bifidobacterium in the caecum. Rebole et al., (2010) also found that adding inulin to the laying hens' diet led to an increase in *Bifidobacterium* in the caecal content.

1.15.2 Effects of prebiotics on poultry performance

(Konosonoka, et al. 2015) carried out a study to investigate the effects of a combination of the dried Jerusalem artichoke as a prebiotic and probiotic bacteria (1 × 10^8 cfu-g-1 *Lactobacillus reuteri* 1 × 10^8 cfu·g-1) fed to broiler chicks from 1 to 42 days old on the productivity of broilers and feed conversion. Their results established that supplementation of the broiler basic feed with 0.5 % dry powder prebiotics in combination with probiotics increased the live weight of the broiler chicks by 2.1 %, the feed consumption for obtaining 1 kg of live weight decreased by 3.2 %, the cholesterol level in the meat of broiler chicks was reduced by 22.7 mg·(100 g)-1, moreover, the quality of the meat was improved by the synbiotic supplements in comparison with the control group. (Nabizadeh 2012) conducted an experiment of 3 levels treatments of inulin (0%, 0.5%, or 1%) for 42 days on broiler chicken to evaluate the effectiveness of this prebiotic supplement on the performance of broiler chickens. Their results showed that live body weight on day 42 was significantly increased when the diets were supplemented with 1% inulin. However liveability, body weight gain, feed intake and feed conversion ratio were not significantly affected in birds fed diets with supplements in comparison with the control group.

1.16 Prebiotic, probiotic and synbiotic use in poultry

Researchers have indicated that use of JA in broiler chickens diet has a beneficial effect on growth performance and suppresses potential pathogens in caeca of broilers. (Kleessen, et al. 2003) evaluated the effect of the fructan-rich Jerusalem artichoke, or topinambur (administered as 0.5% topinambur syrup in drinking water), on cultural numbers of selected caecal bacteria (total aerobes, *Enterobacteriaceae, Bdellovibrio* spp., and *Clostridium perfringens*) and levels of bacterial endotoxins as well as on body weights of broiler chickens for 35 days. They found that administration of JA resulted a significant increase in caecal counts of *B*. *bacteriovorus* and reduced the level of total aerobes, *Enterobacteriaceae,* and C. *perfringens* significantly. Also they observed that on day 35 of the trial period the body weight was increased in the JA treated birds. (Abdel-Hafeez, et al. 2017) conducted a broiler chickens trial to investigate the effects

of probiotic, prebiotic and synbiotic on broiler performance, blood parameters, carcass characteristics, and feed cost of production from 1 to 56 days of age. Group 1 were fed on a control diet while the other groups were given the same control diet supplemented either with a probiotic (Enhancer, USA, *Bacillus licheniformis* and *Bacillus subtilis*) with 1.0, 0.5, and 0.25 kg of prebiotic (Bio-Mos, USA, mannan-oligosaccharides (MOS) derived from the cell wall of certain strains of *Saccharomyces cerevisiae*) group 2, synbiotic (half the amounts of the used probiotic and prebiotic) group 3. They found that chicks fed diets supplemented with probiotic, prebiotic and synbiotic exhibited higher body weight and feed efficiency than chicks fed the control diets. The lowest feed cost per kg of weight gain was observed in the birds fed diets supplemented with synbiotic, probiotic and prebiotic. (Saiyed, et al. 2015) and his colleagues stated that feeding supplement of synbiotic has a beneficial effect over probiotic and prebiotic when used alone. They added prebiotic, probiotic and synbiotic in the broiler diet compared with control, probiotic (of *Lactobacillus acidophilus, Lactobacillus casei, Pediococcus acidilactici, Bacillus subtilis* and *Saccharomyces boulardii*) in the feed 100 g/tonne of feed, prebiotic (of Mannan Oligo-Saccharide in which Mannan and Glucan were included) in the feed at 500 g/tonne of feed, probiotic + prebiotic 100 g/tonne and 500 g/tonne of feed, respectively and probiotic + prebiotic 50 g/tonne and 250 g/tonne of feed. They found that body weight gain was not affected by the supplements significantly but the feed intake was decreased in the synbiotic treatment significantly, meanwhile European performance efficiency factor (EPEF) was improved at all treatments.

1.17 Synbiotic

A synbiotic can be defined as nutritional supplement comprising the mixture of both prebiotic and probiotic ingredients. When synbiotic supplements are consumed, the prebiotics and probiotics may work synergistically in the gut, thereby may give the benefits of both (de Vrese and Schrezenmeir 2008) . One of the purposes of using a synbiotic is to overcome possible survival difficulties for probiotics bacteria. Therefore the rationale to use synbiotics, may be based on observations showing the improvement of survivability of the probiotic bacteria during the passage through the upper intestinal tract (Pandey, Naik and Vakil 2015). In a human study it was claimed that using a

synbiotic has some benefits: 1) Increase the levels of lactobacilli and bifidobacteria, 2) Improve the function of liver, 3) Improve the ability of immunomodulation (Zhang, et al. 2009).

1.17.1 Inulin

(Slavin 2013a)Inulin is one of the most effective and most commonly used prebiotics (Slavin 2013). It is a storage carbohydrate in many plants. It is widely distributed in a variety of plants being present in more than 30,000 vegetable products (Wichienchot, et al. 2011). It occurs in fruits and vegetables, for example chicory, Jerusalem artichoke, globe artichoke, onion, leek, garlic, asparagus, bananas and in the stem of some cereals, such as wheat, oats, soybeans, rye and barley (Slavin 2013b, Mensink, et al. 2015). However, the main sources that are used commercially to extract inulin are usually roots of chicory (*Cichorium intybus*) and tubers of Jerusalem artichoke (*Helianthus tuberosus*) (Kaur and Gupta 2002). Where the industry processes of extraction are similar (Apolinário, et al. 2014). The chemical inulin is not simply one molecule but it is a polydisperse β (2-1) fructan. The chain lengths of these fructans range from 2–60 units, with an average DP of ~10 (Mensink, et al. 2015). Inulin has a specific structure which is the presence of the β-glycosidic bond, with the degree of polymerization (DP) has the range between 2 to 60 (van de Wiele, et al. 2007). Which make it unable to be hydrolysed by the digestive enzymes in the upper gastrointestinal tract of humans and non-ruminant animals like poultry (Buclaw 2016). Unchanged, the prebiotic reaches the large intestine, where it undergoes fermentation and becomes a substrate for some strains of healthy bacteria (Miremadi and Shah 2012).

1.17.2 Jerusalem artichoke as a readily available source of prebiotic in Iraq

Jerusalem artichoke (*Helianthus tuberoses*) is a member of Asteraceae family which is native to North America (Žaldarienė, Jurgita and Judita 2013). Jerusalem artichoke can be grown in different climate conditions, therefore it is cultivated in North America, Europe and Southeast Asia (Puangbut, et al. 2012). All parts of the Jerusalem artichoke plant are used for different applications as well as a food, such as using plant-tops for biomass and animal feed, whilst the tubers can be use as non-food chemical production (Stanly *et al*., 2008). The majority carbohydrate storage plant is as starch whereas in

contrast in Jerusalem artichoke carbohydrate is stored as inulin (Stanly *et al*., 2008). In the industrial production of inulin chicory is used as the major crop (Abou-Arab, Talaat and Abu-Salem 2011). However, the Jerusalem artichoke (JA) tuber has a large amount of inulin approximately 14–19 % (Lingyun, et al. 2007, Saengkanuk, et al. 2011), so it should be a valuable source for inulin production. Also JA is preferable to chicory especially in Iraq as it more widely available and lees expensive. Inulin that is produced from the Jerusalem artichoke tuber has an undesirable flavour and a significant colour which has traditionally made industry neglect JA for inulin production (Srinameb, et al. 2015). Therefore it became in less demand for human which in turn means it can be used as a cheap substance in animal. Also JA is available in Iraq with low cost during winter from October until April.

There are different important nutrients found in artichoke in addition to inulin, such as protein, iron, calcium and potassium (Stanley *et al*., 2008). Gafaar *et al*., (2010) found that Jerusalem artichoke tubers have 7.55% crude protein, 5.72% ash 6.51% crude fibre and 72.99% inulin. In another study on the chemical composition of Jerusalem artichoke by Nadir *et al*.,(2011) they found that the dry matter of artichoke consisted of 5.47% protein, 6.64% ash , 5.88% crude fibre and 77.7% inulin. Whereas, El-Hofi (2005) argued that the dry basis of the tubers of Jerusalem contained 73.50 % inulin, 8.26% crude portion, 5.92% crude fibre 6.82 and ash. In a study on the Iraqi Jerusalem artichoke Alsharafani (2006) found that Iraqi Jerusalem artichoke contains 76.52 % moisture, 0.40% fat, 6.06% protein, 2.24% ash and 9.60% inulin. As JA powder contains inulin therefore it can be used as a nutrient source for beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. Both (Kunová, et al. 2011, Nagpal and Kaur 2011) stated that some lactic acid bacteria are able to use inulin as source of carbon in the media. In addition JA as a raw plant contains other nutrients such as minerals (Lachman 2008) which in turn may be considered beneficial for lactic acid bacteria.

1.18 *Campylobacter* as a target pathogen in poultry production

Campylobacterosis is an acute gastro-enteric disease in humans significant worldwide through its impact on public health (Bless, et al. 2016). It commonly appears that the intestinal environment of all avian species (including wild birds) is favoured by this bacteria (Newell and Fearnley, 2003). However the poultry products are implicated as

the main source of infection in humans: 97% of recorded Campylobacterosis cases in humans are through this route (Wilson et al., 2008). It has been reported that only thirty five colony-forming units (cfu) which need 24 h after entering the broiler chickens gut to be sufficient for the successful establishment of *Campylobacter* infection (Mohan 2015). Regarding the initial age for *Campylobacter* colonization of the chick GIT, there is a wide discrepancy between reported studies. (Potturi-Venkata, et al. 2007) concludes it is not detectable before 10 days post-hatch, while Newell (2002) claims that this organism can be detected at 2-3 weeks post hatch. Nevertheless, there is consensus that *Campylobacter* can spread among birds within a flock quickly. Consequently a flock with *Campylobacter* established in some birds will reach up to 100% positive within a few days and they remain so until slaughter (Stern 2008). For these reasons, it is difficult to control *Campylobacter* presence during processing of poultry meat (Potturi-Venkata, et al. 2007).

Many strategies are currently being used to control or even to reduce the amount of this bacteria in the poultry products but contamination of meat remains a problem (FSA, 2015). One approach is the use of probiotic bacteria to increase the ability of the microbiota in maintaining health of the GIT via the mechanisms described earlier, in particular the capacity of certain strains to compete with *Campylobacter* for the intestinal adhesion sites required for colonisation (Santini, et al. 2010). (Kleessen, et al. 2003) concluded control of intestinal pathogens during the earliest phases of broiler production may be the best strategy for the reduction of human pathogens on processed broiler carcasses. The recent ban on antibiotics in poultry feed has served to focus much attention on alternative methods of controlling the gastrointestinal microbiota.

1.19 Aims and objectives

The overall aim of this study was to evaluate new isolates of *Lactobacillus* as probiotic feed supplements for poultry both when fed alone and with Jerusalem artichoke plant as an affordable source of prebiotic. The objectives set out to meet this aim were:

1. Optimisation of *Lactobacillus johnsonii* delivery to poultry and evaluation of its effects on the inhibition of *Campylobacter*, immune function and performance of meat chicken.
2. Isolation and in vitro evaluation of new strains of *Lactobacillus* from free range poultry as probiotic agents
3. In vivo evaluation of new strains of *Lactobacillus* from free range poultry as probiotic agents in meat poultry
4. Investigation into the effects of Jerusalem artichoke as prebiotic agents in meat poultry when fed alone and in combination with a probiotic supplement.

Chapter 2

Material and Methods

2.1 Introduction

This chapter provides an overview of the studies undertaken and how they correspond to subsequent thesis chapters with a detailed account of the generic materials and methods employed. Five studies were conducted in all (shown in table 2.1), including a pilot screening of naturally occurring *Campylobacter* levels in the local area, two investigations into a novel strain of *Lactobacillus johnsonii* and a major study into the efficacy of a newly developed mix of *Lactobacillus* (LB) isolates with Jerusalem artichoke. Study 1 assessed natural occurrence levels of *Campylobacter* in free range and research-housed poultry. Study 2 determined the optimum method of administering LB to chicks in the pre-starter period (up to d5) on the colonisation of the intestinal tract with the LB. Study 3 utilised the most efficacious method to deliver LB and monitored the level of *Campylobacter* colonisation in birds fed LB for 7 days compared with a control group without probiotic over a 28 day trial period. Study 4 involved isolation and screening of candidate probiotic isolates of lactic acid bacteria and prebiotic (Artichoke Jerusalem) and efficacy assessment *in vitro*. Study 5 investigated the *in vivo* efficacy of the candidate probiotic isolates of lactic acid bacteria when fed alone and in combination with Jerusalem artichoke.

Table 2:1 Description of individual studies conducted

Study	Areas investigated	Chapter
Study 1	Natural occurrence levels of *Campylobacter* in free range and research-housed poultry	3
Study 2 (Coded bird trial LB01)	Effect of differing methods of administering *Lactobacillus* to chicks in the pre-starter period (up to d5) on the colonisation of the intestinal tract with the LB.	4
Study 3 (Coded bird trial LB02)	Level of *Campylobacter* colonisation in birds fed LB for 7 days compared with a control group without probiotic over a 28 day trial period.	4
Study 4	Isolation and characterisation of candidate probiotic isolates of lactic acid bacteria and efficacy assessment *in vitro*	5
Study 5 (Coded bird trial LB03)	Investigation the *in vivo* efficacy of the candidate probiotic isolates of lactic acid bacteria when fed alone and in combination with Jerusalem artichoke.	6, 7 and 8

2.2 Excreta sample collection

Screening for the presence of *Campylobacter* was carried out on excreta samples. Fresh excreta samples with normal appearance were collected into sterile universal tubes using a sterile spatula. Samples from outside the house were collected in the morning to avoid sunlight degradation. During the research trials samples were collected from different pens in triplicate. These samples were immediately placed in an ice box then transferred directly to the microbiology laboratory for preparation and screening. Additionally, fresh excreta from bird vent were collected in swab tubes before transporting on ice to the culture labs.

2.3 Bird sample collection

Caecal samples were collected *post mortem* by placing excised caeca in bags and transporting to the culture labs on ice, where the caeca were opened and samples collected using a charcoal swab.

2.4 Culturing Campylobacter

One gram of excreta was weighed into 9ml of phosphate buffered saline (PBS) then homogenised well by vortexing (Stuart, UK) before serial dilutions down to 10^-5 in PBS were prepared from the sample. 100µl of these dilutions were cultured on CCDA agar (Oxiod, UK) and incubated at 37°C in a microaerobic cabinet (Don Whitley DG250 Anaerobic Workstation, UK) with the following microaerobic atmosphere; 5% O_2, 10% CO_2 and 85% N_2 for 48 hours. After the 48 hour incubation, growth of *Campylobacter* was assessed by enumeration of the small grey colonies on each plate.

2.5 Birds and Husbandry

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. For all trials, birds were sourced from PD Hook Cote hatchery, Oxford, and birds were feather sexed on day of hatch and collected by NTU personnel to reduce travel stress. On commencement of each trial, one day old male Ross 308 chicks were weighed individually to ensure uniformity of size across and within pens, before random allocation to mesh sided pens bedded on wood shavings. The chicks were housed in preheated 0.64m^2 pens in a purpose built, insulated poultry house. The birds were

bedded on clean wood shavings (to a depth of approximately 3cm) and fresh shavings were added into the pens as required. Birds were always allowed *ad libitum* access to the treatment diets and water for the duration of the trial. With care taken to ensure the birds ate and drank as soon as possible. The lighting regimen was as detailed in appendix I, up to Day 6 post hatch, and then a 6hr: 18hr ratio of dark: light was maintained for the rest of the study period, in accordance with commercial practice. Temperature was set at 31°C on day 1 and reduced by approximately 1°C per day until 21°C was reached. Temperatures were recorded daily from both ends of the unit, health checks made twice daily and heating and ventilation adjusted depending on bird behaviour. Fan speed was adjusted to maintain room temperature and humidity. Unhealthy or unusually sized chicks were discarded from the trial on arrival. Birds were individually weighed and only birds between 38 and 46 g were placed. Chicks were weighed by pen on Day 1 and allocated to a dietary treatment on arrival. Commercial guidelines for the care and husbandry of Ross 308 broilers were followed in all studies (Aviagen, 2014). Any mortalities were recorded along with the date and weight of the bird and reason if culled. All birds sampled were euthanized by cervical dislocation as advised in Welfare of Animals at the Time of Killing (WATOK) regulations (DEFRA, 2015).

2.6 Diet Formulation

All trial diets were manufactured on site and fed as mash. The particle size of each diet was uniform, consistent and typical for broiler diets milled through a 3mm screen. The composition and analysis of all the trial diets are provided in the corresponding chapter. When making the diets, each ingredient was individually weighed out and mixed dry for five minutes in a ribbon mixer (Rigal Bennett, Goole, UK) before addition of oil. The diets were then mixed for a further five minutes. Where probiotic supplements were added to the diet, this was undertaken as an additional, final step. For each diet manufacture day, diets without probiotic were manufactured first, and the mixer was cleaned with alcohol and left unused for 5 days before being used for any diet not due to contain a probiotic supplement. The mixer was brushed down at various stages throughout the mixing process to ensure oil clumps were removed. In all studies, diets were randomly allocated to pens within the room, to eliminate any effect of room position.

2.7 Feed Intake measurement

Initially, individual weighed bags of feed were prepared containing precisely weighed feed quantities for each treatment. Each pen of birds were fed from their designated bag throughout the trials. Extra feed was added to the bags if required and the quantity recorded. Uneaten food remaining in the troughs on weigh days was tipped back into the labelled feed bag for each pen, taking care to remove droppings and shavings where necessary. Total feed eaten was calculated as the difference between remaining feed in the bag the amount weighed into the bag. Spillages of feed was not easy to account for spillages were mixed with the droppings and shavings, but spillages were recovered from the floor and added to the remaining feed total – acknowledging that this measure may contribute to inaccurate measurement of feed intake.

2.8 Bird Weights

For all bird trials, chicks were weighed on arrival, and any outside the range of 38-45g 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 using a top pan balance (Mettler Toledo, Leicester, UK) on days specified in each trial protocol (see relevant chapters for details).

2.9 Body Weight Gain

The body weight gain was calculated by the difference between each two periods (weeks).

2.10 Diet chemical analyses

2.10.1 Crude Protein Determination

Protein content of each diet was analysed using the Kjeldahl method (AOAC official method 2001.11) (Peter and Baker 2001, Tahir, et al. 2012). Approximately 1 gram of sample was weighed into distillation tubes (Foss Cat No. 10000155) in duplicate. Both a copper and selenium catalyst tablet (Fisher Scientific, UK) was added to each tube. 12.5 ml of concentrated nitrogen-free sulphuric acid was then added to each tube, and they were heated in a digestion unit (1007 Digester, Foss Tecator, UK) set at 450°C for 45 minutes. Once digestion was complete, the distillation tubes were left to cool for a minimum of 20 minutes and 75ml of distilled water was added to each tube. The tubes were then distilled in a distillation unit (2100 Kjeltec, Foss Tecator, Cheshire, UK) then

50ml of 10M sodium hydroxide was added to the samples, distilled for 3 minutes, the resulting ammonia was expelled into conical flasks containing 2ml 4% boric acid with indicator, causing a colour change from orange to blue. The boric acid was then titrated back to the original colour using 0.1M HCl in a burette and the volume of acid used was recorded. Starch was used as a blank. % nitrogen was calculated by:

1.4 x (V1-V2) x M / W

where:

W= Original weight of sample

V1= Volume of acid to titrate sample

V2= Volume of acid to titrate blank

M=Molarity of acid

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

2.10.2 Extractable Fat Analysis

Samples of diets were analysed for extractable fat content by the Soxhlet method (AOAC official method 2003.05). Approximately 5g of sample was accurately weighed into an extraction thimble. A flat bottomed flask containing approximately 0.25g of anti-bumping granules was accurately weighed. Petroleum ether (150ml) was added to the flask. The thimble was inserted into the bottom of the distillation unit, the distillation apparatus was connected to the condenser and the flask was attached to the apparatus and seated in the heating mantle (set to 40-60°C). The samples were left to extract for approximately 18 hours, then the remaining ether was boiled off on a hotplate and left to evaporate. The flasks were reweighed and extractable fat content was calculated by:

(M2-M1 / M0) x 100 = % extractable fat

Where:

M0=Original weight of sample

M1= Weight of flask plus anti-bumping granules

M2=Weight of flask plus fat and anti-bumping granules

2.10.3 Dry Matter Analysis

Dry matter content of the diet and excreta was analysed by weighing approximately 5-10g of finely ground sample into pre-weighed crucibles. The crucibles were then dried in a drying oven set at 105°C for approximately 4 days, until the weight was constant. The dried samples were then cooled in a desiccator and reweighed.

2.10.4 Mineral Analysis

Mineral content of each diet was analysed by accurately weighing approximately 2-5g of sample, into a pre-weighed ceramic crucible. The crucibles were then placed in a muffle furnace (Nabertherm, B180) for approximately 14 hours at 650°C. The ashed samples were then cooled in a desiccator and reweighed.

2.10.5 Gross energy analysis

Gross energy of the feed and excreta was measured using a bomb calorimeter (Instrument 1261, Parr Instruments, Illinois, USA) according to the (Rutherfurd, Chung and Moughan 2007, Woyengo, Kiarie and Nyachoti 2010). Pellets of feed sample, weighing approximately 1g, 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 calorimeter. The bucket in the bomb jacket was filled with 2 litres of water. Fuse wire (10cm) 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 calorimeter, and the lid of the bomb jacket was shut. Sample weight was entered and the process was started; the calorimeter measured energy produced (MJ/kg) when the pellet is combusted.

2.11 Mucin adherent layer thickness

A section of gastrointestinal tract was analysed for mucin adherent layer thickness by the method used by (Smirnov, et al. 2004). A 1cm section of jejunum was gently flushed with distilled water and then placed in 10g/l Alcian blue (in 160mmol/l sucrose with 50mmol sodium acetate) for 2 hours. The tissue was then washed in 250mmol/l sucrose to remove excess dye. Bound dye was extracted using 10g/l docusate sodium salt overnight at room temperature. The supernatant was then centrifuged at 700 x g for 10

minutes to remove particulate matter and the absorbance read at 620nm (JENWAY, 7315 Spectrophotometer, Bibby Scientific Ltd, UK) against a curve of diluted Alcian blue. Results are expressed as µg Alcian blue released per cm^2 tissue. Two replicate gut samples were analysed per pen from the each bird.

2.12 Immunoglobulin measurement

Immunoglobulins A, M and G were analysed in pooled plasma using ELISA kits specific for chicken plasma from Bethyl laboratories. Plates were coated with antibody specific for the immunoglobulin to be measured and then blocked overnight and washed before diluted samples then these samples were added to the plate and incubated for one hour. HRP conjugated detection antibody was added at an appropriate dilution for an hour before adding a colour reagent 3,3',5,5'-tetramethylbenzidine (TMB) for 15 minutes in the dark, or until colour was sufficiently developed. The absorbance was read on a plate reader at 450nm using a Multiskan plate reader with standard curve values programmed using a 4PL curve fit. QC samples were run to calculate inter assay coefficients of variation (CoV), and all samples were run in duplicate and sample variation used to calculate intra assay CoV.

2.12.1 Serum uric acid measurement

Uric acid content was measured in pooled plasma using an Amplex Red kit (Invitrogen). 5µl of plasma was pipetted into a 96 well plate and the volume of each well made up to 50ul with reaction buffer. A set of uric acid standards from 120 to 10µM were prepared and pipetted onto each plate. The reaction was started by the addition of a reaction mixture containing 50µl of each of Amplex red reagent, uricase and horseradish peroxidase. The plates were then incubated at 37^{o}C for 30 minutes protected from light before measurement on a plate reader at 560nm. Unknowns (sample) were calculated using the standard curve and expressed as mg/dL.

2.13 Isolation and screening of candidate probiotic bacterial isolates and prebiotic (Jerusalem artichoke) and efficacy assessment in *vitro*

2.13.1 Screening and isolation of lactic acid bacteria

Excreta samples were serially diluted down to 10^-10. These dilutions were cultured on de Mann Rogosa Sharpe (MRS) agar (Oxoid) then incubated for 48 hours at 37°C in a

microaerobic atmosphere. After checking for any contamination (clear growth), colonies of interest were selected from each of the highest dilution 10^-8 plates then sub-cultured again on the same media and same conditions to obtain pure cultures. These pure cultures were then further tested as described below. Finally six isolates were chosen for further study.

2.13.2 Morphological and biochemical tests on the isolates

2.13.2.1 Preparing a standard inoculum of isolates

Aliquots of MRS (10ml) broth were inoculated with one colony of each chosen LAB isolate, and incubated at 37°C microaerobically for 24 hours. Cells of isolates were pelleted at 5000 rpm for 20 minutes (Megafuge 16R, Thermos-scientific, Germany), then washed twice with 10 ml PBS and re-suspended in PBS again. The number of viable cells was counted by a viable count and the suspension was diluted to 10^-6 using the absorbance (Optical Density - OD) as an indicator.

2.13.2.2 Stock culture of LAB isolates

Stocks of each individual isolate were prepared by growing the pure cultures that were obtained from section 2.13.2.1 on MRS broth for 24 hours, then the broth was centrifuged(10000rpm/10mints) to obtain a pellet and thereby concentrate the bacterial cells. A stock was made from the concentrated cells and mixed with glycerol at a ratio of 70:30 broth: glycerol in 1ml cryo-vials and stored at -80°C.

2.13.2.3 Gram staining

Gram stain was used to determine the Gram status of the isolates. Isolated cultures were grown in MRS agar at 37°C for 24 hours to obtain fresh cultures for Gram staining. One colony was selected for Gram staining by using the procedure described below (M. L. Kaplan and Kaplan 1933, Bartholomew and Mittwer 1952).

A 0.2µl loop was used to mix a small amount of the colony with sterile water on a microscope slide. The slide was then dried. Colonies were fixed by exposure to a flame 3-4 times for about 5 seconds. Crystal violet was used for staining for 1 minute, then the slide was washed with tap water. Gram's iodine was applied to the slide for 1 minute, the slide was then washed with tap water and acetone/ alcohol applied to decolorise for 5-10 seconds. The counterstain, safranin, was applied for 30 seconds and washed with

tap water. Finally, the slide was dried and light microscopy used to determine if the isolates were potential LAB which are known to be Gram positive.

2.13.2.4 Catalase test

Catalase enzyme activity on hydrogen peroxide was used as a diagnostic tool to assess presence or absence of the enzyme: the formation of gas bubbles (as catalase breaks down hydrogen peroxide into water and oxygen) were deemed to indicate the presence of the catalase enzyme. As Lactic Acid Bacteria (LAB) catalase negative, presence of bubbles indicated isolates were not LAB. Overnight cultures of each single isolate were grown on MRS agar at 37°C under microaerobic conditions. Catalase activity was investigated by 3% hydrogen peroxide solution (one drop) onto a randomly chosen colony. The test was deemed positive if bubbles were observed within 5-10 seconds and negative if not.

2.13.2.5 Production of acids (pH assessment)

Each LAB isolate was grown in 10 ml of MRS broth after being inoculated with one colony of each of the isolates then incubated at 37°C under microaerobic conditions for 24 hours. The pH of the broth was measured by using a pH meter (Mettler-Toledo, UK) for each single isolate. This test was performed in quadruplicate. The pH of the broth after incubation was compared with the initial pH.

2.13.3 Genotypic Identification

2.13.3.1 Identification of *Lactobacillus* genera using 16s rDNA

2.13.3.1.1 DNA extraction

The DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) was used to extract the DNA from the LAB isolates with some modifications described below. One colony, obtained following growth on MRS agar, was inoculated into 10 ml of MRS broth for overnight growth (12h) at 37°C microaerobically. Then, the protocol below was followed: 1.5 ml of culture was placed into a 2ml Eppendorf tube and the cells pelleted and washed twice in PBS by centrifugation at 5000 rpm (Hettich, Germany) for 5 minutes. Afterward the washed cells were lysed by adding 180µl of lysis buffer which was prepared by adding 20mg Lysosome into 1 ml Gram positive buffer (Sigma-Aldrich, UK) incubated at 37°C for 3 hours. Protein was removed the as 25µl of proteinase K and 200ul AL buffer were

added and the tube incubated at 65 °C for 6 hours after mixing by vortex. Then the DNA was bound on the column by adding 200ul ethanol (96-100%) to the lysed cells, mixing thoroughly before applying into the DNeasy Mini spin column. The column was centrifuged at 8000 rpm for 1 min and the flow-through and collection tube were discarded. Then the DNA was cleaned up by placing the spin column in a new 2 ml collection tube, and 500 µl Buffer AW1 added, then centrifuged for 1 min at 8000 rpm. Then column was placed in a new 2 ml collection tube and 500 µl Buffer AW2 added, before centrifugation for 3 min at 14,000 rpm to dry the DNeasy membrane. The column was then placed in 1.5 ml tube and centrifuged for 1 mins at 15000 rpm. Finally the DNA was eluted by placing the DNeasy Mini spin column in a clean 1.5 ml and 50 µl nuclease free water pipetted onto the DNeasy membrane, incubated at room temperature for 5 min, then centrifuged for 1 min at 8000 rpm to elute the DNA. The quantity and quality of DNA was determined using a Nanodrop-2002 Spectrophotometer (Fisher, UK). DNA was stored at -20 °C until further processing.

2.13.3.1.2 Sequencing the DNA

The V4 hypervariable region of the 16S rRNA gene was amplified from genomic DNA using forward primer 515F: GTGCCAGCMGCCGCGGTAA and reverse primer 806R: GGACTACHVGGGTWTCTAAT (Earth Microbiome, 2015) using the 2x KAPA HiFi HotStart ReadyMix, and primers:

PCR amplification 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). This was added into a 96 well plate which was sealed 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. The PCR products were run through 1.5% agarose gel electrophoresis and bands analysed to check the product (amplicon) quality. Were run on 100 volts for 45 mins after loading 2µl of loading dye (Cleaver, MP-250v,UK), and 5µl of amplicon. These were then imaged under UV light (Syngene, G: box, USA). The 16S V4 amplicon was using 20 µl of AMPure XP beads and then incubated at room temperature for 5 minutes. The plate t was placed on a magnetic stand (FastGene MagnaStand, YS, Germany) for 2 minutes or until the supernatant had cleared. The supernatant was then discarded and the beads

washed twice with freshly prepared 80% ethanol before being allowed to air-dry for 10 minutes. The Amplicon was then suspended with 52.5 µl of 10 mM Tris pH 8.5 and gently mixed before incubating at room temperature for 2 minutes with 50 µl of the clear supernatant was transferred to a new 96-well PCR plate.

Index PCR which was performed as follows: 5 µl of DNA was transferred into another new 96-well plate and the Index 1 and 2 primers arranged. The 96-well PCR plate with the 5 µl of re-suspended PCR product DNA was placed in the TruSeq Index Plate Fixture (Illumina, USA) and 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 PCR Grade water. This mixture was gently mixed and the plate was covered with Microseal. Then centrifuged at 1,000 × g at 20°C for 1 minute after which a PCR was performed on a thermal cycler: 95°C for 3 minutes, 8 cycles of: 95°C for 30 seconds 55°C for 30 seconds 72°C for 30 seconds, 72°C for 5 minutes, the hold at 4°C. The Index PCR product was cleaned up as above with: 56 µl of AMPure XP beads added to each well of the Index PCR plate, and gently pipetted mix up and down and incubated at room temperature for 5 minutes. Then the plate was placed on a magnetic stand for 2 minutes or until the supernatant cleared. It was then removed and the supernatant discarded. Then the beads were washed with freshly prepared 80% ethanol twice. Then, carefully, excess ethanol was removed then the beads were air-dried. Next, 27.5 µl of 10 mM Tris at pH 8.5 was added to each well of the Index PCR plate and incubated at room temperature for 2 minutes. Twenty five µl of the supernatant was then carefully transferred from the Index PCR plate to a new 96-well PCR plate.

For validate 1 µl of the final library was run on a tape-station DNA 1000 (Agilent, USA) to verify the size. Library Quantification, Normalization, and Pooling were performed as follows: DNA concentration was calculated in nM, based on the size of DNA amplicons as determined by an Agilent Tape station 1000. Then the library was diluted to 4 nM using 10 mM Tris pH 8.5. Aliquot 5 µl of diluted DNA from each library was pooled; the MiSeq reagent cartridge was removed from -15°C storage and thawed at room temperature. DNA was then 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 minutes 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 (20 pM) PhiX library was added to the tube containing 10 µl denatured PhiX library to result in a 20 pM PhiX. This was then diluted to the same loading concentration as the Amplicon library to get 8mM by mixing 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 hole in the Miseq cartridge then loaded it in the machine using version 3 (300 × 2) chemistry on the MiSeq instrument (Illumina Inc., USA) according to manufacturer's instructions.

2.13.3.1.3 Bioinformatics analysis

The data obtained from sequencing were analysed by Dr Alan McNally at University of Birmingham.

2.13.4 Evaluation the phenotypic characteristics of the isolates

2.13.4.1 Viability of isolates at 42°C

Isolates were tested for their ability to survive at the incubation temperature of 42°C (representing broiler chickens core body temperature). This was performed by spreading 100 µl of standard inoculum (10^6) on MRS plates with each isolate and incubating at either 37°C as a standard or 42°C for 48 hours, in microaerobic conditions. The level of growth was compared to ascertain the viability of the isolates.

2.13.4.2 Tolerance to oxygen

MRS plates were inoculated with overnight growth of isolates and incubated at 37°C for 48 hours at three different levels of oxygen; aerobically in the aerobic incubator (LEEC,

UK), microaerobic atmosphere (Don Whitley DG250 Anaerobic Workstation, UK) and in an anaerobic atmosphere created using anaerobic jars (Thermoscientific, UK) with a gaspack (ANAEROGEN^{TM} COMPACT from Oxoid). After 48 hours, plates were checked for the growth of bacteria and given + for growth and – for no growth and this test was performed in triplicate.

2.13.4.3 Antibiotic resistance

The disc diffusion method (Moubareck, et al. 2005) was used to screen the LAB isolates for antibiotic susceptibility. The test was performed by spread plating the standard inoculum of isolates on MRS plate using cotton swab in three directions to cover all the plate. Then the plates were left to dry for 30 minutes. The antibiotics discs (Oxoid,UK) of Gentamycin 10 (GM), Ampicillin 10 (Ap), Cefmetazole 30(CAZ), Ciprofloxacin 5 (CIP), Cefotaxime (CTX), Tetracycline (TE), Cefoxitin 10 (FOX) and Rifampicin 5(RD) were placed on the plate, with distances around the discs of about 2cm, the plates were incubated at 37°C at microaerobic atmosphere. A clear zone (no growth) around the discs was measured by ruler and recorded. This test was carried out in triplicate and repeated twice.

2.13.5 Assessment of antibacterial activity against *Campylobacter* strains

2.13.5.1 Preparation of cell free supernatant (CFS)

MRS broth (10ml) was inoculated with 200µl of standard inoculum, and incubated for 24 hours. The culture was centrifuged at 15000 rpm for 15 minutes at 4°C then the supernatant was centrifuged again in the same conditions in order to remove all bacterial cells. Finally it was filtered using a 0.2µm filter (Minisart, Germany) and stored at -20°C until use.

2.13.5.2 *Campylobacter* suspension preparation:

Three strains of C*ampylobacter jejuni* were chosen: RM1221 (broiler chickens isolate), NCTC11168 (human isolate) and 01/51 (human isolate). A suspension of each *Campylobacter* strains was prepared by growing of each strain on charcoal-cefoperazone-deoxycholate agar (CCDA) plate for 48 hour at 37°C in microaerobic atmosphere. The bacterial cells were pelleted by centrifugation at 5000 rpm at 4°C for

15 minutes then washed with PBS twice and re-suspended in PBS buffer and diluted to get a 10^6 cfu/ml with PBS.

2.13.5.3 Inhibition test

The supernatant of LAB isolates were used to determine the activity against *Campylobacter* by following the protocol described by (Collado, et al. 2005) with some modifications. CCDA plates were prepared and 1ml of 10^6 cfu/ml *Campylobacter* suspension was spread over the plate surface using a cotton swab and left at room temperature for 15 minutes to dry. A well of 5 mm diameter was created using the base of a sterile 1ml pipette tip in the agar, leaving a distance around each well about 2cm. CCDA plates were then left in a gas jar with Campypack (Oxoid, UK) in the fridge to dry for 2 hours. Wells were filled with 50µl of CFS then left in fridge for 1-2 hours to absorb the supernatant. The plates were incubated in a microaerobic atmosphere at 37°C for 48 hours then the inhibition zone observed and measured in mm using ruler.

2.13.5.4 Detection of hydrogen peroxide production

The LAB isolates were evaluated for production of hydrogen peroxide by culturing bacteria on MRS plates prepared by (Dec, et al. 2014) mentioned. Plates were supplemented with 2.5 mg of 2, 39.5,59-tetramethylbenzidne (TMB); (Sigma-Aldrich) and 0.1 mg horseradish peroxide (Sigma-Aldrich) to each 10 ml of media. Plates were spotted with 20µl of the standard inoculum of isolates and incubated at 37°C microaerobically for 48 hours. After incubation the plates were exposed to air for about 30 minutes. Blue colour in the colonies indicated H_2O_2 production from isolates. This test was repeated twice.

2.13.5.5 Evaluation the ability to survive in the intestinal gut environment

2.13.5.5.1 Tolerance of Bile salts

The sensitivity of isolates to bile salts was performed according to (Ashraf and Smith 2016)Ashraf and Smith, (2016) by supplementing MRS broth with different levels of bile salts. 96 wells plates were used for this purpose, which were filled with 200µl MRS broth with final concentration 10^4 of isolates then and 0%, 0.25%, 0.50%, 0.75%, 1% w/v of bile salts (Ox gall B3883 Sigma-Aldrich ,UK) were added to the wells. In addition, 2 inoculum-free wells of each level of bile salt addition were used as controls. The increase of turbidity in the well was deemed to indicate bacterial growth. The optical density was

monitored (Cytation-imaging readers, Bio-rad- USA) at 600 nm at 0, 1, 2, 3, 4, 12 and 24hours. In addition to non-inoculated broth to compare the density. After 48 hours 100µl of the broth was cultured on MRS agar to check that the turbidity was due to the growth of bacteria.

2.13.5.5.2 Tolerance of Sodium Chloride (NaCl)

Tolerance to sodium chloride (NaCl) was assessed to ensure the LAB isolates were able to withstand presence of salt which is added into the broiler chickens diets at about 0.5% (NRC, 1994). The viability of LAB isolates was performed by culturing in MRS broth containing varying levels of NaCl (sigma, UK). Ten concentrations of NaCl were added to the broth to examine the highest in which the isolates can survive. 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7, 8%, 9% and 10% of NaCl were added to the MRS broth and a 96 well plate were filled with this mixture of 180µl MRS broth (NaCl) and 20 µl of standard inoculum of each isolate. The plates were then incubated at 37°C in a microaerobic atmosphere. The optical density over time was measured at 600mn wavelength using a microplate reader (Cytation-Imaging Reader, Bio-rad, USA) at 0, 2, 4, 6, and 24 hours. A non-inoculated broth was added as well to compare the density of the broth as a standard. Then, after 24 hours 100µl of the broth was cultured on MRS agar to check that the turbidity was due to the growth of bacteria. All tests were carried out in triplicate.

2.13.5.5.3 Tolerance of acid

Acid tolerance was carried out to assess whether the isolates could survive at low pH levels such as the proventriculus pH of 2 (Svihus 2011). The procedure described by (Menconi, et al. 2014) was carried out as follows. A 96 well plate was used for this test and the wells were filled with 180µl of MRS broth which was adjusted to different levels of pH 1, 2, 3, 4, 5 and 6, by use of HCl. Each broth was inoculated with 20µl of standard inoculum. Plates were incubated at 37°C in a microaerobic atmosphere, and the optical density was monitored (CYTATION-imaging readers, Bio-rad, USA) at 600 nm at time points 0, 1, 2, 3, 4 and 5 hours. In addition, a non-inoculated broth was used to compare the density and 100µl of the broth was cultured on MRS agar to check the viability of bacteria. The test was performed in triplicate.

2.13.6 Utilisation of Jerusalem artichoke (Inulin) as source of carbon by LAB isolates.

Readymade culture media usually contains glucose as a carbon source. In this study glucose was replaced with Jerusalem artichoke (JA) plant. Three types of broth were made (Table 2:2) comparing standard broth medium prepared with glucose, a second broth supplemented with commercial inulin (SENSUS, Netherland) in place of glucose and a third type of media supplemented with JA plant. Tubers of Jerusalem artichoke plant were prepared by grinding the dried tuber. A 96 well plate was used for this test, wells filled with the media described above and inoculated with 0.2% of standard inoculum. The growth of isolates was determined at two time points (0 and 24 hours) as previously described. 100µl of the broth was cultured on MRS agar to check the viability of bacteria.

Table 2:2 Composition of standard and prepared media supplemented with commercial inulin and Jerusalem artichoke.

Ingredient	Amount (g/litre)		
	standard	Inulin (pure)	Jerusalem artichoke
Peptone	10.0	10.0	10.0
Meat extract	8.0	8.0	8.0
Yeast extract	4.0	4.0	4.0
D(+)-Glucose	20.0	0.0	0.0
Dipotassium hydrogen phosphate	2.0	2.0	2.0
Sodium acetate trihydrate	5.0	5.0	5.0
Triammonium citrate	2.0	2.0	2.0
Magnesium sulfate heptahydrate	0.2	0.2	0.2
Manganous sulfate tetrahydrate	0.05	0.05	0.05
Tween-80	1 ml	1 ml	1 ml
Inulin	0.0	20.0	0.0
Jerusalem Artichoke	0.0	0.0	20.0

2.13.7 Preparation of broiler chickens dietary supplements of probiotic from isolates

MRS broth were inoculated with 0.2 % of standard inoculum using 500mL bottles and incubated for 16 hours at 37°C microaerobically. Bacterial cells were collected by centrifugation of the broth at 5000 rpm for 20 minutes at 4°C. The cells were washed twice with PBS then homogenised with 100% PBS (W/V) then the mixture of cryoprotectant was added at 100% skimmed milk (W/V) and 5% sucrose (W/V) before

mixing thoroughly by vortex (Stuart, UK). This mixture was incubated at 4°C for 1 hour before freezing to adapt the bacteria for snap freezing in the next step. The tubes of cold isolates were submerged in liquid nitrogen for about 5-10 minutes until frozen. They were then stored at -80°C for 24 hours before drying. A freeze dryer (Bench Top Pro, SP Scientific, USA) was used to dry the mixture of bacterial cells and cryoprotectant which was applied under pressure of about 510 millibar (mb) and -56°C for 12-18 hours until dried. Dried product was then ground using a coffee grinder (Russell, China) and kept in the fridge until to be used in broiler chickens feed. The concentration (CFU) of product was determined; 100mg of organisms powder was added to 900µl PBS then serial dilutions up to 10^-10 were made which were then cultured on MRS agar and incubated at 37°C microaerobically. This product was diluted to 10^-9 with skimmed milk to be used in broiler diet. Skimmed milk was used to dilute the dried product to 10^-9 CFU of each isolate. All diluted products of 6 isolates were mixed in one bottle and stored at 4c.

2.13.8 Assessment of the viability of bacterial cells during the preparation of the broiler chickens feed supplements

The viability of bacteria was checked at several different points during processing: before freezing, after freezing and after drying, viability was determined by viable counts (CFU). 100 mg of dried product was weighed into 900µl PBS and serial dilutions carried out then cultured on MRS agar for 24 hours at 37°C at microaerobic atmosphere, before the CFU was counted. All tests were performed in triplicate.

2.13.9 Preparation of Jerusalem artichoke tubers

Jerusalem artichoke tubers were obtained from the wholesale market in Baghdad, Iraq. Clay, undesirable materials and damaged tubers were removed. The tubers were cleaned with tap water to remove dust and clay that stuck on the tubers. The cleaned tubers were cut into small pieces and sliced to 2-3mm in thickness using a food processer (Kenwood FP126 Food Processor) and were then dried at room temperature for 2 hours. The sliced tubers were then further dried using an air-drier cabinet (locally made) at 60°C for 12 hours. The dried tubers were packed and sealed in double polyethylene bags. The packed, dried JA were then shipped to the UK. In the UK, the dried tuber pieces were packed well in boxes with an appropriate amount of silica gel (Fisher, UK) to keep it dry and avoid moisture absorbance and stored at room temperature. JA were further processed to be added in broiler chickens feed. It was ground in two steps first using

mixer (KENWOOD, UK) to get small pieces then using coffee grinder (Russell, China). Finally the powder was sifted using sieve of mesh size 2mm.

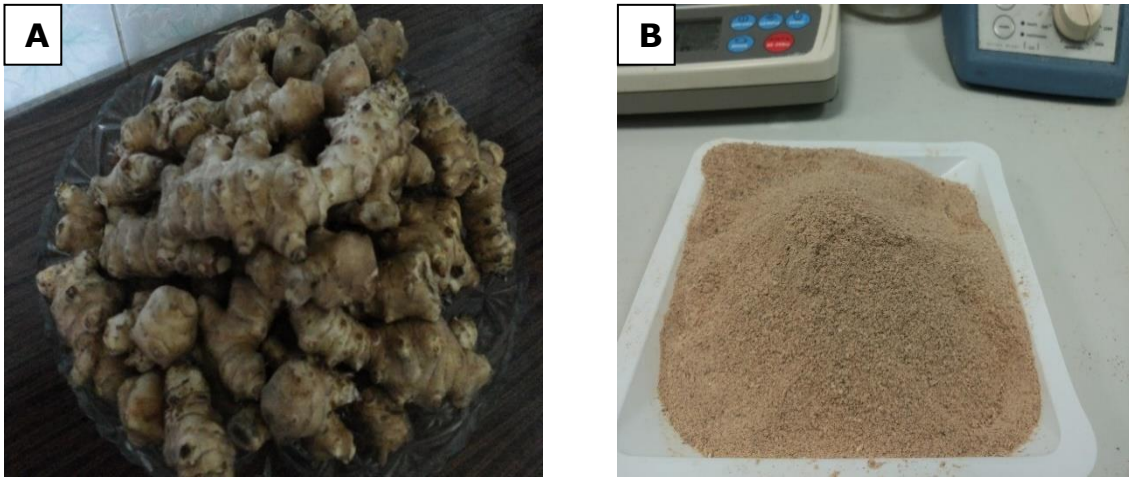

Figure 2:1 Jerusalem artichoke plant used in this study

A: fresh Jerusalem artichoke tuber, B: dried ground Jerusalem artichoke

2.13.10 Determination of the content of inulin in Jerusalem artichoke

The inulin content of the Jerusalem artichoke was determined in duplicate using the Fructan HK Assay Kit and following the protocol recommended by the supplier (Megazyme International, Bray, Ireland) where samples are ground and extracted in hot water. One gram of sample was added to 400 mL of hot (80°C), distilled water which was placed on a hot-plate with a magnetic-stirrer and stirred and heated (80°C) for 20 minutes. The solution was allowed to cool at room temperature then transferred into a 500 mL volumetric flask and the volume was adjusted to the mark with distilled water. The sample was then mixed thoroughly by shaking the flask. The solution was filtered through a Whatman No. 1 (9 cm) filter paper and analysed immediately. Aliquots (0.2 Ml) were analysed (containing approximately 0.1 to 2.0 mg/mL of fructan) by dispensing into the bottom of glass test-tubes (16 x 100 mm). Then, 0.2 mL of solution 3 (sucrase/maltase mixture) was added to the tube and incubated at 40°C for 30 min, and then 0.5 mL of buffer 2 (100 mM sodium acetate buffer, pH 4.5) was added following vigorous stirring on a vortex mixer (called 'Solution A'). 0.2 mL aliquots of Solution A (in duplicate) were added to the bottom of plastic spectrophotometer cuvettes (3 mL volume, 1 cm light path), then 0.1 mL of solution 4 (fructanase solution) was added to the bottom of one cuvette, and 0.1 mL of buffer 2 was added to the second cuvette. The contents were mixed thoroughly and the cuvette was covered with Parafilm. The

covered cuvettes were incubated at 40°C for 30 min in a dry hot block heater to effect complete hydrolysis of fructan to fructose and glucose. The absorbance was read at 340 nm by spectrophotometer (JENWAY, 7315 Spectrophotometer, Bibby Scientific Ltd, UK) at 25°C. The amount of inulin was expressed in terms of fructan concentration

2.13.11 Collecting and preparation of tissue samples

After euthanizing the birds and opening the carcass, the whole Fabricia of Bursa, 5 cm from the middle of ileum and the paired caeca were collected from the birds immediately in sterile tubes and placed in cold polystyrene box about 4C.

2.13.11.1 Preparation of tissue samples for RNA extraction

Tissue from the Fabricia of Bursa and ileum were directly placed in petri dishes. Which were washed with sterile PBS and a cut in pieces of about 0.2- 0.5 cm^2 then stored in cryovials containing 500µl RNAlater buffer (R0901, Sigma, UK) to protect RNA with immediate RNase inactivation, tissue samples were stored overnight at 4°C before being transferred to -80°C for storage until processing for RNA extraction.

2.13.12 Preparation of the samples for total count of LAB and *Campylobacter*

Caeca were separated and one caecum was placed in sterile tube in a cold box (4c) then transported to the lab. When in the lab, approximately 3 grams of content was placed into Eppendorf tubes and stored at -20°C. The second, whole caecum was further processed for culturing.

2.13.12.1 Culturing the content of caeca for LAB

One gram of caecal content was weighed into 9ml of PBS tube then homogenised using a vortex (Stuart, UK) and serial dilutions carried out until 10^-10. The appropriate dilution was cultured in three replicates on MRS agar and incubated for 24 hours at 37°C at microaerobic atmosphere. All colonies that showed similar small, white or creamy appearance were counted.

2.13.12.2 Culturing the tissue of caeca for LAB

From the isolated caeca, one cm^2 of tissue was placed into 9ml of PBS tube, homogenised using a Homogenizer (IKA® T 18 ULTRA-TURRAX® Basic, Brazil) then serial dilutions were carried out until 10^-7 was achieved. Next, the appropriate dilution was

cultured in three replicates on MRS agar and incubated for 24 hours at 37°C at microaerobic atmosphere. Afterward all colonies that showed similar small, white or creamy appearance were counted.

2.13.12.3 Culturing the content of caeca for *Campylobacter*

One gram of caecal content was placed into 9ml of PBS tube, homogenised using a vortex (Stuart, UK) and a serial dilutions carried out until 10^-10. Then the appropriate dilution was cultured in three replicates on CCDA agar and incubated for 24 hours at 37°C at microaerobic atmosphere. Afterward all colonies with tiny, grey appearance were counted.

2.13.12.4 Culturing the tissue of caeca for *Campylobacter*

One cm^2 of caecal tissue was placed into 9ml of PBS tube, then homogenised using a Homogenizer (IKA® T 18 ULTRA-TURRAX® Basic, Brazil) then serial dilutions carried out until 10^-7 was reached. Then, the appropriate dilution was cultured in three replicates aces on CCDA agar and incubated for 24 hours at 37°C at microaerobic atmosphere. Afterward all colonies with tiny, grey appearance were counted.

2.13.12.5 RNA extraction from Fabricius of bursa and ileum

Samples that had been prepared according to methods described in section 2.13.11.1 had RNA extraction performed using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK). Total RNA was purified from broiler chickens tissues following the manufacturer's instructions. Stabilised tissue samples (Bursa of Fabricius and ileum) in RNAlater previously frozen at -80°C were thawed at room temperature and 40mg was weighed (Ohaus, AP110S, Switzerland) into a 2ml eppendorf tube of 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 10000 x g for 15 seconds. Subsequently 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 tubes, using 100 µl RNase-free water, by centrifugation at 1300 x g for 15 seconds.

2.13.12.5.1 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 was 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). Extracted RNA was stored at -80°C until cDNA synthesis.

2.13.12.5.2 cDNA synthesis

cDNA was prepared from 1 µg RNA using the iScript™ cDNA synthesis Kit (BioRad, Hercules, CA). The reaction mix was made using 4µl 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. cDNA synthesis conditions were performed using Thermal Cycler (Techne, TC-4000, UK) using the following conditions:25°C for 5 min, 46°C for 20 min and 95°C for 1 min. The cDNA samples were stored at -20^{o}C until use for qrtPCR.

2.13.12.5.3 Quantitative real-time PCR (qrtPCR)

The mRNA gene expression levels of three cytokines, IFNγ, IL-10 and IL-6, were determined. The primers and house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were chosen as previously described from papers and double checked for target identity using GenBank in the National Centre for Biotechnology Information (NCBI) which are all are listed in table 2:3. The expression level of GAPDH was used for data normalisation. Real-time PCR was performed in microplates. Each sample was subjected to real-time PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. The Ct values of genes of interest were normalised to an average Ct value of the GAPDH (ΔCt) and the gene fold expression of each cytokine was calculated as 2–ΔCt. These expression levels were then used for comparative data analysis.

Table 2:3 Target genes and primers sequences used in this study

RNA TARGET	Primer Sequence	Reference
GAPDH	F-GCCGTCCTCTCTGGCAAAG R- TGTAAACCATGTAGTTCAGATCGATGA	(Mott, et al. 2008)
INF-G	F 5'-GCCCTCCTCCTGGTTTCAG-3', R 5'-TGGCACCGCAGCTCATT -3	(Rothwell, et al. 2004, Kristeen-Teo, et al. 2017)
IL-6	F 5'-CAGGACGAGATGTGCAAGAA-3' R 5'-TAGCACAGAGACTCGACGTT-3'	(Waititu, et al. 2014)
Il-10	F CGGGAGCTGAGGGTGAA R GTGAAGAAGCGGTGACAGC	(G. Li, et al. 2010, Lourenço, et al. 2016)

2.13.12.5.4 Real-time quantitative RT-PCR

The full protocol for Real-Time Quantitative PCR reactions were carried out in duplicate using IQ TM SYBR Green Superscript (BioRad, Hercules, CA) on a BioRad instrument CFX384 (Bio-Rad, USA). Amplification was carried out in a total volume of 20 µl in an Icycler IQ™ PCR 96 well plate (BioRad, Hercules, CA). Reaction mixture was composed of 10 µl IQ SYBR Green supermix (BioRad, Hercules, CA), 2 µl of a primer sets, 4 µl of cDNA mixed with 4 µl H_2O. RT-PCR conditions were 38 cycles 95°C for 3 min, 95°C 15 sec of, 60° C 30 seconds and melting point analysis at 55° C,. PCR products were subsequently stored at -20° C. The MyiQ Single-Color Real Time PCR Detection software was utilized for data analysis instrument CFX384 (bio-rad, USA).

2.13.12.5.5 Analysis of qPCR results by $2-\Delta\Delta Ct$ method

The calculations of $2-\Delta\Delta Ct$ were performed as (Livak and Schmittgen 2001) which included calculating the arithmetic mean of Ct Values then normalizing the values using housekeeping gene to target gene. The values were normalized using control birds $\Delta\Delta Ct$ = ΔCt (Experimental animal) - ΔCt (Control animal). Relative quantity of the target gene calculate of following the equation:

Mean relative fold change = $2-\Delta\Delta Ct$ (where relative fold change is the relative quantity of mRNA transcripts in experimental to that of control).

2.13.13 Gut microbiota analysis

2.13.13.1.1 DNA extraction:

In this step DNA was extracted using a QIAamp fast stool mini kit (QIAGEN, Manchester, UK) and the manufacturer's instruction method was used for DNA extraction with some modification. 200 mg of sample was weighed into in a sterilized 2ml eppendorf tube, then 500µl of fresh lysozyme solution was added, then the samples were incubated at 37°C for 30 minutes with vortex for 15 seconds then the centrifuged for 5 min at 15000 rpm to lyse the cells. Then, to remove the inhibitors from samples, one ml of Inhibit EX Buffer was added and the mixture vortexed continuously until the stool sample was thoroughly homogenized. The mixture was then centrifuged for 1 min at 13000 rpm. In the meantime to remove the protein 15µl of proteinase K was pipetted into a new 2 ml Eppendorf tube. Then, 200µl of supernatant was pipetted from the samples that had been cleaned from inhibitors in previous step into the 2 ml Eppendorf tube containing proteinase K. Then, 200µl of Buffer AL was added and mixed for 15 seconds, then this mixture was incubated at 70°C for 10 min. After incubation 200 µl of ethanol (96–100%) was added to the lysate, and mixed by vortexing for precipitation. Afterward 600µl lysate from the final step was carefully applied to the QIAamp spin column. This was then centrifuged at 13000 rpm for 1 min. Then, the QIAamp spin column was placed in a new 2 ml collection tube, and the filtrate with tube was discarded. The rest of lysate was loaded again to apply all of the lysate on the column. The DNA was then cleaned-up by adding 500 µl of Buffer AW1 into QIAamp spin column. Then, the mixture was centrifuged at 13000 rpm for 1 min. Then, the QIAamp spin column was placed in a new 2 ml collection tube, and the collection tube containing the filtrate was discarded. Carefully, the QIAamp spin column was opened and 500 µl Buffer AW2 was added and centrifuged at 13000 rpm for 3 min. The QIAamp spin column was transferred into a new, labelled 1.5 ml Eppendorf tube and 100 µl nuclease free water was pipetted directly onto the QIAamp membrane. This was incubated for 3 min at room temperature, then centrifuged at 12000 rpm for 1 min to elute the DNA. Finally, the extracted DNA was stored on ice until the concentration of DNA and purity were determined using a Nanodrop-1000 Spectrophotometer.

2.14 Statistical analysis of data

All data was analysed using the SPSS software version 21 or 24 for Windows (IBM Statistics, 2013). After KS testing to test normally distributed, statistical analysis was carried out using either Kristal Wallis independent sample analysis, or one way ANOVA to investigate the effect of dietary treatment on FCR, feed intake and body weight gain (per bird). Treatment means were separated using a Tukey post hoc test and significant level was tested at $P \leq 0.05$.

In the pilot study that conducted to assess the natural occurrence of *Campylobacter* in the local area. Four sites were screened for the levels of *Campylobacter*: the NTU research poultry unit across eight trials and a free range adult layer paddock at Nottingham Trent University and broiler and adult layer free range birds at a commercial farm. The results indicated that there were no *Campylobacter* detected in the NTU poultry research unit during all trials. Also there was no effects of type of feed or broiler chickens age on the prevalence of *Campylobacter*. The commercial farm was negative for *Campylobacter* presence before thinning but it became positive after thinning,

Chapter three:

Evaluation of a new *Lactobacillus* strain as a probiotic agent

3.1 Introduction

One approach to post slaughter reduction of *Campylobacter* contamination is the use of probiotic bacteria to increase the ability of the microbiota of the bird to maintain health of the GIT (Santini, et al. 2010). (Ghareeb, et al. 2012) demonstrated that providing chicks with a probiotic supplement via the drinking water decreased the count of *Campylobacter* in broiler chickens. However, there are several possible ways of supplying probiotics to broiler chickens such as, mist spraying, via feed, oral gavage, application to the vent lip and via drinking water. (Eckert, et al. 2010) stated that administering *Lactobacillus*-based probiotics either by drinking water or feed improved the body weight significantly compared with control treatment, from 15 days of age until 48 days. These authors also reported that *Lactobacillus* via drinking water can improve the body weight and feed conversion of broiler chickens within commercial environments. Institute of Food Research (IFR) microbiologists have isolated and patented a novel strain of *Lactobacillus johnsonii:* FI9785 from broiler chickens. The FI9785 strain was originally found to act as a competitive exclusion agent to control *Clostridium perfringens* in poultry, but more recently the strain was also shown to be able to compete with *Campylobacter in vitro* (Dertli, Mayer and Narbad 2015). This finding prompted the evaluation of FI9785 in live birds, which allowed investigations into whether this bacteria enhances the immune function and improves the GIT health of broiler chickens. Before examining this bacteria in poultry to see whether it has probiotic functionality, the FI9785 strain needed checking for stability outside the bird in possible cryprotect mediums. Hypothesis of the study is that *Lactobacillus johnsonii* are to survive in water and feed and its can keep the viability in the gut of chicks.

The aim of this work was to evaluate the efficacy of a novel strain of *Lactobacillus johnsonii* (LB) isolated and patented by the Institute of Food Research as a probiotic This overarching aim was met via two studies that were designed to determine the efficacy of *Lactobacillus johnsonii* as a probiotic. The first study investigated whether the environmental conditions associated with each proposed delivery were detrimental to survivability. The next study examined the most appropriate method of colonising the intestinal tract of the birds.

3.2 Delivering the LB into chicks gut

The first aim of this investigation was to establish a method for delivery of the novel *Lactobacillus* strain that gives the required consistent colonisation of this strain within target regions of the GIT (primarily caeca and colon). Options evaluated included either through drinking water, mixed into feed or mist spraying the chicks immediately post hatch. The second aim was to verify the impact of probiotic on bird performance and examine probiotic efficacy as a gut health enhancer.

3.2.1 Preparation of probiotic

These works were done by Institute of Food Research (IFR) *Lactobacillus johnsonii* FI9785 cells were proliferated by the on de Man, Rogosa and Sharpe (MRS) broth using a controlled fermenter with a nitrogen gas supply. The harvested cells were collected, centrifuged and freeze dried before mixing with skimmed milk as cryoprotect. Cells were stored at 4^{o}C until use. Batches from different fermentation runs were mixed thoroughly before use in the bird studies.

3.2.1.1 Survivability of *L. Johnsonii* FI9785 in water and feed

L. Johnsonii 9785 had been proliferated as described previously, a pilot study assessing its survivability in two potential delivery mediums (feed and water) was assessed.

Survival of FI9785 in different types of water at room temperature was examined at IFR by taking one gram of dried cells and adding to a set volume of different types of water: fresh hot tap water, fresh cold tap water, water left standing overnight and distilled water. Samples were taken after five different periods of time: 0, 2, 4, 6 and 24 hours before culturing on MRS selective media and microaerobic incubation for 48 hours at 37°C. Colony forming units (CFU) of *L. Johnsonii FI*9785 were numerated according to the morphology of this strain in the suitable dilution. All microbiological analyses were performed in duplicate and the average values were used for statistical analysis.

To test the survival of *L. Johnsonii* FI9785 when stored in broiler chickens feed at room temperature, the procedure below was followed: 1g of dried cells of *L. Johnsonii* was added to 9 gram of diets in different tubes and stored for 10 different periods time 0, 6,

24, 48, 120, 144, 168, 192, 216 and 240 hours. A set volume of PBS was then added to each feed sample before culturing an enumeration as described for water.

3.2.2 Ethics and welfare

As described in chapter 2 (section 2.5).

3.2.3 Bird trial room setup

For each bird trial, treatments were placed around the room in an attempt to avoid cross contamination between treatment pens. Pen distribution in the NTU poultry research unit is shown in figure 4:1. Different nipple drinker lines were also used for each treatment.

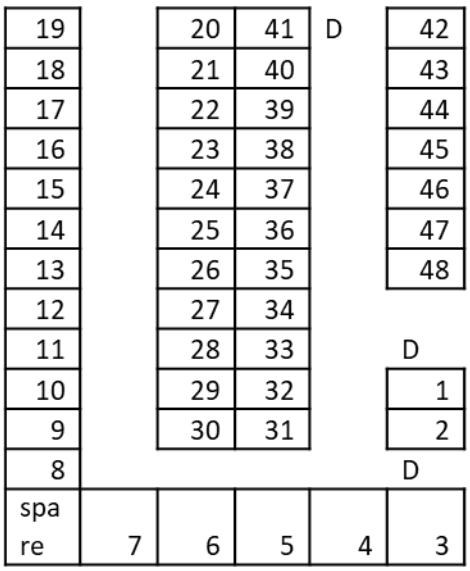

Figure 3:0:1 Pen layout of NTU poultry research unit with entry doors marked 'D'

For both bird trials, the control group were situated under the ventilation inlet so as to reduce the risk of transport of LB between pens via air flow from the treatment pens into the control pens. Diet allocation for bird trial LB01 are shown in figure 4.2

pen	Treatment	Pen	treatment
1	Control	25	
2	Control	26	
3	Control	27	
4	Control	28	
5	Control	29	
6	Control	30	
7		31	Control
8		32	Control
9	LB	33	Control
10	LB	34	Control
11	LB	35	
12	LB	36	
13	LB	37	
14	LB	38	
15	LB	39	
16	LB	40	
17	LB	41	
18	LB	42	Control
19	LB	43	Control
20	LB	44	Control
21	LB	45	Control
22	LB	46	Control
23	LB	47	Control
24	LB	48	Control

Figure 3:0:2 Diet allocation for bird trial LB01

3.2.4 Diet presentation and formulation

Starter diets were made at NTU using previously prepared commercial style mash diets. Diets were mixed to ensure homogeneity and a portion was removed to be mixed with freeze dried *Lactobacillus Johnsonii*, which was premixed with milk powder as a cry-protect. The LB treatment was mixed in a plastic bag to avoid contamination and once mixed the individual pen feed bags were weighed from this one mixed bag. Diets were analysed for nutritional composition as described in chapter 2 section 2.10).

3.2.5 Trial design for LB01

80 male Ross 308 birds were divided at day 1 post hatch into four groups (treatments) with 5 birds per pen and four replicate pens per treatment. The study was conducted for 10 days with treatments as below:

A) Control (birds fed basal feed free of LB with standard rearing)

B) Spray (birds sprayed at the hatchery with a suspension of LB)

C) Feed (birds fed LB supplied as a feed additive until day 7 then fed basal diet)

D) Water (birds supplied with LB via drinking water until day 5).

All pens were given water which had been stood overnight to remove chlorine via bell drinkers for the first 5 days of the study.

3.2.5.1 Feed preparation and delivery

A basal diet was mixed from previously manufactured trial diets formulated to meet nutritional requirements for the age and strain of the birds. The diet was weighed into individual bags for each pen for all diets apart from the feed treatment which was manufactured as described in section 3.2.4.

3.2.6 Treatments

A - Control treatment: No lactobacillus was added to this diet

B - Feed treatment: 100g of freeze dried LB cells were weighed and mixed into 2kg of basal diet in a plastic bag before 500g weighed out into individual bags, one for each replicate pen on this treatment.

C - Water treatment: batches of the water treatment was made up on a daily basis, using water de-chlorinated as previously described for the first 5 days of the study. Freeze dried LB (10g) was added per 300ml water and mixed before dispensing into the appropriate bell drinkers.

D - Spray treatment: birds were sprayed at the hatchery in their cardboard container and transported in a separate vehicle to the other birds to avoid cross contamination. The spray treatment was made up in de-chlorinated water using 40g of LB in 500ml. This was dispensed from a spray bottle until all birds were all visibly wet and the bottle was then reweighed to allow the dosage to be calculated. Each bird received 1.7ml of LB treatment. Care was taken to maintain the temperature in the transport vehicle to avoid chilling the chicks.

3.2.6.1 Collecting and preparation of bird trial LB01 samples

Body weight, feed intake and FCR were recorded on day 5 and 10 on a per pen basis as described in chapter2. Eight birds from each treatment were euthanized at day 5 and 10 post hatch. Crop, jejunum, ileum, caeca and colon digesta were collected from each bird and place in a zipped bags. All tissues collected were placed in a polystyrene box with ice and transferred to IFR to be cultured on the same day by me with help from the IFR

team. At IFR the fresh tissues were aseptically opened in sterile petri dishes and one gram of tissue with tissue content were diluted 10-fold by weight in PBS and mechanically homogenized using a homogenizer. The samples were then serially diluted in PBS and appropriate dilution was added to the MRS agar before following the culture process previously described for enumeration of *lactobacilli*.

3.2.7 Statistical analysis

All data analysis was carried out using SPSS v 22. After KS testing to confirm normality, treatments effects were compared to control using one way ANOVA with a tukey post hoc test for all parameters measured. Significant difference between means was declared at ($P \leq 0.05$).

3.3 Results

3.3.1 Measurements parameters of trial LB01

Figure 3:3 shows survival of *L. Johnsonii* FI9785 in different types of water over time at room temperature. The results for tap water left overnight are similar to distilled water or better. This indicates tap water left standing overnight is the best water-based delivery medium but fresh hot or cold tap water seem to have no great detrimental effect on the viability of *L. Johnsonii* FI9785.

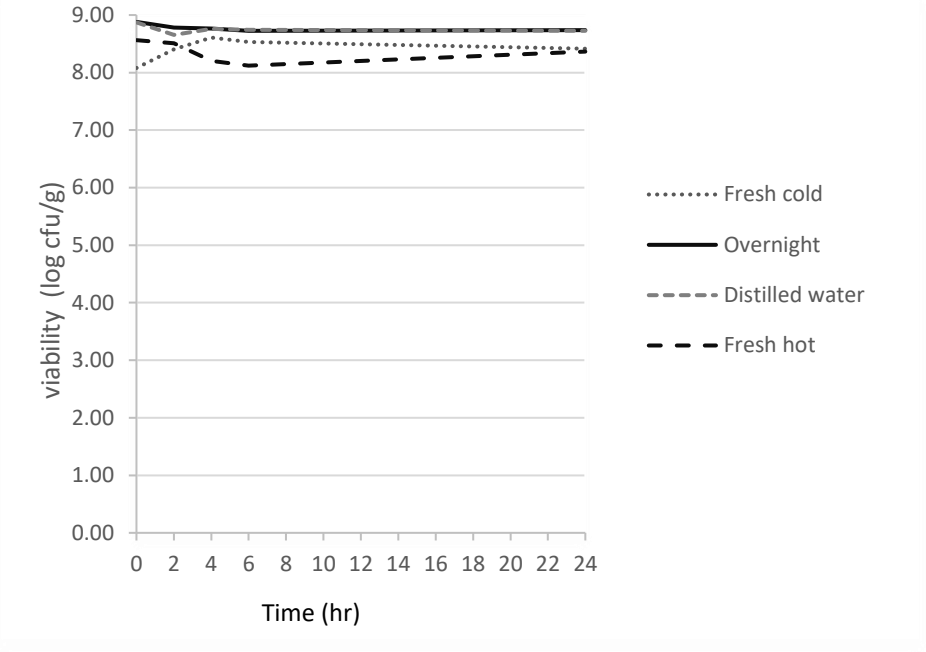

Figure 3:3 Viability of *L. Johnsonii* FI9785 in different types of water at room temperature over 24 hours period.

[Data presented a logarithms of Colony forming unit of LB (*L. Johnsonii* FI9785)per gram of water (log cfu/g)]

Figure 3:4 shows that for feed, the count of LB remained in the log 8 order of magnitude which indicates that there was no major degradation in the CFU of *L. Johnsonii* when stored up to 240 hours (10 days) at room temperature in broiler chickens feed.

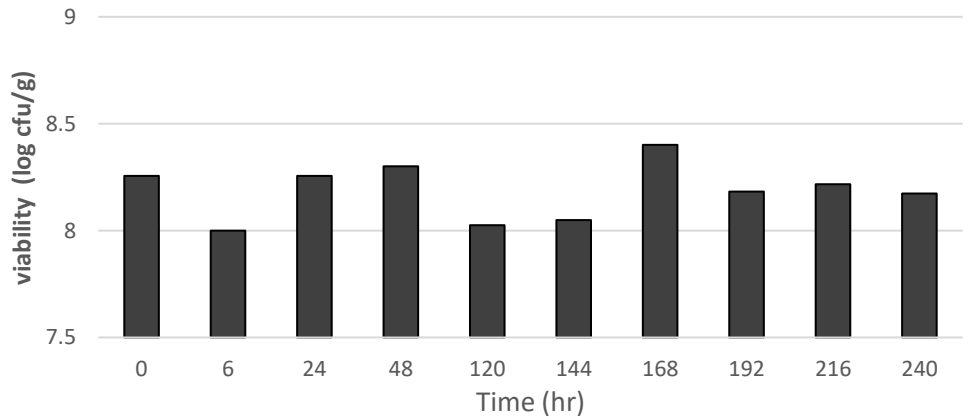

Figure 3:4 Viability of *L. Johnsonii* FI9785 in the broiler chickens diet at room temperature (25°C).

3.3.2 Enumeration of *L. johnsonii* in the intestinal bacteria of broiler chickens

Figure 4.5 shows that there were differences between treatments in *L. johnsonii* colonisation of different parts of broiler chickens GIT at day 5.

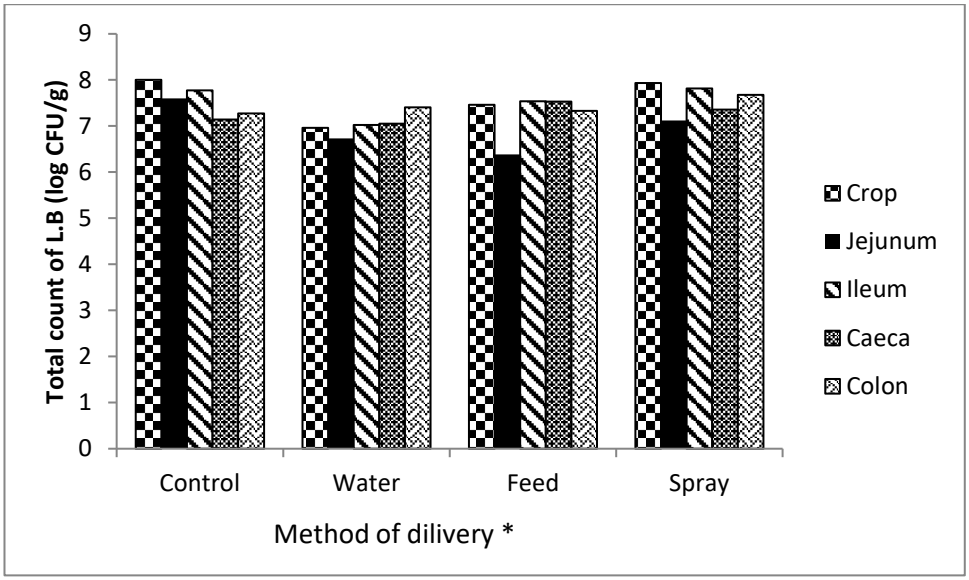

Figure 3:5 Day 5 *L. Johnsonii* colonisation of different broiler chickens tissues.

* control (feed –LAB), water, feed and spray (+LAB)

Figure 4.6 shows the colonisation of *L. Johnsonii* of different broiler chickens tissues at day 10 and indicates that the there was no different in the level of *L. Johnsonii* colonisation of the caeca (There are no error bars as not provided by IFR).

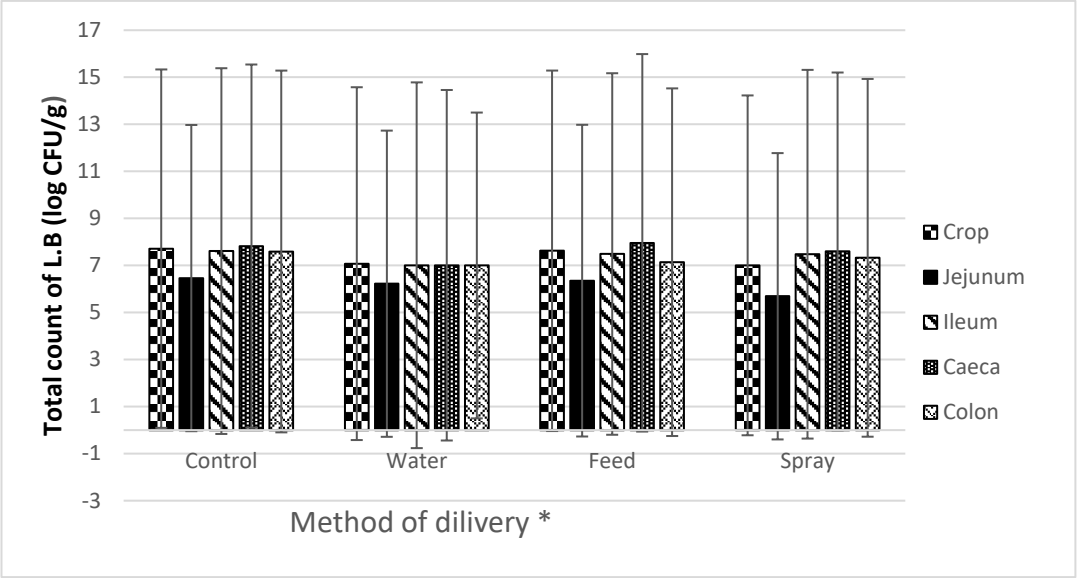

Figure 3:6 LB01Day 10 *L. johnsonii* colonisation of different broiler chickens tissues with S.E.

* control (feed –LAB), water, feed and spray (+LAB)

Tables 4:1 show the effects of LB delivery method on weekly body weight body weight gain, feed intake and FCR during 1-5 and 5-10 days of age, and cumulatively. Table 4:1 illustrates that there were significant differences between the effects of the four treatments on the body weight and body weight gain at day 5 of age, with supply via water producing a substantial increase in body weight gain compared to any other treatment. Also this table shows no difference between treatments in weight gain but significant overall effects of LB on feed intake by day 10 as the birds in the treatment groups consumed higher than control group. However, from table 4.1 the improved performance associated with water supply of probiotic was transitory and the cumulative treatment effect was lost by day 10, and similarly, the altered feed intake was also not apparent when considered cumulatively.

Table 3:1 Effect of *Lactobacillus* delivery method on bird performance from day 5 to day cumulative at 10 LB01

Performance	Treatments				*P value*
	Control	Spray	Water	Feed	
Body weight (g) day 1	49±1.46	47±2.13	47±1.87	48±2.10	0.78
Body weight(g) day 5	89±1.52^{a}	87±2.64^{a}	98±1.91^{b}	89±3.17^{a}	0.02
Body weight gain(g) day 1-5	39±0.27^{a}	39±1.79^{a}	51±0.87^{c}	41±1.74^{ab}	<0.01
Feed intake(g) day 1-5	61±13.38	54±4.47	60±4.79	58±5.25	0.92
FCR* day 1-5	1.21±0.03	1.37±0.13	1.15±0.08	1.40±0.09	0.20
Body weight(g) day 10	187 ±8.02	183 ±7.52	207 ±9.73	183 ±13.03	0.29
Body weight gain(g) day 5-10	97 ±7.94	96 ±5.40	107 ±8.50	93 ±9.73	0.96
Feed intake(g) d 5-10	158 ±8.78^{a}	192 ±5.27^{ab}	190 ±16.34^{ab}	243 ±2.73^{b}	0.01
FCR 5-10	1.64 ±0.10	1.89 ±0.0	1.8 ±0.20	2.18 ±0.14	0.28
Body weight gain(g) day 0-10	137±8.82	135±7.38	160±11.19	135±12.28	0.27
F I(g)D0-10	269±28.13	282±14.76	290±19.83	339±6.62	0.11
FCR 0-10	2.01±0.32	1.979±0.11	1.83±0.16	2.39±0.16	0.41

(Differing superscript letters within one column denote means are significantly different at $p < 0.05$ level). *FCR: feed conversion ratio

3.4 Discussion

Figure 4:3 shows survival of *L. Johnsonii* FI9785 in different types of water over time at room temperature. The results for tap water left overnight are similar to distilled water. This is likely due to evaporative loss of chlorine. Although fresh hot or cold tap water seem has no great effects on the viability of *L. Johnsonii* FI9785. However, tap water left standing overnight was chosen for practical reasons as the best way of efficiently delivering the *L. Johnsonii* bacteria to the birds via their drinking water. For feed viability, results showed that there was no major degradation in the CFU of L. *Johnsonii* when stored up to 240 hours (10 days) at room temperature in broiler chickens feed. This indicates that this strain is able to survive when mixed and stored in broiler chickens diets with no detrimental effects.

Results of bird trial LB01 use of different delivery routes resulted in apparently no significant differences between treatments in colonisation of different parts of broiler chickens GIT compared to control at day 5. However, as the data provided by IFR was without S.E, this did not allow rigorous investigation into the differences among treatments. The level of *L. Johnsonii* colonisation of different broiler chickens tissues at day 10 of bird trial LB01 indicates that there was no different among all methods of delivering feed was the optimum method for delivering probiotic bacteria.; Therefore, food will be chosen as a delivery method for this strain of *L. Johnsonii* as it the easiest way in the broiler chickens farm. Interestingly, there was substantial colonisation by *L. Johnsonii* of the early GIT regions of the in control birds, without obvious cause. It is unclear what happened, but there are two possible hypotheses; firstly there may be contamination of these birds by the FI9785 strain. The rationale behind this theory is the high level of this bacteria found in the crop and, because the crop is at the beginning of the broiler chickens GIT, which mean that the high level of LB present from the first part of gut this is suggest that contamination via the feed. Another hypothesis is that this strain is not IFR *FI*9785 strain but a wild *Lactobacillus Johnsonii* strain which cannot be visually discriminated from *FI*9785. The rationale behind this theory is that, because of the level of LB was higher in the control (which was not given the probiotic) and in the spray delivery method (which was given only one dose) than in the water and feed treatments. Moreover, in the crop of control and spray method, which is the section of

GIT in the beginning of feed journey, the level of LB was high, which means these birds were still receiving LB at the time of sampling. In contrast, in the birds of the feed and water treatments, which were delivering LB at the time of sampling, the level of LB was low.

However it was difficult to consider either of these hypothesises without identifying and confirming by PCR the precise strain of *L. Johnsonii* found in the control fed birds. As this line of work was discontinued by IFR and no primers for *L. Johnsonii* FI9785 were available, it as was not determined whether the control-fed birds in trial LB01 were contaminated with the IFR strain or simply colonised by other wild type *L. Johnsonii* strains.

Bird trial LB01 investigations into the effects of LB delivery method on weekly body weight, body weight gain, feed intake and FCR during 1-5 and 5-10 days of age, and cumulatively indicated that there was no significant effects of adding LB to feed until day 10 of age. The lack of bird performance differences between the various LB delivery routes mean that the decision over optimum delivery route could be based on intestinal colonisation levels alone.

3.5 Conclusion

In conclusion, while both feed and water present viable routes for delivering probiotic supplements, delivery via feed avoids issues associated with water sanitisation and also provides the highest level of intestinal colonisation. The efficacy of the novel LB strain *L. Johnsonii* FI9785 indicate it promotes a protective immune response but does not appear to improve bird growth performance in a low pathogen environment. A major challenge for this work was the apparent contamination of the control samples with *L. johnsonii*. The differences that found between treatments do not support contamination, but as the strain could not be identified without appropriate primers for PCR, this cannot be verified.

Chapter Four:

Evaluation of a new *Lactobacillus* strain as a probiotic feed additive for poultry

4:1 Introduction

The gastrointestinal tract (GIT) is a very important organ for maintaining health, as the majority of pathogens affecting poultry enter through the GIT with feed or water, and absorption of nutrients is also controlled by the health status of the GIT (Celi, et al. 2017). The mucosal layer of the GIT covers the epithelial surface and acts as an interface between the external and internal environment. Its function is as a medium for protection, lubrication and transport between the epithelial cells and lumen (Perry 2006). The predominant component of mucin is mucin-type glycoprotein, which is produced by the mucus- secreting goblet cell (Smirnov, et al. 2004). *Campylobacter* colonised broiler chickens can persist with a high rate of colonisation until the slaughter stage and beyond (Svetoch and Stern 2010). Any residual *Campylobacter* associated with the carcass post-slaughter presents a risk to human health (Public Health England, 2016).

One approach to post slaughter reduction of *Campylobacter* contamination is the use of probiotic bacteria to increase the ability of the microbiota of the bird to maintain health of the GIT via the mechanisms described earlier (Santini, et al. 2010). (Ghareeb, et al. 2012) demonstrated that providing chicks with a probiotic supplement via the drinking water decreased the count of *Campylobacter* in broiler chickens. However, there are several possible ways of supplying probiotics to broiler chickens such as, mist spraying, via feed, oral gavage, application to the vent lip and via drinking water. (Eckert, et al. 2010) have stated that administering *Lactobacillus*-based probiotics either by drinking water or feed improved the body weight significantly compared with control treatment, from 15 days of age until 48 days. (Eckert, et al. 2010) also reported that *Lactobacillus* via drinking water can improve the body weight and feed conversion of broiler chickens within commercial environments. Institute of Food Research (IFR) microbiologists isolated and patented a novel strain of *Lactobacillus johnsonii:* FI9785 from broiler chickens. The FI9785 strain was originally found to act as a competitive exclusion agent to control *Clostridium perfringens* in poultry, but more recently the strain was also shown to be able to compete with *Campylobacter in vitro* (Dertli, et al. 2015). This finding prompted the IFR team to approach NTU in order to test FI9785 in live birds, which allowed investigations into whether this bacteria enhances the immune function and improves the GIT health of broiler chickens. Before examining this bacteria in

poultry to see whether it has probiotic functionality, the FI9785 strain needed checking for stability outside the bird in possible cryprotect mediums. The hypothesis of this study are to minimise the *Campylobacter* in the broiler gut and to improve the performance.

The aim of this work was to evaluate the efficacy of a novel strain of *Lactobacillus johnsonii* (LB) isolated and patented by the Institute of Food Research as a probiotic in meat broiler chickens. This overarching aim was met via three studies that were designed to determine the efficacy of feeding *Lactobacillus johnsonii* as a probiotic to broiler chicks. A subsequent bird trial utilised the most efficacious method to deliver LB and monitored the level of *Campylobacter* colonisation in birds fed LB for 7 days compared with a control group without probiotic over a 28 day trial period.

4.1 Delivering the LB into chicks gut

The first aim of this investigation was to establish a method for delivery of the novel *Lactobacillus* strain that gives the required consistent colonisation of this strain within target regions of the GIT (primarily caeca and colon). Options evaluated included either through drinking water, mixed into feed or mist spraying the chicks immediately post hatch. The second aim was to verify the impact of probiotic on bird performance and examine probiotic efficacy as a gut health enhancer.

4.1.1 Ethics and welfare

For each bird trial husbandry and ethical clearance procedures were carried out as described in chapter 2 (section 2.5).

4.1.2 Bird trial room setup

For each bird trial, treatments were placed around the room in an attempt to avoid cross contamination between treatment pens. Pen distribution in the NTU poultry research unit is shown in figure 4:1. Different nipple drinker lines were also used for each treatment.

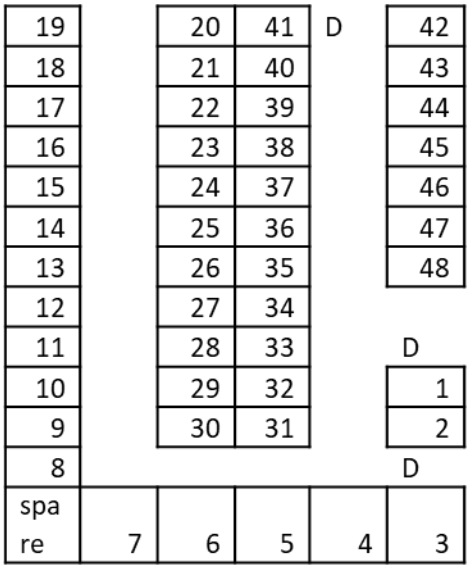

Figure 4:4:1 Pen layout of NTU poultry research unit with entry doors marked 'D'

For both bird trials, the control group were situated under the ventilation inlet so as to reduce the risk of transport of LB between pens via air flow from the treatment pens into the control pens. Diet allocation for bird trial LB02 are shown in figure 4.2.

pen	Treatment	Pen	treatment
1	Water	25	
2	Water	26	
3	Water	27	
4	Water	28	
5		29	
6		30	
7		31	
8	Feed	32	
9	Feed	33	
10	Feed	34	
11	Feed	35	
12		36	
13		37	
14		38	
15		39	
16		40	
17		41	
18		42	Spray
19		43	Spray
20	Control	44	Spray
21	Control	45	Spray
22	Control	46	
23	Control	47	
24		48	

Figure 4:4:2 Diet allocation for bird trial LB02

4.1.3 Diet presentation and formulation

Starter diets were made at NTU using previously prepared commercial style mash diets. Diets were mixed to ensure homogeneity and a portion was removed to be mixed with freeze dried *Lactobacillus Johnsonii*, which was premixed with milk powder as a cry-protect. The LB treatment was mixed in a plastic bag to avoid contamination and once mixed the individual pen feed bags were weighed from this one mixed bag. Diets were analysed for nutritional composition as described in chapter 2 section 2.10).

4.1.4 Trial design for LB02

144 male Ross 308 birds were divided at day 1 post hatch into two groups (treatments) with birds were fed either a control or test diet containing used the feed delivery of LB and compared this head to head with a control diet to monitor effects on gut colonisation of both LB and *Campylobacter* and to monitor bird performance up to d28. One replicate was a pen containing 4 or 5 birds on d1 (total 72 birds per treatment group). The sides of the control pens were covered at day 3 with plastic sheeting to minimise cross-contamination between treatments. In the pens with 5 birds (odd numbered pens), one bird was culled on D5 to assess the colonization of LB.

4.1.5 Diet presentation and formulation

Treatments were Control (birds fed basal diet throughout) and test (birds fed basal diet containing *lactobacillus* at 50g per kg feed for first 7 days). Diets were fed to the birds as a starter feed for days 1-20 and grower feed for days 21-28 with feed and water available *ad libitum* throughout the trial.

A basal diet was mixed from previously manufactured trial diets formulated to meet the age and strain of the birds using a 100kg ribbon mixer for a minimum of 5 minutes. A grab sample was taken during the feed weighing prior to the trial for analysis. Diet was prepared fresh on d4 to allow treatment to continue until d7. From d21, all birds were fed a commercial grower pellet until d29. Growth performance assessments for bird trial LB02

Mean bird weight, feed intake and FCR per pen were determined on d1, d7, d14, and d28 as described in chapter 2 (section2.5). At the end of the performance measurement period (28 days) the European Production Efficiency Factor (EPEF) was calculated, based on the age of broilers at euthanasia (days), the average live body weight (kg / head),

viability (%) and feed conversion ratio: Liveability (%) x live body weight at end trial (kg) x 100 x age of end trial (days) x feed conversion ratio.

4.1.6 Collecting and preparation of bird trial LB02 samples for gut microflora analysis

At day 14 post hatch, nine chicks per treatment were euthanized and gut microflora samples collected and assessed as described below. Remaining birds were euthanized at the end of the trial (day 29). Culturing for trial LB02 was conducted across two sites: the IFR microbiology laboratories in Norwich, and also at the NTU microbiology laboratories at their Clifton campus. For NTU cultures, caeca samples were collected post mortem by placing excised caeca in bags and transporting to the culture labs on ice, where caeca were opened and samples collected using a charcoal swab. Additionally, fresh excreta from the pens were swabbed or collected in swab tubes before transporting on ice to the culture labs. Immediately upon arrival at the microbiology laboratories, 1g of gut contents from the ileum and caecum samples from each chick were aseptically removed and used for the assessment of gut microflora population changes using standard microbiology (culture techniques) as described in Section 2.13.12.2. Preparation of samples for culturing involved differing serial dilutions for caecal and excreta sampling respectively, before adding 20 µl from the appropriate dilution to petri dishes of selective media and incubation under microaerobic conditions for 48 hours at 42^{o}C. Colonies of *Campylobacter* were enumerated in individual plates, then, the mean calculated to give a final count value for each bird sample.

Additionally, on day 29, *L. Johnsonii* culturing was performed at IFR. Upon arrival at IFR, tissues were aseptically opened in sterile petri dishes and one gram of tissue (including digesta content) was diluted 10x w/v in PBS and mechanically homogenized. The samples were then serially diluted in PBS for enumeration of lactobacilli. An appropriate dilutions were cultured on MRS and under microaerobic conditions at 37^{o}C for 48 hours.

4.1.7 Collecting and preparation of bird trial LB02 samples for immune parameters

Immediately post mortem, blood samples were collected and pooled from 2 birds per pen into EDTA tubes and multiple aliquots of plasma stored at -20^{o}C for IgA and IgM and uric acid concentrations. Finally on the day of sampling, mucin layer thickness in jejunal

tissue was assessed by excision of a 1 cm section of jejunum with mucin content measured as described in chapter 2 section 2.11. Determination of IgA and IgM concentration in the plasma was subsequently determined using ELISA kits (Bethyl Laboratories) as described at chapter 2 section 2.12. Plasma uric acid concentration was also determined from another plasma aliquot via Amplex Red uric acid kits (Invitrogen) as described at chapter 2 section 2.12.

4.1.8 Statistical analysis for both Bird trial LB02

All data analysis was carried out using SPSS v 22. After KS testing to confirm normality, treatments effects were compared to control using one way ANOVA with a tukey post hoc test for all parameters measured. Significant difference between means was declared at ($P \leq 0.05$).

4.2 Results

4.2.1 Measurements parameters of trial LB02

From figure 4:7 it can be seen that there was no significant difference between the two groups in the CFU of *Campylobacter,* although there was a numerical reduction in *Campylobacter* colonisation of birds fed the probiotic.

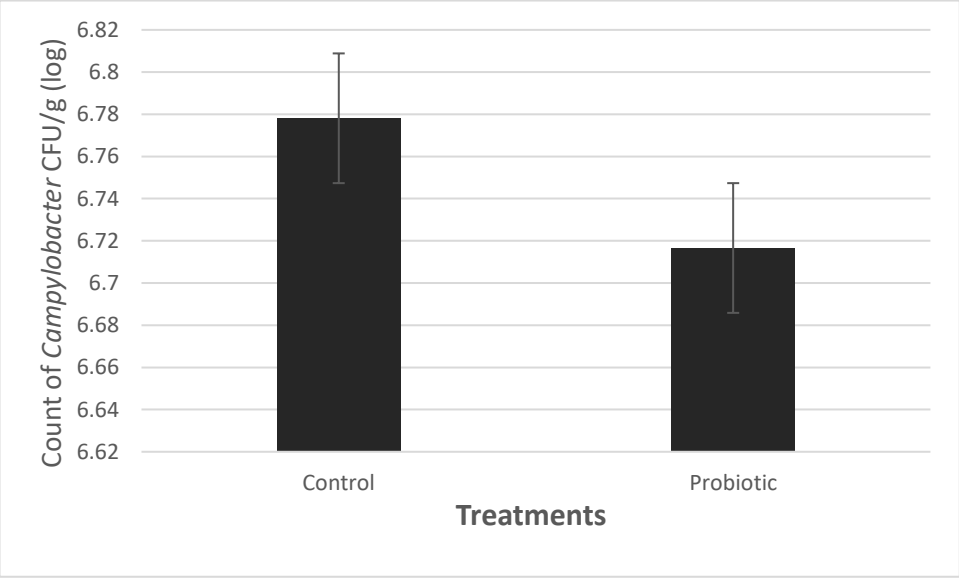

Figure 4:3 Mean values with SE of caecal colonisation of *Campylobacter* in caeca of control and *L. Johnsonii* probiotic-fed birds at day 28 (LB02).

Tables 4:1 also show the probiotic had no significant effects on the productive performance of birds in this trial up to three weeks of age.

Table 4:1 Effect of probiotic on week 1, 2, 3, 4 and cumulative bird performance

Performance	Treatment		P value
	Control	Probiotic	
Body weight (g) day 1	35.61±0.69	35.9±0.79	0.674
Body weight (g) day 7	113.1±4.38	114.75±3.62	0.774
Body weight gain (g) 7	77.49±3.81	78.84±3.17	0.787
Feed intake (g) 7	150.23±15.68	121.59±3.89	0.095
FCR 7	1.94±0.17	1.56±0.06	0.059
Body weight (g) day 14	321.09±12.0	329.32±11.3	0.623
Body weight gain (g) 14	207.98±8.23	214.56±8.20	0.575
Feed intake (g) 14	311.73±15.22	305.55±9.98	0.737
FCR 14	1.52±0.09	1.44±0.04	0.435
Body weight (g) day 14	321.09±12.08	329.32±11.30	0.623
Body weight (g) day 21	688.09±28.60	676.4±22.61	0.751
Body weight gain (g) 21	367.09±18.26	347.08±14.12	0.396
Feed intake (g) 21	497.97±22.15	486.73±22.37	0.724
FCR 21	1.36±0.02	1.42±0.07	0.425
Body weight (g) day 21	688.09±28.60	676.4±22.61	0.751
Body weight (g) day 28	1233.39±41.02	1154.95±26.66	0.121
Body weight gain (g) 28	545.29±18.443^{a}	478.55±7.942^{b*}	0.003
Feed intake (g) 28	837.09±20.20	802.29±8.32	0.127
FCR 28	1.551±0.04^{b}	1.681±0.02^{a}	0.019
Cumulative body weight	1233.39± 41.02	1154.95± 26.66	0.121
Cumulative body weight gain	1197.77±40.70	1119.05±26.181	0.116
Cumulative Feed intake	1797.03±41.69	1716.17±27.09	0.116
Cumulative FCR	1.511± 0.02	1.542± 0.03	0.48

(Differing superscript letters within one column denote means are significantly different at $p \leq 0.05$ level). *FCR: feed conversion ratio, ± values indicated SE

Table 4:1 shows that there was no significant effect ($p \leq 0.05$) of probiotic in the diets on the productive performance of the broiler chickens in week 4, apart from body weight

gain and FCR. In week four of age the body weight gain significantly (p≤0.05) decreased in the LB treatment birds. This degradation in body weight began in week 3 but was not significant (p≤0.05). Also table 4:1 illustrates that the probiotic has no significant (p≤0.05) effects on overall productive performance of the birds over 4 weeks.

Uric acid concentration in serum was recorded as a measure of antioxidant status (Cohen, Klasing and Ricklefs 2007). Table 4:9 shows there were no significant effects of probiotic addition on uric acid concentration in the blood plasma of birds, indicating that the probiotic does not appear to exert anti-oxidative effects on birds. Thickness of mucin layer in the jejunum (expressed as µg Alcian blue released per cm tissue) was significantly (*P*<0.05) increased from 32.06µg to 43.85µg. Also, table 4:9 shows concentration of IgA in the plasma blood of probiotic treated birds was increased more than two-fold, but IgM was not significantly altered.

Table 4:2 Effect of probiotic on uric acid, IgA and IgM in serum and jejunal mucin thickness

	Uric acid (µg/dl) ±SE	IgA (µg/ml) ±SE)	IgM (µg/ml) ±SE)	Mucin thickness* (µg) ±S.E.)
Control	10.28 ±0.595	234.7 ±21.65	410.4 ±73.39	32.06 ±1.351
Probiotic	9.47 ±0.527	624.5 ±37.82	358.9 ±66.81	43.85 ±3.262
P Value	0.314	<0.001	0.693	0.002

*Alcian blue released per cm tissue, ± values indicated SE

4.3 Discussion

Results of bird trial LB02 indicated that there was no significant difference between two groups in the CFU of *Campylobacter,* probably because the unit was negative for *Campylobacter* before sampling so *Campylobacter* did not establish well in the broiler chickens GIT, as this bacteria needs time to colonise the gut of birds (Newell 2002). In addition, the closed mesh barriers between the pens do not allow birds to move from pen to pen to spread *Campylobacter* around the unit. It is also possible that insufficient birds were sampled to account for inherent variability between individual birds. For this reason, caecal samples from additional birds already archived from this trial were cultured in an attempt to increase replication to ascertain whether observed numerical differences actually represent significant differences between the two treatment groups. Unfortunately, culturing of previously frozen samples resulted in cfu levels many orders of magnitude lower than fresh culturing, so this line of investigation was redundant.

Performance table 3.1 shows the probiotic had no significant effects on the productive performance of birds in this trial up to three weeks of age. This may be due to the non-commercial rearing conditions of the NTU poultry unit (low stocking density, high standards of husbandry and low pathogen exposure). Also, the NTU feeding system creates a high level of spillage resulting in falsely high feed intakes, particularly during week 1.

Table 3:1 shows that there was no significant effect ($p<0.05$) of probiotic in the diets on the productive performance of the broiler chickens in week 4, apart from body weight gain and FCR. In week four of age the body weight gain significantly ($p<0.05$) decreased in the LB treatment birds. This degradation in body weight began in week 3 but was not significant ($p<0.05$). In addition, this decline in body weight gain also correlated with the body weight at day 28 as they were both decreased during week 4 in the LB treatment, while the FCR has increased significantly ($P<0.05$) in the probiotic treatment. Many other research publications show probiotics do not improve the body weight or affect performance (Lutful Kabir 2009) but it is unusual to find a negative effect of probiotic usage on weight gain. It is possible that the probiotic bacteria has competed with the birds on the food, hence this birds consumed more feed which caused the

increasing FCR or it may be the skimmed milk has negatively affected digestibility of the feed, although there is no reported literature supporting this theory.

Table 3:1 illustrates that the probiotic has no significant ($p<0.05$) effects on overall productive performance of the birds over 4 weeks, which is in keeping with many other reported findings; possibly because our poultry unit invokes relatively benign microflora to colonise the GIT of newly placed chicks, so addition of the probiotic does not lead to substantial alteration.

Results of LB02 showed that there were no significant effects of probiotic addition on uric acid concentration in the blood serum of birds, indicating that the probiotic does not appear to exert anti-oxidative effects on birds. However, there are some indications that the probiotic exerts an immune response in the birds: thickness of mucin layer in the jejunum (expressed as µg Alcian blue released per cm tissue) was significantly ($P<0.05$) increased from 32.06µg to 43.85µg. Also, table 4:9 shows concentration of IgA in the plasma blood of probiotic treated birds was increased more than two-fold, but neither IgG nor IgM were significantly altered. A similar Ig response to probiotic supplementation of meat chicken was also reported by (Haghighi, et al. 2006), who found that oral gavage of chicks at day 1 post hatch with three strains of probiotic (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis)* resulted in increased IgG and IgA in all probiotic-fed birds.

The increased mucin layer thickness suggests this strain of *L. Johnsonii* has ability to stimulate the goblet cells to increase the production of mucin, which may play a role in protecting the epithelium from attachment by pathogenic bacteria. These heightened mucosal immune responses concur with several other studies suggesting probiotics enhance the immunity of broiler chickens. A similar increased mucin layer thickness associated with including a probiotic in chicken feed was found by (Forte, et al. 2018) where it was found that dietary inclusion of *Lactobacillus acidophilus* resulted in increased ileal mucin layer thickness. However the performance data derived from LB02 are not in agreement, as (Forte, et al. 2018) found bird body weight and feed intake of probiotc-fed birds in their study was improved, while in this study the was no significant effect on these parameters.

Other researchers also report mixed growth performance responses to use of probiotic supplements. (De Cesare, et al. 2017) found that dosing chicks with *Lactobacillus acidophilus* D2/CSL (CECT 4529) improved body weight was at day 15 of age. In contrast, (Olnood, et al. 2015) used four strains of *Lactobacillus* (tentatively identified as *Lactobacillus johnsonii, Lactobacillus crispatus*, *Lactobacillus salivarius* and an unidentified *Lactobacillus* sp) in broiler chickens diets and found there were no significant differences on the body weight, feed intake and body weight gain at day 21 of the age of broiler chickens.

4.4 Conclusion

In conclusion, studies into the efficacy of the novel LB strain *L. Johnsonii* FI9785 indicate it promotes a protective immune response but does not appear to improve bird growth performance in a low pathogen environment. A major challenge for this work was the apparent contamination of the control samples with *L. johnsonii*. The differences that found between treatments do not support contamination, but as the strain could not be identified without appropriate primers for PCR, this cannot be verified. The subsequent studies were carried out without any form of collaboration, and focussed on in house development of a potential probiotic supplement and prebiotic support via Jerusalem artichoke as a source of inulin.

Chapter five:

Assessment of Lactic acid-bacteria isolates derived from the intestines of apparently healthy free range poultry as a potential probiotic agent for broiler with Jerusalem artichoke as a potential prebiotic

5.1 Introduction

Probiotic supplement has been defined as a live microbial feed supplement which has a positive effect on the health of an animal through altering the balance of intestinal microflora (Fuller 1989). Lactic Acid Bacteria (LAB) are one of the most important microorganism groups in the intestine of animals as well as humans. Species within the genera of *Lactobacillus* and *Bifidobacterium* are the predominant microorganisms in gastrointestinal microbiota in the human and animal (Guarner and Malagelada 2003). *Lactobacillus* is the most commonly selected genus to be used as a probiotic in humans as well as in animals (Moreira, et al. 2005, McCoy and Gilliland 2007). Lactobacilli are classified as Gram-positive, catalase-negative, non-sporulating, anaerobic fermentative bacteria, which are acid-tolerant and have particular sensitivity to oxygen (Kleerebezem and Hugenholtz 2003). *Lactobacillus* strains can be used as probiotic agents in the early life of chicks to improve the gut microflora, and are commercially available as feed supplements for poultry (O'Dea, et al. 2006). However, there is a substantial cost involved in the purchase of commercially-derived probiotics from multinational companies, which could be avoided if strains of *Lactobacillus* that appear to maintain intestinal health in poultry could be isolated and cultured on a more regional level. Also there are a many studies that have confirmed the activity of probiotic to minimise the *Campylobacter* spp. in the broiler (Santini, et al. 2010) which in turn can produce fewer contaminated broiler chickens products for consumers.

If a LAB strain is to be used as a successful probiotic it must exhibit some important characteristics. First of all the strain should have an antagonistic activity against pathogenic bacteria. In addition a candidate bacteria should be able to survive not only in the gastrointestinal environment (Fontana, et al. 2013) but must also maintain their viability during processing and storage. Requirements during the processing steps include: viability after freezing and drying, and ability to survive in environments containing different levels of oxygen. Key features of survival in the gastro-intestinal tract include; tolerance to strongly acidic pH, contact with bile salts and sodium chloride; and the bacteria should able to survive at 42°C temperature (chicken core body temperature) (Giloh, Shinder and Yahav 2012).

Inulin is a non-digestible plant oligosaccharide that can be used as a prebiotic to selectively stimulate growth of *Lactobacillus* and *Bifidobacterium* while limiting growth of non-beneficial bacteria in the intestine, which leads to improve the host health (Gibson, et al. 2004, Coudray, et al. 2005). Jerusalem artichoke is a plant that is successfully grown widely around the world under different climate environments, which is renowned for storing a relatively high amount of inulin (McLaurin, Somda and Kays 1999).

The aim of this study was to isolate strains of LAB from the excreta of apparently healthy broiler chickens that have been shown to be *Campylobacter* free, despite exposure in a free range management system and to then screen these isolates for the essential attributes of probiotic bacteria as well as to test their inhibitory activity against *Campylobacter* which is a known human pathogen commonly isolated from the chicken intestine. A secondary aim was to source and prepare Jerusalem artichoke plant material as a source of inulin and to assess its potential as a prebiotic. Hypothesis of this works are the isolates of LB from apparently healthy, mid-aged outdoor pet chickens are potential probiotic agent and also JA plant is good source for prebiotic in broiler chicken feed.

5.2 Method

5.2.1 Screening the chicken for *Campylobacter* spp.

Details in section 2.4

5.2.2 Screening and isolation of lactic acid bacteria

As explained in section 2.13.1

5.2.3 Identification of isolates

5.2.3.1 Morphological and biochemical tests on the isolates

Details in section 2.4

5.2.3.1.1 Gram staining

Gram stain was used to determine the Gram status of the isolates from fresh growth as explained in section 2:13:3:2.

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5.2.3.1.2 Catalase test

Catalase test was performed to check if isolates are catalase negative or positive (catalase producer or not) by using 3% hydrogen peroxide solution.

5.2.3.1.3 pH of growth broth

pH of broth was measured by using pH meter (Mettler-Toledo, UK). The pH of the broth after incubation was compared with the initial pH of broth.

5.2.3.2 Genotypic Identification:

5.2.3.2.1 DNA extraction

Details in the section 2:13:3:1:1

5.2.3.2.2 Gene sequencing (16S rDNA)

Details in the section 2:13:3:1:2

5.2.4 Assessment of antibacterial activity against Campylobacter strains.

As explained in section 2:13:5

5.2.5 Detection of hydrogen peroxide production

Details in the section 2:13:5:4

5.2.6 Evaluation the phenotypic characteristics of the isolates as probiotic agent to survive in the intestinal gut environment

5.2.6.1 Viability of isolates at (relatively high temperature)

Details in the section 2:13:4:1

5.2.6.2 Tolerance to oxygen

Details in the section 2:13:4:2

5.2.6.3 Antibiotic resistance

Details in the section 2:13:4:2

5.2.6.4 Tolerance of Bile salts

Details in the section 2:13:5:5:1

5.2.6.5 Tolerance of Sodium Chloride (NaCl)

Details in the section 2:13:5:5:2

5.2.6.6 Tolerance of acid

Details in the section 2:13:5:5:3

5.2.7 Utilization of Artichoke Jerusalem (Inulin) as source of carbon by LAB isolates.

Basal MRS broth medium was used as a standard and compared with media in which the glucose was replaced with either the same amount of artichoke powder or commercial inulin (SENSUS, Netherland). The growth of isolates was determined by measuring the absorbance (optical density ($O.D_{600}$)) using plate reader (CYTATION-imaging readers-Bio-rad, USA) at different time points: zero, 12 and 24 hour.

5.2.8 Preparing the isolates as probiotic (chicken feed supplements)

The cells of bacteria, grown in broth media, were pelleted and washed with PBS then resuspending in PBS, skimmed milk and sucrose which then mixed thoroughly by vortex and freeze drying .

5.2.9 Preparation of product as feed supplement

Dried product was diluted to 10^9 cfu with skimmed milk to be use in broiler chickens feed.

5.2.10 Assessment the viability of bacterial cells during the preparation of broiler chickens feed supplements

Details in section 2:13:8

5.2.11 Preparation of Jerusalem artichoke tubers

Details in section 2:13:9

5.2.12 Determination of the content of inulin in Jerusalem artichoke plant

Details in section 2:13:9:10

5.3 Results

5.3.1 Screening the broiler chickens for *Campylobacter* spp. and *Lactobacillus* spp.

The results confirmed that all collected samples from the broiler chickens at Brackenhurst animal unit were *Campylobacter* free. The morphologic characteristics confirmed that they are probably LAB, therefore 6 isolates that has the characteristic of LAB (white or creamy) from highest dilution were selected for further tests Anupama and Sharma (2017).

5.3.2 Morphological and biochemical tests on the isolates

5.3.2.1 Catalase, Gram stain and acid production

Table 5:1 shows that all LAB isolates were Gram positive, catalase negative and rod-shaped. All selected isolates decreased the pH of the broth media below pH 6 which as initial pH (before incubation). Lowest pH was in the broth of isolate 6 (pH 3.42) while the highest pH was of isolate 1 (pH 4.03).

Table 5:1 Morphological characteristics of selected lactic acid bacteria isolates

Isolates	pH of broth	Gram stain	Catalase test	Cells shape
1	4.03	+	-	Rod
2	3.75	+	-	Rod
3	3.72	+	-	Rod
4	3.97	+	-	Rod
5	3.50	+	-	Rod
6	3.42	+	-	Rod
Initial media pH	6.5			

5.3.3 Evaluation the phenotypic characteristics of the isolates as probiotic agent to survive in the intestinal gut environment

All isolates were able to grow at both temperatures 37°C and 42°C (broiler chickens intestine temperature). All showed ability to grow at all three different levels of oxygen. Either with or without oxygen in addition to low level of oxygen.

5.3.3.1 Identification of LAB

5.3.3.1.1 Genetic identification analyses of isolate (16S rRNA)

16s rRNA was used to identify the genus and species of the 6 isolates. This technique was able to identify the isolates to species level. Table 5:2 shows that at genus level all isolates were belonging to the *Lactobacillus* genus. While at the species level, isolates 1, 2, 3 and 5 belong to species *Lb reuteri* while isolate 4 was *Lb fermentum* and isolate 6 was *Lb salivarius*.

Table 5:2 Genus and species of selected LAB isolates identified by 16S

ID of Isolate	Genus	Species
1	*Lactobacillus*	*reuteri*
2	*Lactobacillus*	*reuteri*
3	*Lactobacillus*	*reuteri*
4	*Lactobacillus*	*fermentum*
5	*Lactobacillus*	*reuteri*
6	*Lactobacillus*	*salivarius*

5.3.3.1.2 Susceptibly of LAB isolates to antibiotics test

Antibiotics discs were used examine the susceptibly of these 6 isolates toward a range of antibiotics. Table 5:3 shows that all LAB isolates were sensitive to antibiotics; Ampicillin, Rifampicin and Cefotaxime. While antibiotics ciprofloxacin and ofloxacin have no action toward the LAB isolates as there was no zone around the disc. The susceptibility of isolates to gentamicin was low or negative as the zone of inhibition was small. Tetracycline had variable activity toward the isolates, as the highest sensitivity was of the isolate 5 and 1, while isolates 2, 3, 4 and 6 all seem have similar zone of sensitivity toward Tetracycline.

Table 5:3 Prevalance of resistance of LAB to selected antibiotic

Isolate	Antibiotics						
	Gentamycin	Ampicillin	ciprofloxacin	Tetracycline	Rifampicin	Cefotaxime	Ofloxacin
L. reuteri	+	++++	_	++	+++	++++	_
L. reuteri	+	++	_	_	++	++++	_
L. reuteri	+	+++	_	+	++	++++	_
L. fermentum	-	++++	_	_	++	++++	_
L. reuteri	+	+++++	_	+++	+++	++++	_
L. salivarius	-	+++++	_	+	++	+++	_

-no zone, +less 2mm, ++5mm, +++7mm, ++++ 9mm, ++++11mm

5.3.3.1.3 Antimicrobial activity of lactic acid bacteria isolates

The antimicrobial activity of the 6 isolates was tested to investigate their activity towards pathogenic bacteria. Three different strains of *Campylobacter* were chosen as indictors. Table 5:4 and figure 5:1 show that all isolates have an inhibitory activity towards the three strains of *Campylobacter*. It seems that the highest activity was for isolate 6 against all three strains of *Campylobacter*. While the lowest activity was of isolates 3 and 4 toward 01/51 strain.

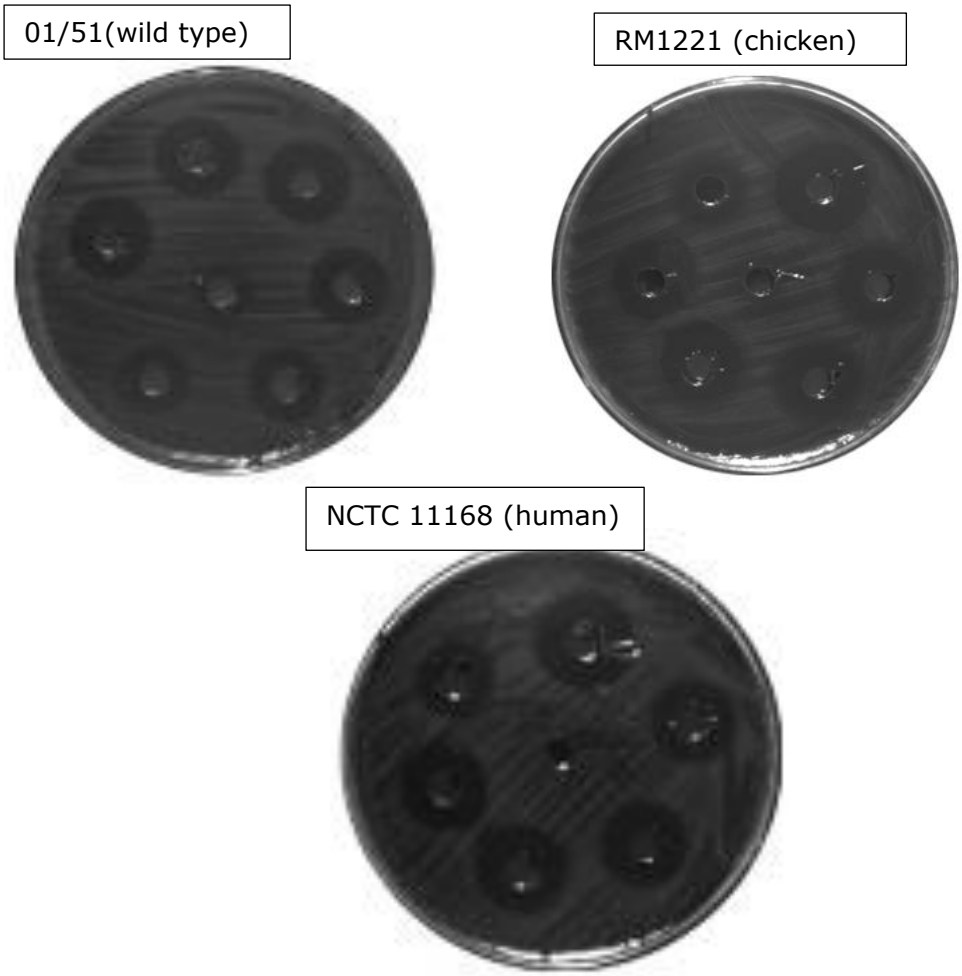

Figure 5:1 Inhibition zone of *Campylobacter jejuni* by cell –free supernatant of LAB isolates

Campylobacter strains are; left top 01/51, right top RM1221 and the bottom is NCTC 11168. Wells of 6 isolates supernatant on each plate from left top to right top; strain1 (L. reuteri), strain2(L. reuteri), strain 3 (L. reuteri), strain 4 (L. fermentum), strain 5(L. reuteri), strain 6(L. salivarius) 1, 2, 3, 4, 5 and 6 while middle well is the control (only MRS broth)

Table 5:4 Antimicrobial activity of LAB isolates cell-free supernatant toward three strains of *Campylobacter* performed by agar well diffusion method

Isolates	Inhibition activity against strains of *Campylobacter jejuni*		
	RM1221	01/51	NCTC 11168
1	+*	++	++
2	++	++	++
3	++	+	++
4	++	+	++
5	++	++	++
6	+++	+++	+++

* Diameter of inhibition zone + 4-8 ++ 10-12mm, +++ 13-15

5.3.3.1.4 Hydrogen peroxide production

All isolates were tested for production of H_2O_2 which is consider as antimicrobial substance that is produced by *Lactobacillus* bacteria. This was done by detecting the blue halo around the colony on the plate using supplemented agar media. Results show that Isolates 1, 3, 4 and 6 were positive for H_2O_2 production as there was a halo of blue colour around the colonies of isolates. While isolates 2 and 5 were negative of H_2O_2 production as there was no halo around the growth (data not shown).

5.3.3.1.5 Tolerance of isolates to Bile salts

The sensitivity of LAB strains to bile salts was tested on MRS broth containing different levels of bile salts. Figure 5:2 present the survivability of LAB isolates in the supplemented media with 0, 0.25, 0.50, 0.75 and 1% of bile salts. This figure show that all isolates were able to survive and grow in the inoculated medium with different levels of bile salts. Level zero was as standard to compare the growth in the MRS broth as figure present the results.

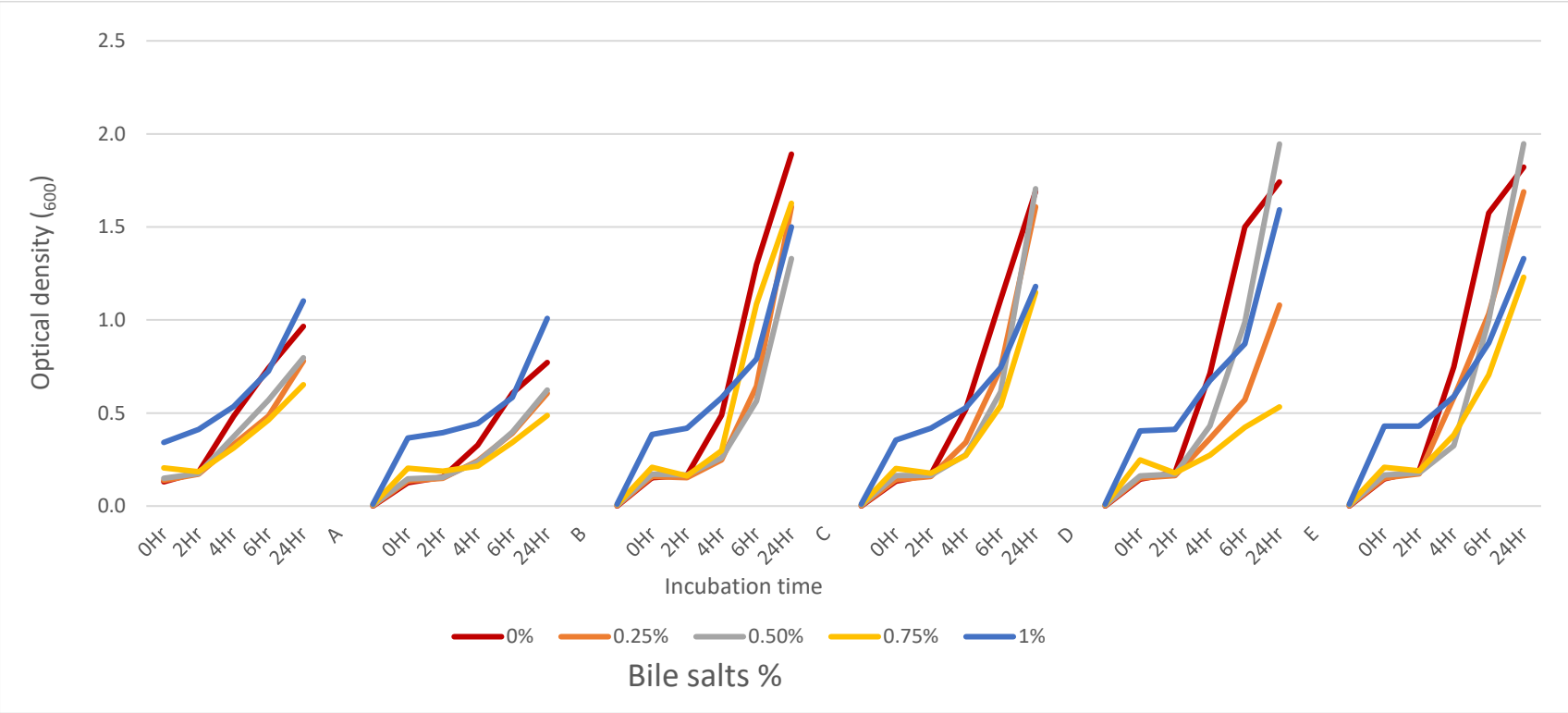

Figure 5:2 Survival of LAB isolates in the media supplemented with 0, 0.25, 0.50.075 and 1% of bile salts.

A: strain1 (L. *reuteri*), B: strain2 (L. *reuteri*), C: strain 3 (L. *reuteri*), D: strain 4 (L. *fermentum*), E: strain 5(L. *reuteri*), F: strain 6(L. *salivarius*)

5.3.3.1.6 Tolerance of isolates to sodium chloride (NaCl)

Figure 5:3 show that in general the growth of bacteria decreased as concentration of NaCl increased. However it seems that the growth of isolates was unaffected significantly at the concentrations of 1 and 2% of NaCl which was close to the standard media (0%) as the absorbance (O.D) were similar. In the other levels of NaCl the growth of bacteria was decreased when the concentration of NaCl increased. At 3% the growth was less but still close to standard. At concentrations 4 and 5% there was delay of growth until 6 hours then growth increased rapidly by 24 hour. While at level 6% there was minimal growth, a high levels (7-10%) seem that viability was much lower.

5.3.3.1.7 Tolerance of isolates to acidic media

Isolates were examined whether they could grow and survive in the low level of pH in the intestinal gut of broiler chickens. Figures 5:4 and table 5:5 show that all isolates can grow at pH 4, 5 and 6, although, growth at pH 4 was less. At pH 2 and 3 growth low but they were still viable as table 7 shows.

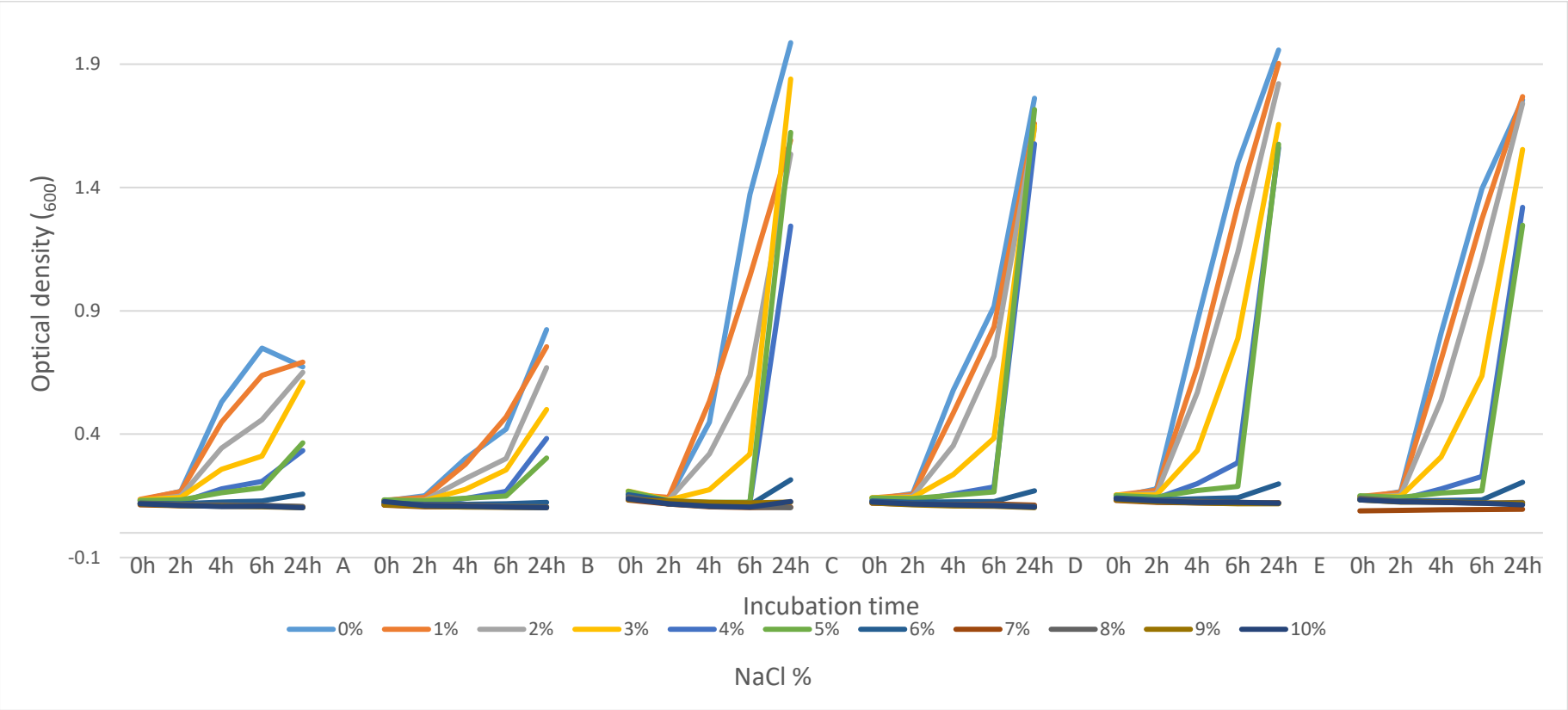

Figure 5:3 effect of NaCl in the media on the viability of LAB isolates at levels 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%.

A: strain1 (L. *reuteri*), B: strain2(L. *reuteri*), C: strain 3 (L. *reuteri*), D: strain 4 (L. *fermentum*), E: strain 5(L. *reuteri*), F: strain 6(L. *salivarius*)

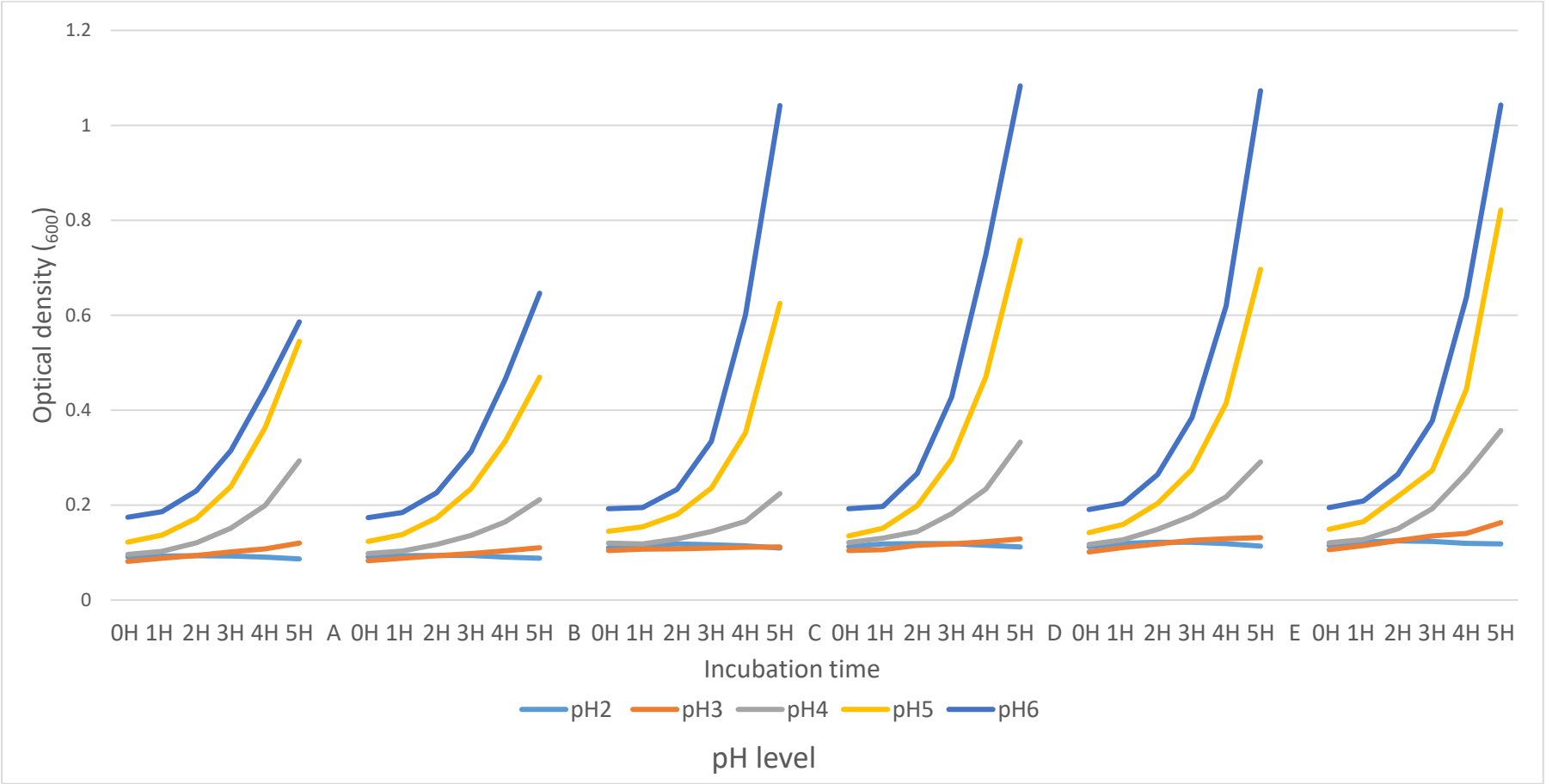

Figure 5:4 Growth of LAB isolates in MRS broth varying pH over different time points.

*A: strain1 (L. *reuteri*), B: strain2(L. *reuteri*), C: strain 3 (L. *reuteri*), D: strain 4 (L. *fermentum*), E: strain 5(L. *reuteri*), F: strain 6(L. *salivarius*)

Table 5:5 viability of LB isolates from the acidified broth

Isolate	Growth of isolates from the broth (pH)				
	2	3	4	5	6
1	+*	+	++	++	++
2	+	+	++	++	++
3	+	+	++	++	++
4	+	+	++	++	++
5	+	+	++	++	++
6	+	+	++	++	++

*+ low growth, ++ good growth

5.3.4 Utilization of Jerusalem artichoke (JA) plant by isolates

The isolates were screened for their ability to use the JA plant as a carbon source for their growth. Three supplements were used in the broth media, glucose (standard), Inulin and Artichoke, number of bacteria increased over 24 hours of incubation for all isolates. Figure 5:5 show that the count of bacteria was increased after 24 hours on the broth that supplemented with artichoke instead of glucose. The growth of isolates on inulin broth was the lowest while the highest growth was in the broth of artichoke.

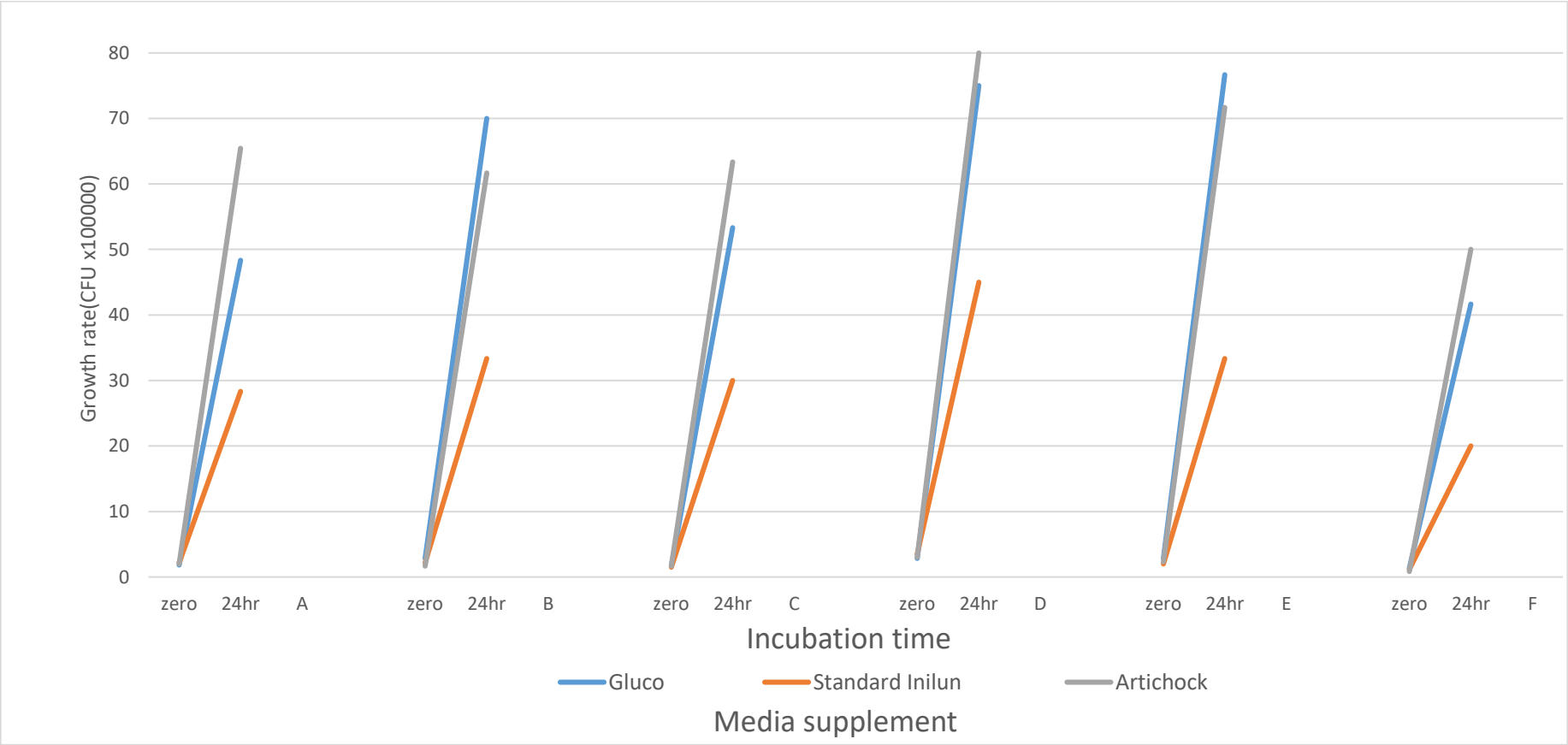

Figure 5:5 growth rate ($O.D_{600nm}$) of LAB isolates in media containing different carbon source; prepared with glucose-base (standard), inulin (commercial) and Jerusalem artichoke plant.

*A: strain1 (L. *reuteri*), B: strain2 (L. *reuteri*), C: strain 3 (L. *reuteri*), D: strain 4 (L. *fermentum*), E: strain 5(L. *reuteri*), F: strain 6(L. *salivarius*)

5.3.5 Viability of bacterial cells during preparation of probiotic product

Isolates were examined for viability during processing to ensure they retained viability at different stages of production as feed supplements. As figure 5:6 shows that there was no big drop in the survival of all LAB isolates during different steps of processing. It seems that the drying has the greatest influence on the viability, however the biggest drop of just 1.5 logs was seen for isolate 3.

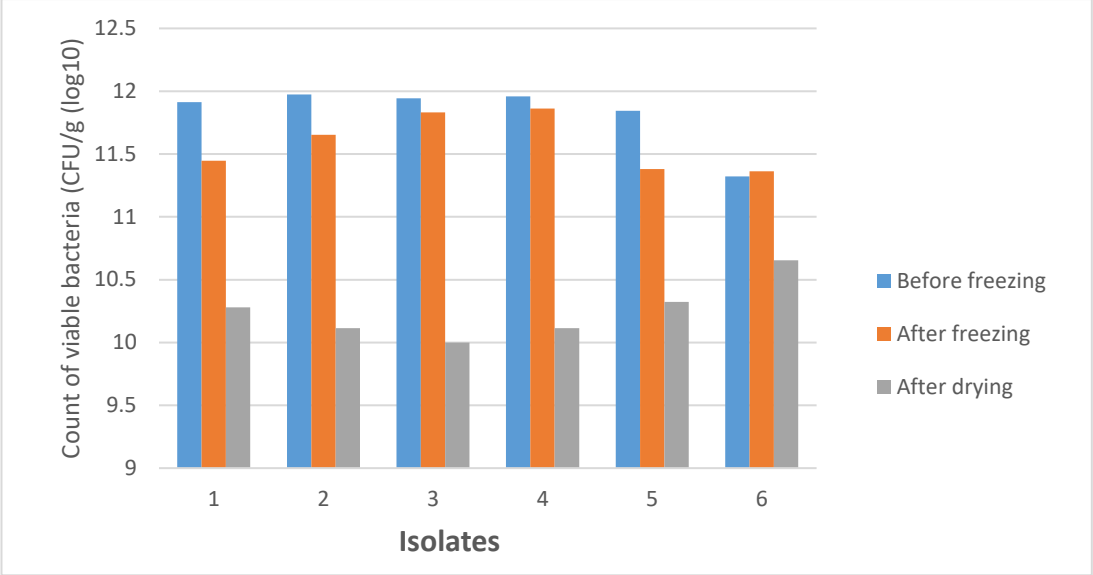

Figure 5:6 Effects of preparation of isolates probiotic product on the viability of bacterial cells**.**

* strain1 (L. *reuteri*), strain2 (L. *reuteri*), strain 3 (L. *reuteri*), strain 4 (L. *fermentum*), strain 5 (L. *reuteri*), strain 6(L. *salivarius*)

5.3.6 Inulin content of Jerusalem artichoke and utilisation in the culture media

Inulin content was measured in the dry JA plant to determine the concentration of inulin in the Iraqi JA. The concentration of inulin was 52% of the dry matter of tuber.

5.4 Discussion

Lactobacillus isolates were isolated from *Campylobacter*-free chicken and examined to be used as a probiotic agent in broiler chickens feed. Six isolates were selected for further investigation. In addition JA plant was examined to be used as a prebiotic supplement in broiler chickens. Evaluation of the isolates included three steps: First was the morphologic and phenotypic properties of the isolates to identify them. Secondly, the isolates were examined for their activity toward *Campylobacter* and their survival *in vitro* in environments created to mimic the stresses faced by the organisms during passage through the intestinal gut of broiler chickens. Finally, isolates were examined for viability during preparation as feed supplements. Jerusalem artichoke was also examined for its ability to be used as a carbon source for *Lactobacillus*.

The morphology and biochemical tests confirmed that all isolates were thought to be LAB which allowed for further tests. Genetic examination confirmed that the isolates all belonged to the *Lactobacillus* genus with three species identified: *Lb reuteri*, *Lb fermentum* and *Lb salivarius*. All the three species of *Lactobacillus* are common species in the broiler chickens gut and they have all been used previously as probiotic organisms to improve the health and performance of broiler (Olnood, et al. 2015, Shokryazdan, et al. 2017). Further validating use as a probiotic agent in this study.

All LAB isolates were inhibitory against the three chosen species of *Campylobacter*. This result agree with the findings of others studies (Santini, et al. 2010), in which *Lactobacillus isolates from chicken* were able to inhibit the growth of *Campylobacter*. Also (Ghareeb, et al. 2012) found that *Lb*. *salivarius* and *Lb*. *reuteri*, *Enterococcus faecium* and *Pediococcus acidilactici* did inhibit the growth of *Campylobacter jejuni*. Some of them are able to produce H_2O_2 which is one important mode of action of LAB to be used as probiotic as an antimicrobial agent against pathogenic bacteria (Servin 2004). All isolates in this study remained viable at low pH (2 and 3) for up to 5 hours. They were also able to survive with additional bile salts at the levels 1% in the media for 24 hours and viability has maintained 6% on NaCl.

The results of the current study are consistent with other research (Jin, et al. 1998, Hassanzadazar, et al. 2012) which found that *Lactobacillus* isolates from chicken or humans have tolerance to a simulated gut environment. The concentration of inulin in the Iraqi JA was 52% of the dry matter of the tuber. This level of inulin in the dried plant is considered as a concentrated supplement, also it is acceptable as the JA will be added in small amount in the feed of broiler chickens. In addition using the dried plant matter is easier for handling and storage and it can be added to and mixed with other supplements. The LAB isolates were able to use JA powder as a source of carbon. This result confirms the findings of (Kunová, et al. 2011, Nagpal and Kaur 2011) as they found that some Lactic Acid Bacteria are able to use inulin as source of carbon in the media. Also the growth of LAB in the media containing the JA was higher than in the standard media (glucose) and this may be because the powder was a raw plant which contained other nutrients such as minerals (Lachman 2008). Also it seems that growth in media with pure inulin (Frutafit®) was lower than with other supplements, so the growth on inulin from artichoke was better than on the pure inulin. This level of inulin in the plant made the Jerusalem artichoke powder an attractive feed supplement in the broiler chickens diets.

5.5 Conclusion

The aim of this study was to screen LAB isolates from chicken for their possible use as candidate probiotics. All tests that have been carried out on these isolates can confirm that the isolates have the characteristics of lactic acid bacteria and they have an activity toward *Campylobacter* as a pathogenic bacteria indictor. Also they belong to the genus of *Lactobacillus* and its species that have already been used as probiotics in human and animal. In addition isolates are able to keep their viability during freezing and drying and they can survive in the environments of gastrointestinal gut of broiler chickens. This evaluation suggests that these isolates that were isolated from chicken can be used as a potential probiotic agent in the diets of chicks. It also seems that the artichoke plant is good source for inulin to be used in chicken as a prebiotic. As all six candidate probiotic strains appeared to be viable candidates for potential use in broiler chickens diets, all were therefore taken forward for assessment in vivo in bird trial LB03.

Chapter six:

The influences of prebiotic, probiotic and synbiotic supplements on the performance of broiler chickens

6.1 Introduction

Currently there is a growing interest of scientific research on the role of probiotics, prebiotics and synbiotic as effective alternatives to the use of antibiotics in animal nutrition (Alloui, Szczurek and Swiatkiewicz 2013, Cheng, et al. 2014). Additionally, several researchers have discussed the effect of probiotic, prebiotic and synbiotic on poultry performance.

Gut microflora contribute to the digestion, absorption and the metabolism of dietary carbohydrates, protein, lipids and minerals and the synthesis of vitamins. Therefore any interruption that occurs in the balance of gut microbiota is likely to affect nutrient digestion and absorption which in turn, could affect bird health and performance. Gut microflora play an important role in nutrition and health status of the host as it has been extensively reported that intestinal microflora balance is beneficially influenced by prebiotic and probiotic (Kim, et al. 2011, Semova, et al. 2012, Daliri and Lee 2015).

Supplementation with probiotics and prebiotics has been used to improve the performance of broiler chickens. (Shokryazdan, et al. 2017) reported that feeding probiotic (*Lactobacillus salivarius*) has led to achievement of live body weight of meat chickens at 42 days of age which were higher significantly ($P \leq 0.05$) than control fed birds. (Mountzouris, et al. 2010) observed that diets containing 10^8 cfu probiotic/kg increased the body weight of broilers significantly ($P<0.05$) comparing with control group without probiotic. (Mookiah, et al. 2014) showed that use of prebiotic brand (IMO) which contains monnaologosaccharide , probiotic of 11 *Lactobacillus* strains and combination of both (synbiotic) in poultry feed significantly ($P<0.05$) improved body weight gain of broiler chickens at 22-42 and 1-42 days of age, and feed conversion compared with control group.

This experiment aimed to study the effects of dietary supplements of prebiotic (Jerusalem artichoke), probiotic (novel *Lactobacillus* isolates) and synbiotic (combination of both) in the chicken feed on the performance during different ages. The specific hypothesis for this chapter is that the isolates could be a good resource as probiotic agent to be used in chicken feed.

6.2 Method

6.2.1 Trial design

6.2.1.1 LB03 Trial design

216 male Ross 308 broilers were ordered by NTU from PD Hook and collected from their Cote (Oxford) hatchery. The birds were divided at day 1 post hatch into six groups (treatments) with the birds that fed a control diets by using the feed delivery of LB and compared this control diet to monitor effects on gut colonization of both LB and *Campylobacter,* immune function and to monitor bird performance at day 7, 21 and 42. Husbandry and ethical clearance procedures were carried out as described in chapter 2 section 2.5. One replicate was a pen containing 6 birds on d1 (total 36 birds per treatment group). Diet allocation is shown in appendix H. In the pens with 6 birds. The unit was divided and sealed into two sides: probiotic and non-probiotic, using plastic sheeting to minimise cross-contamination by *Lactobacillus* isolates (probiotic).

6.2.2 Diet mixing and sampling Diet Formulation

All trial diets were manufactured on site and fed as mash. A basal diet was mixed from previously manufactured trial diets formulated to meet the age and strain of the birds. A grab sample was taken during the feed weighing prior to the trial for analysis. The composition and analysis of all the trial diets are provide in the corresponding chapter 2 section 2.6. To avoid any cross contamination all non-probiotic (prebiotic) diets were manufactured, weighed and removed from the feed room before making probiotic diets. Equipment used for mixing of probiotic diets were flushed with wheat (then wheat discarded) cleaned thoroughly and sprayed with hycolin and left for 7 days before manufacture of non-probiotic diets.

5kg of diet was weighed into individual bags for each control pen. For the treatment pens, an appropriate quantity of freeze dried LB cells to meet the concentrations specified in table 6:1 were weighed and mixed into a plastic bag then weighed out for each treatment pen. Supplements were mixed with small amount of feed then added and mixed with the weekly allocated basal diet volume.

The 6 dietary treatments for LB03 are shown in table 3:, with 6 replicate pens per treatment. Birds were all fed a starter mash until d14 then a commercial grower pellet until d28 then finisher until 42. The dietary treatments for LB03 are shown in table 6:1. Briefly, birds were fed the following diets: T1 (Control - birds that fed basal diet); T2 (prebiotic - birds fed 5% JA powder); T3 (prebiotic - birds fed 10% JA powder); T4 (probiotic - birds fed mix of isolates of Lactobacillus(LB) at level 10^9 CFU/kg; T5(synbiotic - birds fed basal diet +LB+5% JA); T6 (synbiotic - birds fed basal diet +LB+10%JA).

Table 6:1 Dietary treatments for bird trial LB03

Diet	Treatment
Control(T1)	standard feed throughout
Prebiotic (T2)	Artichoke prebiotic level 1(5 g/kg feed)
Prebiotic (T3)	Artichoke prebiotic level 2(10 g/kg feed)
Probiotic (T4)	10^9 CFU of each of 6 isolates of *Lactobacillus*
Synbiotic (T5)	Probiotic +prebiotic level 1
Synbiotic (T6)	Probiotic +prebiotic level 2

6.2.3 Birds and Husbandry

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. Commercial guidelines for the care and husbandry of Ross 308 broilers were followed in all studies (Aviagen, 2008). 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, 2007) and the Animal Scientific Procedures Act (ASPA, 1986).

6.2.4 Feed Intake

Feed intake was measured as explained in section 2.7

6.2.5 Bird Weights

Bird weight was measured as explained in section 2.8

6.2.6 Feed conversion ratio (FCR) was calculated as follows

FCR (kg feed/kg gain) = cumulative feed intake (kg)/total weight gain (kg);

6.2.7 Body weight gain (BWG)

BWG (grams on period) = BW (g) at the end of each week - BW (g) in first of the week. Viability (%) = chicks remaining at the end of period (%);

6.2.8 European Production Efficiency Factor was calculated as follows:

European Production Efficiency Factors (EPEF) EPEF = [Viability (%) X BW (kg) / age (day) X FCR (kg feed/kg gain)] X 100

6.3 Results

Tables 6.2 shows the effects of supplements of prebiotic, probiotic and synbiotic on the weekly body weight of birds fed each diet. While some differences in weekly mean bird body weight were identified, a consistent response was not observed across all weeks. As expected, no significant differences in bird body weight were observed on day 0. At day 7 only probiotic treatments affected the body weight which was 118.51g in the T4 comparing to 134.82g in the control. By day 7 a significant decrease in body weight of chicks fed diet T4 (probiotic alone) was observed. However, this negative effect on bird body weight was lost at day 14 where there was no significant difference between treatments, although it should be noted that T4 remained numerically the lowest value on day 21, a significant decrease in body weight was observed in all birds fed diets containing probiotic either alone or in combination with the prebiotic supplement (diets T4, T5 and T6). On day 28, no significant differences in body weight were observed but interestingly at this time point, the errors terms associated with each mean value began to differ substantially, and the variability in error terms remained for the rest of the study.

Table 6:2 Effect of prebiotic, probiotic and synbiotic on weekly live body weight (g), of broiler chicken (Mean ± standard error).

Day	T1	T2	T3	T4	T5	T6	P value
D0	40 ±0.70	39 ±0.66	39 ±0.50	38 ±0.98	38 ±0.80	37 ±0.81	0.326 L*(0.63) Q (0.69)
D7	134 ±4.37	134 ±4.63	124 ±7.80	118 ±5.34	128 ±5.49	123 ±5.53	0.294 L(0.101) Q (0.306)
D14	343 ±16.43	338 ±16.50	310 ±18.75	298 ±25.03	330 ±25.52	322 ±24.43	0.674 L(0.445) Q (0.267)
D21	809[a] ±29.17	804 [a] ±33.13	791[a] ±33.03	645[b] ±44.73	681[b] ±34.74	627[b] ±50.41	0.002 L(0.001) Q (0.884)
D28	1298 ±39.62	1256 ±33.78	1177 ±59.61	1185 ±78.48	1241 ±52.03	1127 ±83.45	0.411 L(0.091) Q (0.747)
D35	2052 ±57.67	2006 ±46.85	1878 ±59.57	1965 ±107.84	1976 ±69.79	1783 ±87.21	0.164 L(0.039) Q (0.793)
D42	2885 ±73.80	2791 ±77.35	2667 ±81.25	2807 ±120.82	2843 ±96.13	2550 ±98.61	0.138 L(0.086) Q (0.674)

(a, b) data with the same superscript in the same row are not significantly different (P≤0.05).

T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed Basal diet +LB+10%JA. L, linear, Q, Quadratic.

Tables 6:3 shows the effects of supplements of prebiotic, probiotic and synbiotic on the weekly body weight gain of birds fed each diet on day 7 and day 14 no significant differences were observed between diets. On day 21, a significant decrease in body weight gain (corresponding to the previously described decrease in body weight for these treatments) was observed in all birds fed diets containing probiotic either alone or in combination with the prebiotic supplement (diets T4, T5 and T6). In contrast however, on day 28, significant differences in body weight gain were observed as interestingly, the body weight gain for birds fed diets T4, T5 and T6 was higher than for birds fed the other treatments. On day 35 and 42 a numerical reduction was observed in body weight gain of birds fed diet T6 compared to other diets, but this

effect were not significant. Overall cumulative body weight gain was not significantly different between treatments but numerically reflected the pattern of growth seen for each dietary treatment.

Table 6:3 Effect of prebiotic, probiotic and synbiotic on weekly body weight gain (g) of broiler chicken (Mean ± standard error).

Body weight gain	T1	T2	T3	T4	T5	T6	P value
D7	94 ±4.13	94 ±4.15	85 ±7.54	80 ±5.61	90 ±5.23	85 ±4.90	0.354 L(0.161) Q(0.309)
D14	208 ±12.27	204 ±13.90	186 ±13.71	197 ±15.47	201 ±21.67	199 ±19.50	0.942 L(0.688) Q (0.3)
D21	466 [a] ±29.80	465 [a] ±17.57	481 [a] ±21.84	347 [b] ±27.23	350 [b] ±16.85	304[b] ±30.57	0.002 L(0.03) Q (0.233)
D28	489[a] ±40.92	452[a] ±15.37	405[b] ±31.33	539[a] ±47.15	560[a] ±22.81	500[a] ±34.26	0.012 L(0.066) Q (0.456)
D35	754 ±24.51	750 ±17.18	701 ±8.67	780 ±48.94	735 ±27.51	656[b] ±31.62	0.063 L(0.071) Q (0.187)
D42	832 ±18.61	785 ±58.44	788 ±28.37	842 ±31.17	867 ±40.36	766 ±49.08	0.452 L(0.92) Q (0.625)
Cumulative	2845 ±73.43	2752 ±77.38	2628 ±81.26	2769 ±120.38	2805 ±95.42	2512 ±98.03	0.133 L(0.088) Q (0.67)

(a, b) data with the same superscript in the same row are not significantly different (P≤0.05).

T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed basal diet +LB+10%JA.

Table 6:4 shows the effects of supplements of prebiotic, probiotic and synbiotic on the weekly and cumulative feed intake of birds fed each diet on days 7, 14, 21 and 28

and 35, no significant differences in feed intake were observed. However, at day 42, a substantial, significant increase in feed intake was observed for all birds fed diets containing the probiotic, either alone or in combination with the prebiotic supplement (diets T4, T5 and T6). For day 42, feed intake (FI) was 1981.74, 2129.31, 2131.89g for diets T4, T5 and T6 respectively compared to 1545.71 gram for control-fed birds. This resulted in a significantly higher cumulative feed intake for birds fed diet containing low levels of symbiotic treatment (T5) compared to all other diets: feed intake (FI) was 5298.84, 5060.83, 4926.27g for diets T4, T5 and T6 respectively compared to 4583.61g for control-fed birds

Table 6:4 Effect of prebiotic, probiotic and synbiotic on feed intake (g) of broiler chicken (Mean ± standard error).

Day	Treatments						P value
	T1	T2	T3	T4	T5	T6	
D7	120 ±3.15	118 ±9.39	120 ±10.84	112 ±9.02	120 ±3.54	123 ±6.26	0.943 L(0.815) Q (0.519)
D14	312 ±11.80	315 ±14.48	301 ±16.70	281 ±25.22	280 ±20.41	299 ±17.05	0.643 L(0.214) Q (0.457)
D21	505 ±18.56	479 ±24.73	478 ±26.86	484 ±30.28	507 ±28.68	455 ±37.77	0.810 L(0.518) Q (0.898)
D28	913 ±40.54	931 ±62.73	894 ±56.69	896 ±47.65	972 ±33.63	917 ±31.99	0.866 L(0.716) Q (0.842)
D35	1186 ±32.66	1215 ±36.89	1128 ±25.09	1168 ±48.26	1288 ±106.85	1132 ±42.17	0.353 L(0.985) Q (0.855)
D42	1545^{b} ±93.92	1459^{b} ±15.12	1434^{b} ±36.38	1981^{a} ±50.77	2129^{a} ±58.74	2131^{a} ±208.96	0.004 L(0.02) Q (0.225)
Cum.	4583^{b} ±171.34	4520^{b} ±108.60	4358^{b} ±131.40	4926^{b} ±190.95	5298^{a} ±153.21	5060^{b} ±266.22	0.006 L(0.001) Q (0.443)

(a, b) data with the same superscript in the same row are not significantly different (P≤0.05).

T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed basal diet +LB+10%JA.

Tables 6:5 shows the effects of supplements of prebiotic, probiotic and synbiotic on the weekly and cumulative feed conversion ratio (FCR) of birds fed each diet. Table 4 also shows the European Production Efficiency Factor (EPEF) associated with each diet. On day 7 and day 14 no significant differences in FCR were observed between diets. On day 21, a significant deterioration in FCR was observed in all birds fed diets containing probiotic either alone or in combination with the prebiotic supplement (diets T4, T5 and T6). On day 28 and day 35, no differences between treatments were observed. On day 42, a substantial, significant deterioration in FCR was observed in all birds fed diets containing the probiotic, either alone or in combination with the prebiotic supplement (diets T4, T5 and T6), which corresponded to the substantial increase in feed intake recorded during this week for birds fed these diets. Cumulative FCR reflected the effects observed in week 6: a substantial, significant deterioration in all birds fed diets T5 and T6. The calculated EPEF values for birds fed diets containing the probiotic, either alone or in combination with the prebiotic supplement (diets T4, T5 and T6) were also poorer that all other diets.

Table 6:5 Effect of prebiotic, probiotic and synbiotic on feed conversion ratio of broiler chicken and EPEF (Mean ± standard error).

FCR	Treatments						
	T1	T2	T3	T4	T5	T6	P value
D7	1.27 ±0.04	1.26 ±0.10	1.41 ±0.07	1.41 ±0.08	1.35 ±0.07	1.36 ±0.09	0.475 L(0.094) Q (0.85)
D14	1.51 ±0.05	1.56 ±0.09	1.65 ±0.10	1.38 ±0.05	1.42 ±0.07	1.45 ±0.06	0.663 L(0.768) Q (0.373)
D21	1.11^{b} ±0.09	1.03^{b} ±0.03	0.99^{b} ±0.04	1.41^{a} ±0.06	1.45^{a} ±0.06	1.52^{a} ±0.07	0.00 L(0.03) Q (0.076)
D28	1.91 ±0.12	2.08 ±0.18	2.39 ±0.23	1.72 ±0.18	1.75 ±0.10	1.88 ±0.16	0.82 L(0.204) Q (0.41)
D35	1.58 ±0.03	1.62 ±0.07	1.61 ±0.04	1.51 ±0.06	1.66 ±0.04	1.67 ±0.06	0.118 L (0.06) Q (0.23)
D42	1.86^{b} ±0.11	1.92^{b} ±0.18	1.83^{b} ±0.06	2.36^{a} ±0.08	2.47^{a} ±0.07	2.66^{a} ±0.21	0.00 L (0.004) Q (0.094)
Cumulative	1.61^{b} ±0.04	1.65^{b} ±0.05	1.66^{b} ±0.05	1.78^{b} ±0.03	1.89^{a} ±0.05	2.01^{a} ±0.06	0.00 L(0.043) Q (0.079)
EPEF	426.44^{a} ±15.55	405.81^{a} ±19.85	383.92^{a} ±18.42	376.33^{a} ±19.78	359.25^{b} ±19.75	302.74^{b} ±13.93	0.001 L(0.003) Q (0.34)

$^{(a, b)}$ data with the same superscript in the same row are not significantly different (P≤0.05).

T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed basal diet +LB+10%JA.

6.4 Discussion

The aims of this study were to investigate the effects of dietary supplementation with prebiotic, probiotic and synbiotic on the chicken performance. The performance of control-fed birds in this study was and other trials that have been conducted at poultry unit at NTU, data were in a similar range, indicating the data in trial LB03 is a reasonable representation of the level of performance that could be expected for this strain of bird raised in the NTU poultry unit. Interestingly, field data gathered from Iraq in 2018 (Rashaad, personal communication, 1 Feb2018) (see appendix K) on Ross 308 male performance was similar to that achieved at NTU.

The results of the present study showed that overall there was no significant combined, linear and quadratic ($p \leq 0.05$) effects of supplements of prebiotic (JA) on the body weight, body weight gain, feed intake, feed conversion ratio and European Production Efficiency Factor. Meanwhile there were negative effects of probiotic either with or without prebiotic on the performance of chicken. Body weight was decreased at day 7 and 21 in the treatment 4 (probiotic), the early effect at day 7 may be as a results of addition of *Lactobacillus* strains in the chicken diet. This results were in line with those of (Panda, et al. 2000), as these authors also did not find any significant difference in the BWG of chickens that given feed containing probiotics L. *acidophilus* and *Streptococcus faecium* compared with control. Awad, et al. (2009) also found that adding supplement of probiotic has no effect on the body weight at day 35 along with body weight gain, but they found that synbiotic supplementation has affected these performance significantly ($p \leq 0.05$). Other authors reported no effect from any form of pre- pro or symbiotic supplementation: Sarangi, et al. (2016) observed that using prebiotic, probiotic and synbiotic in feed of chicken did not affect the body weight, feed intake and FCR until day 42 of age. (Salehimanesh, et al. 2016) reported that using additives of prebiotic, probiotic and synbiotic in the broiler rations did not significantly BW and BW gain. However, (Saiyed, et al. 2015) added prebiotic, probiotic and synbiotic in the broiler diet, and stated that these additives affected the body weight gain positively, as gain was significantly higher in the treatments in the first week, although there were no effects of these supplements from week 2 to 6. While most authors have reported no effect, in the current study

at day7 (week1) probiotic diets negatively affected the body weight and at day 21 and 42 both probiotic and synbiotic treatments negatively affected the body weight. (Abdel-Hafeez, et al. 2017) found that chicks fed diets supplemented with probiotic, prebiotic and synbiotic either with or without feed restriction exhibited higher body weight and feed efficiency than chicks fed the control diets.

Overall in this study, undesirable effects on the chicken performance were associated with dietary treatments containing the *Lactobacillus* strains as feed consumption increased, and body weight declined, which resulted in a high FCR values. These negative results may be due several different reasons; first, as a result of supplement itself, which may be due to the competition for nutrients between these probiotic and the birds themselves, as this bacteria require nutrients such as simple carbohydrates and minerals (Wasielewski, et al. 2016). Another possibility is that the species of LB used in the pro- and synbiotic diets directly cause weight loss: there were 4 strains of *Lactobacillus* that were *reuteri*, which Fak and Backhed (2012) indicated may initiate loss of weight when administered to humans.

6.5 Conclusion

This study revealed that prebiotic of Jerusalem artichoke has no effects on the performance of chicken of this study at all ages. However prebiotic has been used in this study in high level relatively (5 and 10%) so, a lower level may be need to be used to investigate the effects on the chicken performance. It is clear that the probiotic supplements alone or mixed with prebiotic has negatively affected the performance of broiler but the effects varied with bird age and levels of prebiotic mixed with probiotic. Effects of probiotic and synbiotic which led to the degradation in the body weight and increased feed intake and FCR may have been due to the strain action of the bacteria that were selected as candidate probiotic strains. Finally use of prebiotic supplements in chicken feed needs further research investigating both higher or lower supplementation levels and different ages of chicks to gain more information about it. Also these candidate probiotic strains should be studied using lower levels in the diet and finally, isolates should be examined separately not as mixture. In order to gain a deeper understanding into the modes of action behind the effects associated with these supplements, gut microbial investigations were undertaken next.

Chapter seven:

Effects of additives of prebiotic, probiotic and synbiotic into on the caeca microbiota in broiler

7:1 Introduction

Gut microbiota play an important role in animal growth, digestibility and nutrient absorption (Celi, et al. 2017), and can modulate the immunity of the host (Purchiaroni, et al. 2013). There are many factors that can manipulate this community in the gut for instance, age, diet, stress, rearing, and environmental factors (Wei, et al. 2013; Ranjitkar, et al. 2016). Prebiotic, probiotic and the mixture of both (synbiotic) substances have all been used in chicken feed as feed additives. The use of probiotics in chicken feed has been shown to maintain the gut microflora (Daliri and Lee 2015). Also (Kim, et al. 2011) observed that dietary supplementation with prebiotic Fructooligosaccharides (FOS) increased the diversity of *Lactobacillus* in the chicken gut. Both inulin and FOS are prebiotic agents (Kelly 2008) which are fermented by certain lactic acid bacteria and *Bifidobacterium*, which in turn have been attributed to being beneficial to the host as potential probiotic microorganisms (Kaplan and Hutkins 2003, Roberfroid 2007). Nabizadeh (2012) found that the addition of inulin to feed may increase *Bifidobacterium* numbers and decrease *E. coli* numbers in the caecal content of chicken. Also, probiotic isolates from poultry such as lactobacilli were also found to ferment FOS (Saminathan, et al. 2011).

One of the valuable benefits of manipulating the intestinal microbiome is that microflora in the gut can contribute to resistance to pathogenic bacteria and decrease infection in the gastrointestinal tract of chicken (Vieira, Teixeira and Martins 2013). Hence, considerable efforts have been invested in studying probiotics and their application in human and animal health have led to the knowledge that probiotic therapy can be a prudent intervention strategy. Use of probiotics to control and prevent pathogenic bacterial contamination and infection both in poultry and in humans from species such as *Campylobacter*, *Escherichia coli, Salmonella* spp. And *Clostridium* spp. is based on the advantages derived from manipulation of the gut microflora (La Ragione, et al. 2004; Murry, Hinton J and Buhr 2006, Alemka, et al. 2010). Until recently, many microbial groups present within a microbiome remained undetected due to the limitations of standard classical microbiological methods. The limitations associated with culturing may be due to species-species interdependence in certain situations, and or due to a lack of knowledge of the particular nutritional

requirements of these non-culturable microbes (Muyzer 1999); (Lu, et al. 2003b). The development of modern molecular approaches make it possible to identify different bacterial populations in environmental samples without cultivation (Harmsen, et al. 2000). Using the 16s rRNA gene sequencing technique makes the study of the poultry microbiota more comprehensive and gives more information about the species breadth of these communities (Wei, et al. 2013). In the hypothesis of this study are that the prebiotic and probiotic could manipulate the caeca microbiota positively and minimise the level of campylobacter in the gut of broiler.

This study aimed to investigate the effects of the Jerusalem artichoke plant as an inulin-based prebiotic as well as newly isolated strains of *Lactobacillus* as probiotic and a mixture of them (synbiotic) on the *Campylobacter* count in the caeca of chicken. Both traditional colony counts were carried out on the caecal content at day 1, 7, 21 and 42, as well as a culture-independent method based on 16s rRNA gene sequencing at day 7 and 42.

7.1 Methods

Caeca were collected from trial LB03 birds post mortem and then stored at 4 °C prior to culturing and DNA extraction.

7.1.1 Sample preparation

As explained in section 2:13:3

7.1.1.1 DNA extraction

As explained in section 2:13:3:1:1

7.1.1.2 Sequencing the DNA

As explained in section 2:13:3:1:2

7.1.1.3 Bioinformatics analysis

As explained in section 2:13:3:1:3

7.2 Results

7.3 Prevalence of *Campylobacter* spp. in the poultry unit and chicken gut

All the caeca collected during the trial for all treatments were screened for the presence of *Campylobacter* at 7, 21 and 42 days of the trial in addition to day 1 of age before feeding the chicks all of which were negative. In addition the poultry unit was screened before the trial started and this was also free of *Campylobacter*.

7.4 Microbial composition of the caeca

7.4.1 Culture-dependent method

7.4.1.1 Microbial composition of the caeca at day 1

At day 1 chicks before treatment were screened for lactic acid bacteria (LAB) and *Campylobacter* in the caeca. The colony counts (CFU) as shown in table 7:1 were between 10.7 to 11.07 $\log_{10}$. Meanwhile chicks were free of *Campylobacter* at this age. All colonies counted were similar in colour creamy/white and in size similar size.

Table 7:1 Total count of LAB and *Campylobacter* in the caeca of chicks at day one

Samples	Log CFU
Sample 1	10.7
Sample 2	11.07
Sample 3	10.84
Sample 4	11
Mean	10.92

Four caeca samples were chosen randomly from chicks at day 1.

7.4.1.2 Microbial composition of the caeca at days 7, 21 and 42

Table 7:2 shows that, on day 7, as expected there was a higher concentration of LAB in the digesta than that found associated with the tissue, indicating the large proportion of LAB is adherent to caecal tissue. The levels of LAB in the caecal content and tissue did not change significantly ($p \leq 0.05$) by the addition of prebiotic, probiotic, and synbiotic, although the low level synbiotic treatment showed a trend (P=0.056)

toward lower LAB colonization in tissue. However LAB in tissue was linearly affected by the treatments. Also, there was no *Campylobacter* detected in the caeca for any of the treatments.

Table 7:2 Effect of prebiotic, probiotics and synbiotic feed supplements on the microbial composition of the tissue and contents of the caeca at day7 mean of CFU (log10 per gram of sample).

Samples	Treatments						P value[2]
	T1[1]	T2	T3	T4	T5	T6	
LAB in tissue	6.69 ±0.39	6.68 ±0.61	6.74 ±0.36	6.19 ±0.37	6.13 ±0.49	6.30 ±0.52	0.056 L(0.017) Q(0.813)
LAB in digesta	10.59 ±0.71	10.79 ±0.48	10.84 ±0.10	10.71 ±0.29	10.42 ±0.56	10.46 ±0.53	0.194 L(0.27) Q(0.244)
Campylobacter spp. in digesta and tissue	Nil	Nil	Nil	Nil	Nil	Nil	Nil

[1]T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed basal diet +LB+10%JA. Data are shown as mean of CFU log ± S.E (n=6) in comparison to those from controls. [2] P value indicates significant difference compared to control at (P≤0.05).

Table 7:3 shows that, on day 21 the levels of LAB in the caecal content and tissue were slightly lower than levels found on day 7. As on day 7 LAB levels in both caeca content and tissue did not change significantly (p≤0.05) by the addition of prebiotic, probiotic, and synbiotic but it was significant linearly in the content. Also, there was no *Campylobacter* detected in the caeca for any of the treatments at all studied ages.

Table 7:3 Effects of supplements of prebiotic, probiotics and synbiotic on CFU of tissue and content of caeca at day 21 mean of CFU (log10 per gram of sample).

samples	Treatments						P value[2]
	T1[1]	T2	T3	T4	T5	T6	
LAB in tissue	5.33 ±0.39	5.15 ±0.34	5.32 ±0.15	5.66 ±0.46	5.07 ±0.23	5.49 ±0.52	0.129 L(0.489) Q(0.983)
LAB in digesta	9.77 ±0.59	9.64 ±0.19	9.56 ±0.49	9.06 ±0.81	9.08 ±1.01	8.67 ±1.14	0.166 L(0.009) Q(0.737)
Campylobacter spp. in content and tissue	Nil	Nil	Nil	Nil	Nil	Nil	

(1)T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6(synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of CFU log ± S.E (n=6) in comparison to those from controls. [2] P value indicates significant difference compared to control at (P≤0.05).

Table 7:4 shows that on day 42 the levels of LAB in the caecal content and tissue were intermediate between those recorded for day 7 and 21. On day 42 LBA levels did not change significantly (p≤0.05) by the addition of prebiotic, probiotic, and synbiotic and there was no *Campylobacter* detected in the caeca for any of the treatments.

Table 7:4 Effects of supplements of prebiotic, probiotics and synbiotic on CFU of tissue and content of caeca at day 42 data shown in mean of CFU (log10 per gram of sample).

Samples	Treatments						P value[2]
	T1*	T2	T3	T4	T5	T6	
LAB in tissue	5.92 ±0.62	6.20 ±0.88	5.90 ±0.73	5.61 ±0.32	5.81 ±0.70	5.83 ±0.60	0.807 L(0.414) Q(0.792)
LAB in digesta	10.12 ±0.44	9.83 ±0.53	9.96 ±0.44	9.93 ±0.43	10.05 ±0.35	9.96 ±0.57	0.921 L(0.844) Q(0.779)
Campylobacter spp. in content and tissue	Nil	Nil	Nil	Nil	Nil	Nil	

[1]T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of CFU log ± S.E (n=6) in comparison to those from controls. [2] P value indicates significant difference compared to control at (P≤0.05).

7.4.2 Population of microbiota in the caeca Culture-independent method

Molecular level screening (metagenomics) via 16s rDNA was used in this analysis to determine the microbiota profile in the caeca of chicken fed all treatments and control diets.

7.4.2.1 Quality of the sequencing run

The quality of sequencing data stated that a total of 31,488,628 raw sequencing reads were generated which is relatively fair enough as Illumina recommended that the total reads should between 44-50 million for the kits that used in this study, however, they stated that some factors such as sample quality and type can affect the number of reads. The quality score (%≥ Q30) 88.32% which relatively high as the kit supplier (Illumina) recommended that the quality score should be above 70% for the kit that been used in this study (Illumina, 2018).

7.4.2.1.1 Bacterial abundance in the caeca content

Figure 7:1 shows relative abundance of the top 7 genera in the caeca content of chicken trial, as determined by 16s rDNA and only the percentage above For the genus level only those with a greater than 2% abundance were chosen to be discussed abundance of genera were chosen to discuss in addition to Bifidobacterium and Escherichia/Shigella as they are important genera which is considered as pathogenic bacteria.

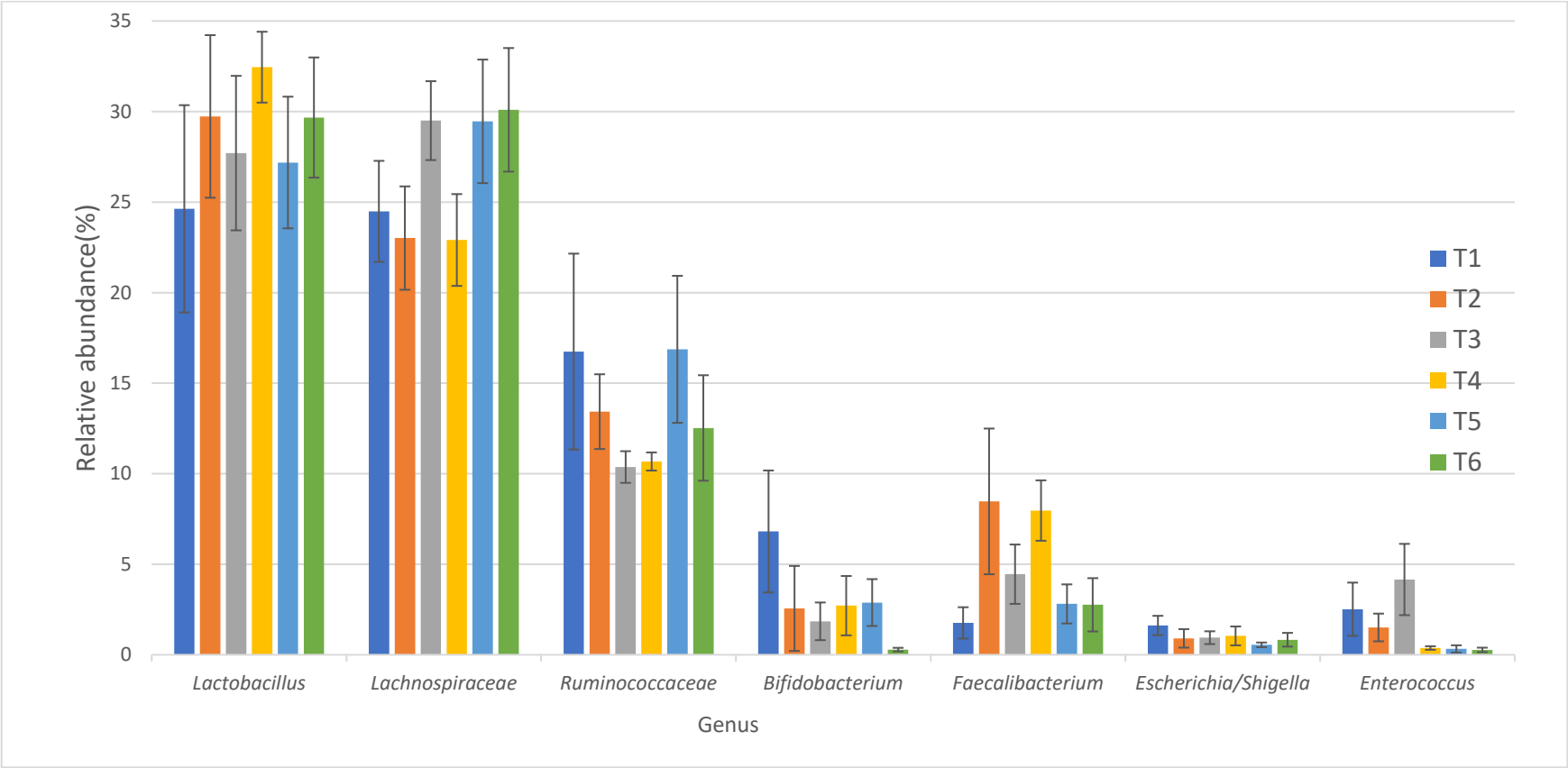

Figure 7:1 Means of Relative abundance (± S.E) of the 7 dominating genera in the caecal contents of control birds and those treated with the various feed supplements at day 7.

*T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6(synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of abundance bars of S.E (n=6) in comparison to those from controls. Stars indicate significant difference compared to control at (P≤0.05).

The effects of supplements on the phyla of *Firmicutes* and *Proteobacteria* were variable. From tables 7:5 and 7:6, it can be seen that from the error bars that the variation between individual replicates was high and the differences between mean values were not significant at ($p \leq 0.05$) for many of the results. At day 7 *Firmicutes* phylum was increased in the treatments of supplements. However the increment was significant ($p \leq 0.05$) at T2 (5%prebiotic), T3 (10% prebiotic) and T6 (10% prebiotic + probiotic) meanwhile in treatments T4 (probiotic) and T5 (5%prebiotic+probiotic) differences were not enough to reach significant difference to control ($p \leq 0.05$). In contrast the *Proteobacteria* phylum also appeared affected by the additions but it was decreased in the treatments compared with control however differences were not significant ($p \leq 0.05$). Meanwhile, no significant effects were observed at day 42 on the *Firmicutes* phylum. Moreover, the abundance of *Proteobacteria* not affected by the supplements at both days.

Table 7:5 Means of Relative abundance (± S.E) of *Firmicutes* and *Proteobacteria* phylum in the caeca content of control and treatment at day7.

Phylum	Treatments						
	T1*	T2	T3	T4	T5	T6	P value
Firmicutes	$88.57^{(b)}$ ±1.62	$95.14^{(a)}$ ±0.91	$95.24^{(a)}$ ±0.94	$93.97^{(b)}$ ±1.05	$93.74^{(b)}$ ±0.57	$95.71^{(a)}$ ±0.85	0.04
Proteobacteria	8.901 ±0.20	5.658 ±0.60	2.314 ±0.42	3.927 ±0.32	3.063 ±0.72	2.954 ±0.35	0.07

* T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus* (LB) at level 10^9 CFU/kg. T5 (synbiotic) birds fed basal diet +LB+5% JA. T6 (synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of abundance ± S.E (n=6) in comparison to those from controls. Different in superscript in the same row indicates significant difference compared to control at (P≤0.05).

Table 7:6 Means of Relative abundance (± S.E) of *Firmicutes* and *Proteobacteria* phylum in the caeca content at day 42

Phylum	Treatments						P value
	T1*	T2	T3	T4	T5	T6	
Firmicutes	92.13 ±2.92	92.62 ±3.28	89.91 ±2.81	94.81 ±0.66	94.36 ±1.53	95.89 ±0.87	0.07
Proteobacteria	0.76 ±0.20	1.29 ±0.59	1.46 ±0.41	1.35 ±0.32	1.50 ±0.72	1.16 ±0.34	0.08

* T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus* (LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of abundance ± S.E (n=6) in comparison to those from controls.

At the genera level shown in figures 7:1 and 7:2, *Lactobacillus* was the most abundant genus in the caeca of all groups (both control and treatments) with a relative abundance ranging from 24% - 32%. There was no significant difference ($p \leq 0.05$) in the abundance of *Lactobacillus* between any of the treatments and the control. There was a differences in the relative abundance of Genera of *Bifidobacterium* spp., *Lachnospiraceae*, *Enterococcus*, Ruminococcaceae, and *Escherichia*/*Shigella* however they were not significantly affected by the supplements at day 7. Also there was no difference in abundance of *Bifidobacterium* spp., *Lachnospiraceae*, Ruminococcaceae, and *Escherichia*/*Shigella* at day 42 (figure 7: 2). Meanwhile at day 7, the abundance of *Faecalibacterium* was affected by some of the supplements: it was significantly ($p \leq 0.05$) higher in T2 (5% prebiotic) and T4 (probiotic) than in control-fed birds. At the day 42 (figure 7: 2), it seems that the variability in the replicates was higher than at day 7. Meanwhile, *Erysipelotrichaceae* genus was affected significantly ($p \leq 0.05$) by the supplements as it was decreased in all treatments comparing with control. Here in this study, at age 42 the community was shifted significantly ($p \leq 0.05$) of some genera, as *Enterococcus* disappeared at day 42 while *Blautia* and *Erysipelotrichaceae* did not appear among the most abundant genera at day 7 but came to prominence as the birds grew older (Figure 7:2).

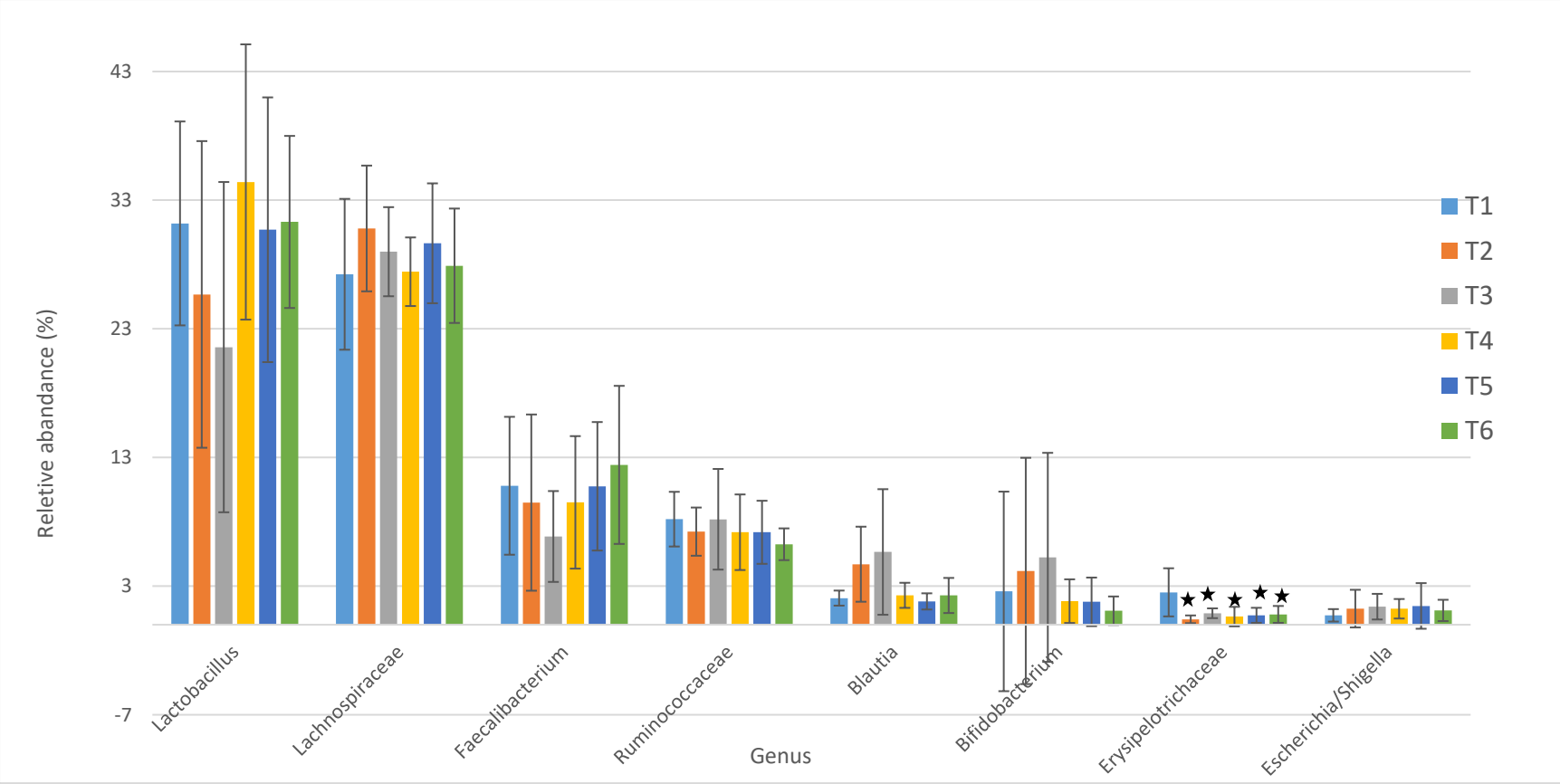

Figure 7:2 Means of Relative abundance (± S.E) of the 7 dominating genera in the caecal contents of control birds and those treated with the various feed supplements at day 42.

*T1 (Control), birds that fed basal diet. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6(synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of abundance bars of S.E (n=6) in comparison to those from controls. Stars indicate significant difference compared to control at (P≤0.05)

7.5 Discussion

The aim of this study was to investigate the influence of dietary supplementation of a prebiotic (Jerusalem artichoke tuber), probiotic 6 isolates of *Lactobacillus* and a combination of both (synbiotic) on caecal microflora profile of broiler chickens. The Lactic Acid Bacteria (LAB) were present in relatively high numbers in the caecal contents of chicks at day one, which continued throughout the trial and it seems there was no significant changes in number at all treatments and ages. The LAB were grown on MRS agar as selective media and because MRS media can grow wide range of LAB genera (Oxoid, 2017) which include *Lactobacilli*, *Lactococci*, *Enterococci*, *Streptococci*, *Leuconostoc* and *Pediococci* (Pessione 2012) in addition to *Bifidobacterium* (Sule, et al. 2014). Hence, may be all of these genera were counted which gave similar count in general.

Throughout the trial chicks that were screened for *Campylobacter* were also negative. This may be because of the cleaning and disinfectant regimen that is used in the NTU poultry unit. The number of LAB in the digesta and tissue the caeca were not affected by the prebiotic, probiotic and synbiotic supplements of at ages 7, 21 and 42 days. The reason for this is likely due to the fact that MRS media was not selective enough for *Lactobacillus* and allowed the growth of a wide variety of LAB. As seen from the figure 7:1 and 7:2 and table 7:5 and 7:6 the *Firmicutes* phylum was in high abundance in all birds. This phylum includes all genera of LAB and when grown on the one media they are morphologically similar, therefore they are all considered to be LAB. The culture-dependent results from the caecal content and tissue showed that there was no difference between control and treatments in the numbers of LAB. Therefore perhaps all treatments have similar numbers of colonies but not all of them were belong to same species or even same genus of LAB.

This absence of an effect of dietary treatment on the numbers of lactobacilli present in the caeca of broilers is in agreement findings from many other microbial studies in chicken. Olnood, et al. (2015a), who also fed a novel probiotic four strains of *Lactobacillus* (tentatively identified as *Lactobacillus johnsonii, Lactobacillus crispatus*, *Lactobacillus salivarius* and *Lactobacillus* sp.) In the broiler feed, there was not a significant ($p \leq 0.05$) effect on the LAB in the caecal content. Also Dibaji, et al. (2014) found similar results when they added a synbiotic which consisting of (*Enterococcus faecium* + fructo-oligosaccharides to chicken feed. In contrast, Dibaji, et al. (2014) found that, by adding probiotic containing different strains of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* to chicken feed, it was possible to increase the total number of *Lactobacillus* significantly in treatments comparing with control. However it is important to note that in the current study counting included all colonies grown on the plate that were similar in the size and colour. This may have introduced some inaccuracies as the edge of each colony was not easy to recognise without use of a microscope.

The results of the Culture-Independent Method (CIM) are shown in the figures 7: 1 and 7: 2. At both 7 and 42 days of age the *Firmicutes* was the most dominant phylum in the caeca of the chicks. Most of the bacteria within the *Firmicutes* phylum are considered to be Butyrate producers in the gut microbiota of chicken (Varmuzova, et al. 2016b), which correlates to the health of the host. In this study *Firmicutes* was significantly ($p \leq 0.05$) in higher abundance than in the control at day 7 for all of supplements treatments. Here it seems that the prebiotic supplements with (synbiotic) or without probiotic have affected the abundance of the *Firmicutes* however probiotic alone could not manipulate this phylum at day 42. It is suggested therefore that the prebiotic has encouraged the bacteria belonging to *Firmicutes* phylum to flourish.

Results of this study revealed that relative abundance of *Lactobacillu*s and *Bifidobacterium* were not affected by the supplements at both days 7 and 42 which is in agreement with the results of many other studies. Fukata, et al. (1999) found that addition of gut content to chicken feed did not bring about differences in lactobacilli or *Bifidobacterium* in chicken caeca at day 7 or day 21. This finding is not

in agreement with Nabizadeh (2012) who found that the addition of inulin to the chicken feed increased the count of *Bifidobacterium* in the caeca. Also, Shang, et al. (2010), when they added the inulin to layer hen feed, reported that lactobacilli were not affected by this addition but Bifidobacterium level was increased in the treatments. (Samal, et al. 2015) found that adding 6% of JA powder into rat feed improved the total count of *Bifidobacterium* in the caecum. (Rebole, et al. 2010) found that adding inulin to the laying hens' diet led to an increase in *Bifidobacterium* in the caecal content.

At day 7, the abundance of organisms within the *Faecalibacterium* genus increased in all treatments but it was only significant ($p \leq 0.05$) in T2 (5%prebiotic) and T4 (probiotic). This increase in the abundance of these bacteria may be because of these supplements made the environments preferable for *Faecalibacterium*. These results are consistent with (Park, et al. 2016*)* who found that when adding prebiotic-based Mannanoligosaccharide (MOS) the abundance of the *Faecalibacterium* genus was increased in the treatment compared with the control. *Faecalibacterium* is also known as one of the butyrate-producing genera (Wang, et al. 2016, Egshatyan, et al. 2016, Pryde, et al. 2002). Butyrate has been shown to have anti-inflammatory activity (Van Immerseel, et al. 2010, Celasco, et al. 2014). Findings of this study were in agreement with (Ramirez-Farias, et al. 2009); (Wang, et al. 2017) when they used prebiotic and probiotic respectively.

Blautia is a genus belong to the phylum *Firmicutes* which has been traditionally believed to carry genes related to polysaccharide metabolism which is thought to enhance the efficiency of energy harvesting by the host (Kasai, et al. 2015). During this metabolism, acetate is also produced (Kettle, et al. 2015, Turroni, et al. 2016), which has been shown to improve intestinal defence and protects the host against lethal infection (Fukuda, et al. 2011). However not all published support this mechanism. The results from this trial are in agreement with what (Krumbeck, et al. 2015) found when they used a prebiotic (galactooligosaccharides) in humans as they observed an increase in the *Blautia* genus. Findings of this study are not in agreement with the findings of (van Zanten, et al. 2014) who found that the addition of a synbiotic to human food did not increase the abundance of Blautia, but actually

brought about a decrease compared with the non-treated control. The genus *Erysipelotrichaceae* was decreased in the caecal content at day 42. The importance of these bacteria is in inflammation which is related to disorders of the gastrointestinal tract in humans (Chen, et al. 2012, Dinh, et al. 2015). Findings of this study were in agreement with (Neveling, et al. 2017) when they added probiotic strains that were isolated from chicken which consisted of *L. crispatus*, *L. gallinarum*, *L. johnsonii*, *L. salivarius*, *Enterococcus faecalis* and *Bacillus amyloliquefaciens* to the chicken diet found that degradation in the abundance of this *Erysipelotrichaceae* genus, while (Tanner, et al. 2014) found that using FOS in swine feed increased the abundance of *Erysipelotrichaceae.* Meanwhile at day 42 abundance of genus of *Erysipelotrichaceae* was decreased in the treatments of supplements compared with control so it may be concluded that these supplements modified the gut microflora in a mildly positive manner, as researchers found that this genus gives indictor for inflammation (Palm, et al. 2014), hence as in this study these supplements caused a degradation in this bacteria. Finally it seems that the interaction between prebiotic and probiotic has no effect of the level of this genus.

The abundances of many genera were modified in the current study (either increased or decreased) but often they were not changed to reach the declared point of significant difference, which may be due to variation among replicates which is shown in size of the error bars (S.E). Stanley, et al. (2013b) studied the microbiota in the chicken individually of each single bird of three trials which were similar in feed and all conditions. They identified that there was a variation from batch to batch across the three trials and in addition they found that the variations were large within each trial. Hence, it seems individual bird to bird variation is normal in the gut microbiota of chicken. Therefore such studies need large number of replicates to minimise the impact of variation among individuals. Another option would be to study each single individual separately, as large variation in the caecal microflora of chicken still occurs regardless of the conditions of bird experiments.

7.6 Conclusion

This study was conducted to evaluate the effects of prebiotic (JA powder), probiotic (6 isolates of *lactobacillus*) and synbiotic (mix of pre and probiotic) supplements on the caecal microbiota of the chicken. Caecal content of chicken at all ages were negative for *Campylobacter,* which did not allow investigation into of the efficacy of the supplements in reducing colonisation of the chicken gut by *Campylobacter* in. To rigorously investigate the effects of supplements on the pathogen, it is better to challenge the birds by directly introducing the pathogenic bacteria to get more applicable results. This study revealed that it is difficult to do this kind of investigation on pathogenic bacteria in poultry without directly challenging the chicken - even though this experiment was done in the summer, when the prevalence of *Campylobacter* is likely to be higher, and the biosecurity regime in the unit was intentionally reduced to match levels akin to poor practice on a commercial poultry farm. Also, the study confirmed that using a culture-based method is a suitable to get the profile of gut microbiota. Meanwhile, the molecular-based method appeared an appropriate method but the number of replicates must be high enough in order to improve the confidence in the results.

Despite the limitations described above and lack of significant differences between control and treated birds for the reasons discussed earlier some keys alteration to the microbiome were associated with all treatments. The post-hatch increases in *Firmicutes* phylum and *Faecalibacterium* genus has some advantages for subsequent growth as both are considered to be butyrate producers. Meanwhile at day 42 abundance of genus of *Erysipelotrichaceae* was decreased in the treatments of supplements compared with control so it may be concluded that these supplements modified the gut microflora in a mildly positive manner pre-slaughter. From these findings it may be concluded that addition of prebiotic, probiotic and synbiotic have positively manipulated the microflora in the gut of chicken. The impact of the altered microbiota on the local and systemic immune function was investigated subsequently in order to gain a broader understanding of how the supplements affect overall health status.

Chapter eight:

Effect of dietary prebiotic, probiotic and synbiotic supplement on the immune function

8:1 Introduction

Commensal bacteria are in close contact with cells of the gut-associated immune system. Modulation of the immune response may occur as a result of interactions between host cells and bacteria or their structural components (Macpherson, et al. 2000). Dietary supplementation of probiotics, prebiotics, or synbiotic has been shown to manipulate or maintain the intestinal microbiome in both human and animal studies (Mookiah et al., 2014). This can cause a shift in the GIT population in favour of beneficial bacteria (e.g. *Lactobacillus spp.* and *Bifidobacterium spp*.), which in turn can positively affect immune function (Isolauri, et al. 2001, Rafter, et al. 2007), therefore, these supplements can be used to enhance immune activity (Kamada, et al. 2013). Cytokines are secreted proteins released by cells to communicate and act as signal molecules to activate and regulate the immune response. Shang, et al. (2015) claim that using fructo-oligosaccharide (prebiotic) in chicken can upregulate the expression of IFN-γ, IL-10 and IL-6.

There are two ways for supplements to impact on cytokine modulation - directly and indirectly. Firstly, supplements may act directly through their actions on the gut-associated lymphoid tissue, and the second possible route is indirect, as they can alter the intestinal tract microflora in a manner that enhances the abundance of key microorganisms that themselves directly affect immune function in the gut.

Furthermore, a balance of commensal bacteria in the gut can work as an efficient barrier against pathogen colonization. In addition, it can produce metabolic substrates like short chain fatty acids (LeBlanc, et al. 2017) and vitamins, and stimulate the immune system in a non-inflammatory manner (Kamada, et al. 2013). Therefore, there is a correlation between the composition of the colonizing microbiota and variations in immunity. Also, Yitbarek, et al. (2015) found that when using synbiotic in the chicken feed will upregulate IFN-γ compared with control.

These cytokines plays a critical role in mucosal surfaces exposed to a dense population of microorganisms to maintain homeostasis and respond efficiently to pathogenic challenges. Cytokines are commonly used as biomarkers to evaluate the impact of feed additives on the host immune response (Wigley and Kaiser 2003,

Kaiser, et al. 2006). INF-γ, IL-10 and IL-6 are important cytokines influencing health of the gastrointestinal tract INF-γ and IL-6 (pro-inflammatory modulators) and IL-10 (attenuation of inflammatory response) therefore studying the cytokine profile offers insight into understanding how pre- and probiotic supplementation may affect immune functions the in chicken. Modern molecular methods like Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) enable measurement of the relative abundance of messenger RNA for different cytokines from relatively small sample volumes (Amsen, de Visser and Town 2009).

This chapter reports on the immune parameters studied in bird trial LB03, which was conducted as described in chapter 6. The objective of this study was to investigate the potential effects of pre, pro and synbiotic supplementation in the feed of chicken on immune functions by measuring the expression of INF-γ, IL-6 and IL-10 in ileum and bursa. These cytokines have been chosen as they are considered as an important marker in responses to bacterial infection as pro-inflammatory or anti-inflammatory cytokines (Kaiser, et al. 2000, Mühl and Pfeilschifter 2003, Amsen, et al. 2009, Isolauri, et al. 2001, Rafter, et al. 2007, Mookiah, et al. 2014, Brisbin, et al. 2008b, Macpherson, et al. 2000). The specific hypotheses for this chapter are as follows: INF-γ and IL-6 gene expression will be up-regulated in response to these supplementation in both ileum and Bursa of Fabricius, and concurrently IL-10 gene expression will be reduced in each tissue.

8.1 Methods

8.1.1 Trial design

This investigation uses material from bird trial LB03 as described in chapter 6.

8.1.2 Rationale for selection of target tissues:

Caeca from all birds in the trial were used to study the microbiota profile in the gut as the microbiota profile was considered a key investigative parameter for the overall research aim. The tissue preparation requirements for assessing gene expression of cytokines and profiling microbiota directly conflict, as for gene expression, fresh tissue should be excised and processed as soon as possible after killing the birds using a chemical protectant to preserve the mRNA. In contrast, for microbiota profiling, it is essential to minimise exposure to air and immediately freeze the samples to arrest all biological activity. This makes it difficult to collect content and tissue from same caeca so therefore ileum was chosen as the closest site in the intestine to the caeca to study the impact of pre, pro and synbiotic supplementation on some aspects of immunity. Also, the bursa of Fabricius was chosen as the unique gland in birds considered to be the site of critical development of the B-cell lymphocytes (Ratcliffe 2006).

8.1.3 Collection of the tissues

On bird trial days 7, 21 and 42 post hatch, one bird per replicate pen was euthanized. Tissues from ileum and bursa of Fabricius has excised immediately post-mortem and stored in RNAlater at -80 °c until further processing for RNA extraction (detailed in chapter 2).

8.1.3.1 RNA extraction

The process described in chapter 2, was followed to extract RNA from both tissue sources.

8.1.4 cDNA synthesis

Full details described in chapter 2

8.1.5 RT-qPCR

Primers were chosen from papers (Rothwell, et al. 2004, Mott, et al. 2008, G. Li, et al. 2010, Waititu, et al. 2014, Lourenço, et al. 2016, Kristeen-Teo, et al. 2017) and checked for target identity using GenBank from the National Centre for Biotechnology Information (NCBI). The full protocol undertaken is explained in chapter 2.

8.2 Results:

8.2.1 Quality and quantity of extracted RNA

There was no significant difference in RNA quality or quantity between the treatment groups. Checking RNA integrity is a critical step before cDNA synthesis to ensure that DNA is removed for successful mRNA quantification by RT-qPCR (Imbeaud, et al. 2005). In addition, the majority 260/230 ratios were also found to be in the acceptable range of 2.0-2.2, which is used as a secondary measure of nucleic acid purity (see appendixes E and F).

8.2.2 The effect of prebiotic, probiotic and synbiotic supplements on the mRNA expression of IFN-γ, IL-10 and IL-6 in the ileum tissue of chicken.

Figure 8:1 shows that in the ileum, there were no significant differences in expression of IFN-γ between treatment at day 7 and day 42, which showed a high level of variability between replicates (n=6). However, at day 21, all supplemented groups showed a significant ($P \leq 0.05$) reduction in IFN-γ expression compared to the control group ($P \leq 0.01$).

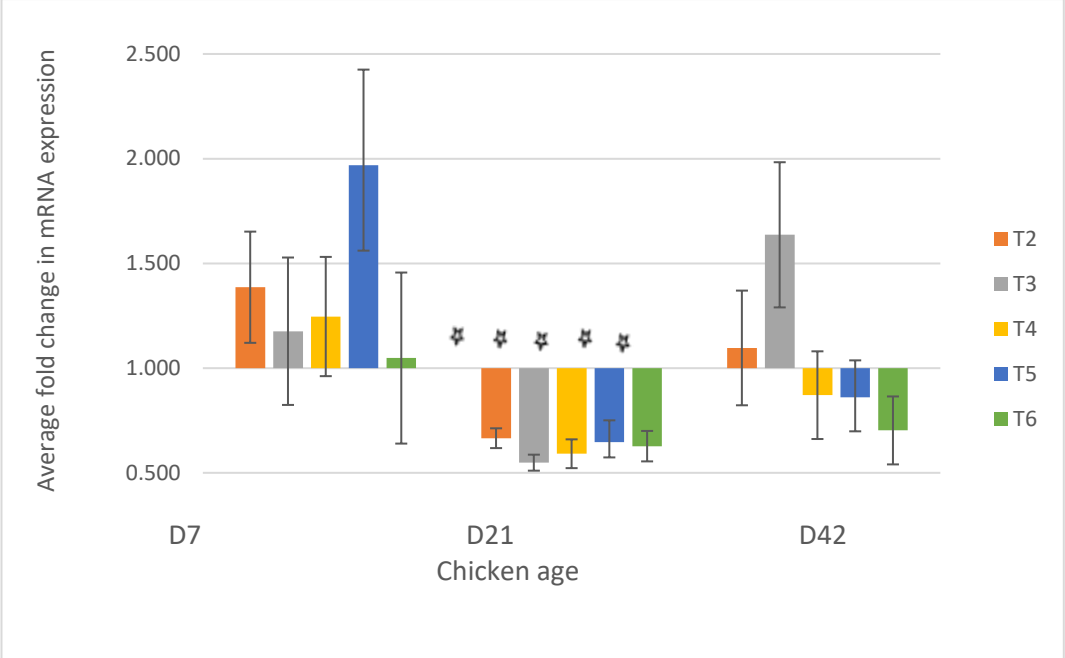

Figure 8:1 Fold change of IFN-γ expression in the ileum tissue at days 7, 21 and 42 of the age of chicks fed prebiotic, probiotic, and synbiotic. T1 (control).

T1 (Control), birds that fed basal diet displayed as 1 on axis. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of fold change (2^-ΔΔCt) ± S.E (n=6) in the mRNA level of cytokines in comparison to those from control. (*) indicates significant difference compared to control at (P≤0.05).

Figure 8:2 shows the effects of pre, pro, and synbiotic supplements on the fold change of IL-10 expression in the ileum tissue at days 7, 21 and 42. The level of IL-10 was significantly (P≤0.05) low in the ileum tissues of birds at ages 7 and 21 days for all treatments apart from T4 (probiotic) at day 7 and T2 (5% prebiotic) at day 21 as the differences were not significant (P≤0.05) at these treatments. At day 42 of age, the supplements have no effects on the levels of IL-10 expression, as the levels in the prebiotic and probiotic were close to the level of all treatments and control. However, IL-10 expression in tissues from birds fed the synbiotic with the high level of prebiotic (10%) was lower than the control, however, it was not significant (*P*≤0.05).

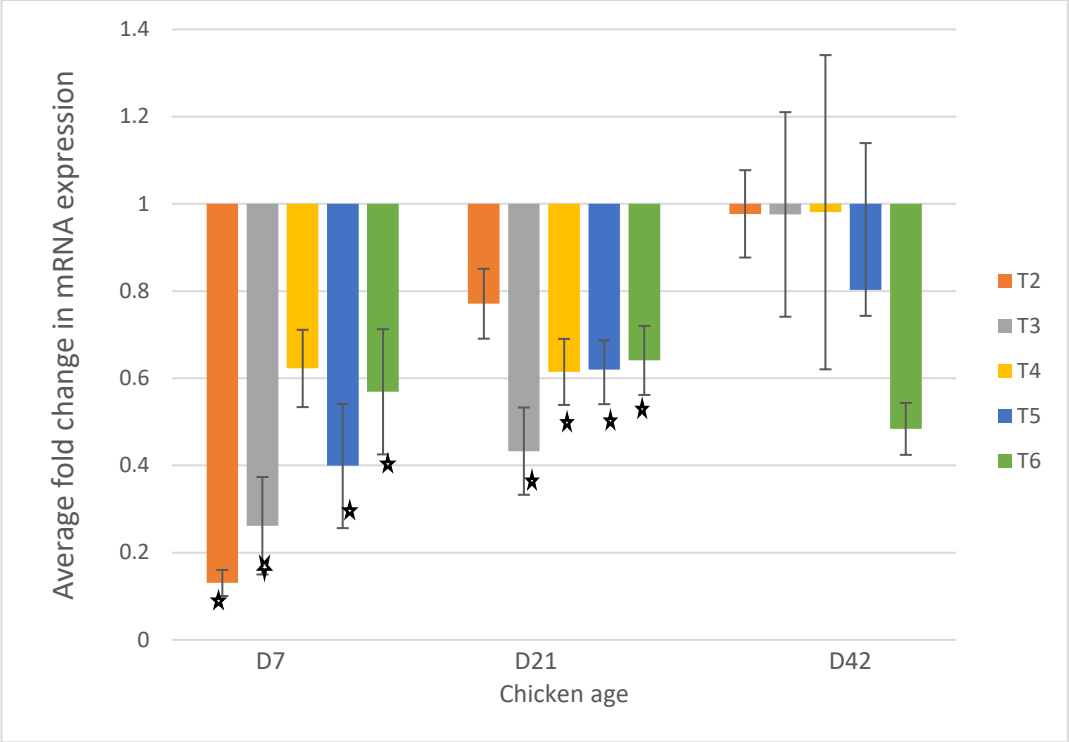

Figure 8:2 Fold change of IL-10 expression in the ileum tissue at days 7, 21 and 42 of the age of chicks fed prebiotic, probiotic, and synbiotic.

T1 (Control), birds that fed basal diet displayed as 1 on axis. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6(synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of fold change (2^-ΔΔCt) ± S.E (n=6) in the mRNA level of cytokines in comparison to those from control. (*) indicates significant difference compared to control at (P≤0.05).

Figure 8:2 shows that in the ileum, there were no significant differences between treatment at day 21 and day 42 in the fold change of IL-10 expression of the ileum tissue. Which showed a high level of variability between replicates (n=6). However, at day 7, all supplemented groups showed a significant (P≤0.05) different in IL-6 expression compared to the control group (P≤0.01) although this was consistent across all the supplemented groups.

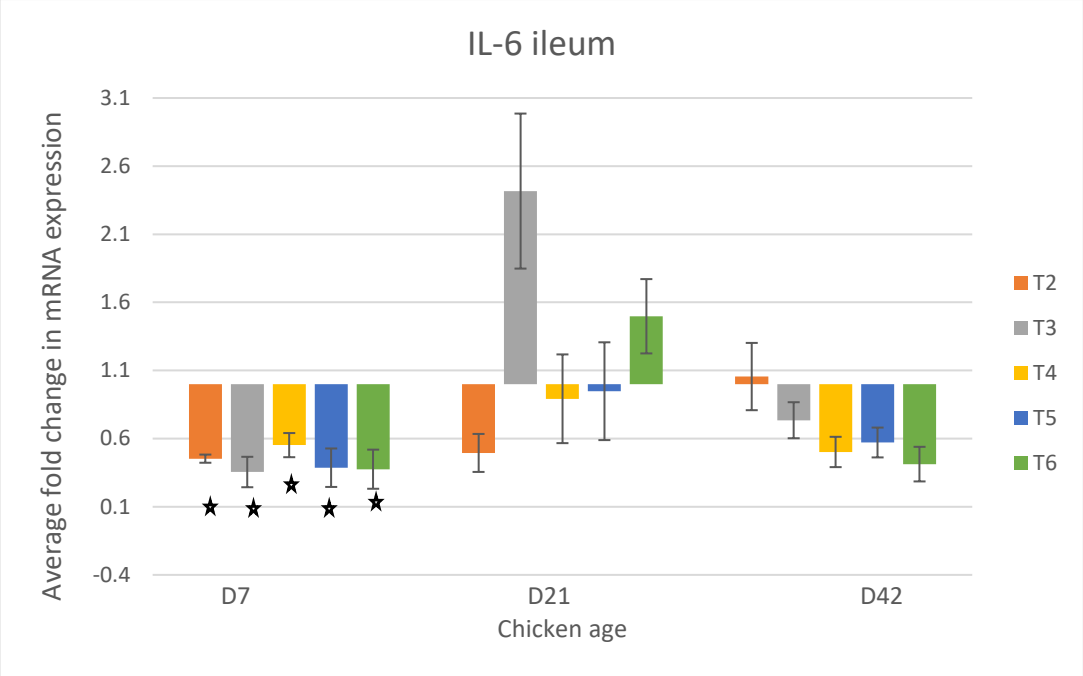

Figure 8:3 Fold change of IL-6 expression in the ileum tissue at days 7, 21 and 42 of the age of chicks fed prebiotic, probiotic, and synbiotic.

T1 (Control), birds that fed basal diet displayed as 1 on axis. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of fold change (2^-ΔΔCt) ± S.E (n=6) in the mRNA level of cytokines in comparison to those from controls. (*) indicates significant difference compared to control at (P≤0.05).

8.2.3 The effect of prebiotic, probiotic and synbiotic supplements on the mRNA expression of IFN-γ, IL-10 and IL-6 in the bursa of Fabricius tissue of chicken.

Figures 8:4, 8:5 and 8:6 show the effects of adding prebiotic, probiotic and synbiotic to the diet of chicken at ages 7, 21 and 42 days on the IFN-γ, IL-10 and IL-6 expression in the bursa of Fabricius tissue. Results show that dietary supplements of pre, pro and synbiotic have no effect (P≤0.05) on the level of all these cytokines in the tissue of the bursa at all studied ages of chicken compared to control-fed birds.

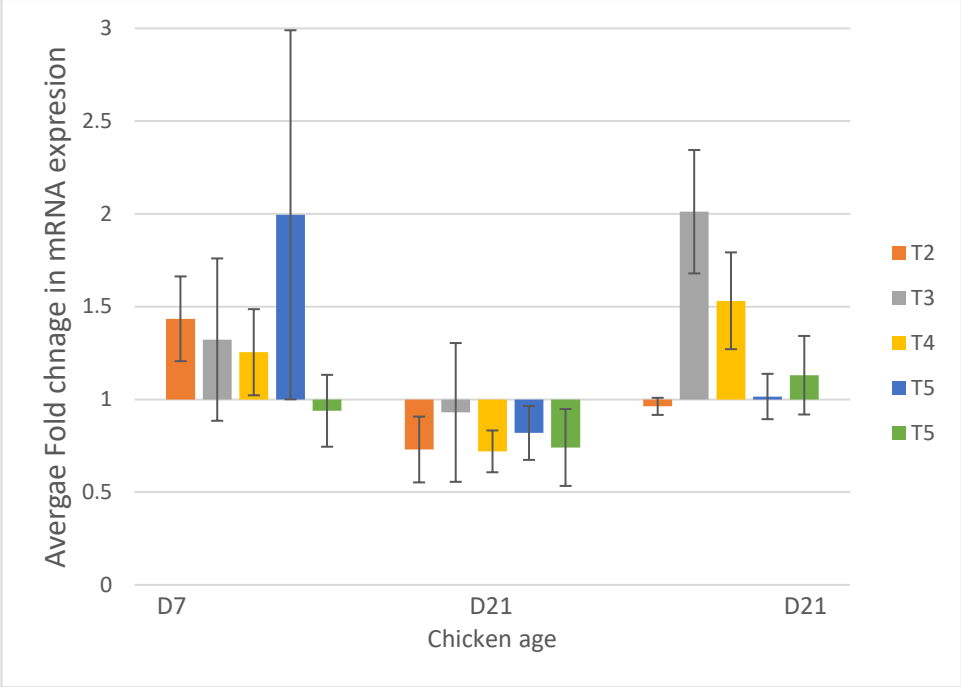

Figure 8:4 Fold change of IFN-γ expression in the Bursa tissue at days 7, 21 and 42 of the age of chicks fed prebiotic, probiotic, and synbiotic.

T1 (Control), birds that fed basal diet displayed as 1 on axis. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of *Lactobacillus*(LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6(synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of fold change (2^-ΔΔCt) ± S.E (n=6) in the mRNA level of cytokines in comparison to those from control.

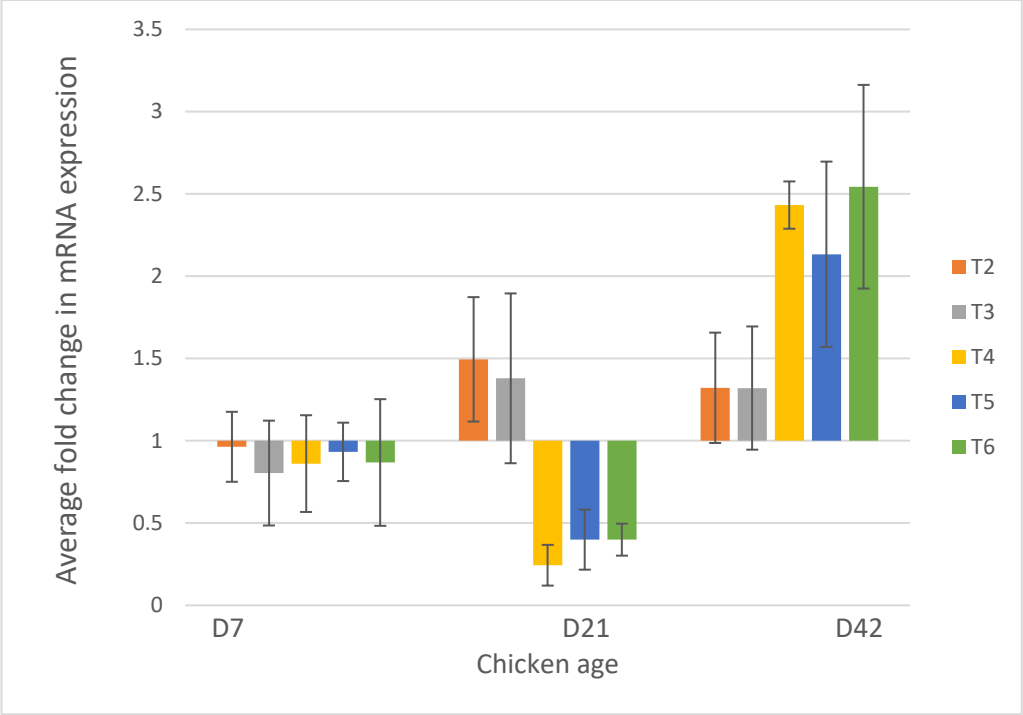

Figure 8:5 Fold change of IL-10 expression in the Bursa tissue at days 7, 21 and 42 of the age of chicks fed prebiotic, probiotic, and synbiotic.

T1 (Control), birds that fed basal diet displayed as 1 on axis. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4(probiotic) birds fed mix of isolates of Lactobacillus(LB) at level 10^9 CFU/kg.T5(synbiotic) birds fed basal diet +LB+5% JA.T6(synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of fold change (2^-ΔΔCt) ± S.D (n=6) in the mRNA level of cytokines in comparison to those from controls

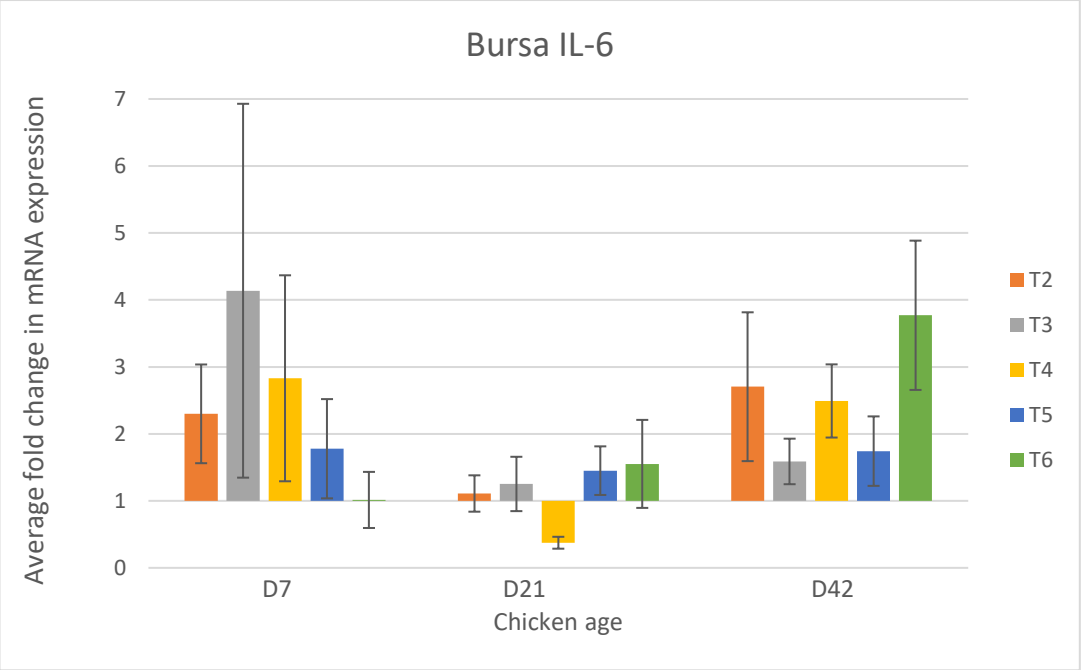

Figure 8:6 Fold change of IL-6 expression in the Bursa tissue at days 7, 21 and 42 of the age of chicks fed prebiotic, probiotic, and synbiotic.

Vertical axis reset to 1 the value of T1, T1 (Control), birds that fed basal diet displayed as 1 on axis. T2 (prebiotic) birds fed 5% JA powder. T3 (prebiotic) birds fed 10% JA powder. T4 (probiotic) birds fed mix of isolates of Lactobacillus (LB) at level 10^9 CFU/kg.T5 (synbiotic) birds fed basal diet +LB+5% JA.T6 (synbiotic) birds fed Basal diet +LB+10%JA. Data are shown as mean of fold change (2^-ΔΔCt) ± S.E (n=6) in the mRNA level of cytokines in comparison to those from control.

8.3 Discussion

8.3.1 Quality and quantity of extracted RNA

Using intact RNA is a key element for the successful application of modern molecular biological methods, like RT-qPCR or microarray analysis. Unlike RNA is highly unstable and susceptible to RNAse degradation ubiquitously present in the environment. Starting with low quality of degraded RNA may strongly compromise the results of downstream applications which are often labour-intensive, time-consuming and highly expensive. The ratio of 260/280 is commonly used as an indicator of the purity of RNA in relation to DNA contamination. For this trial, the majority of extracted RNA were found in the acceptable range (1.8-2) (Biotek.com, 2017). There was no significant difference in RNA quality or quantity between the treatment groups. Checking RNA integrity is a critical step before cDNA synthesis to ensure that DNA is removed for successful mRNA quantification by RT-qPCR (Imbeaud, et al. 2005). In

addition, the majority 260/230 ratios were also found to be in the acceptable range of 2.0-2.2 which is used as a secondary measure of nucleic acid purity. Therefore, the samples passed the quality checks required to be used for cDNA synthesis and qPCR analysis. In addition, RNA extraction provided good yield with a concentration of RNA at minimum yield was 100 ng/µl.

8.3.2 Interferon-gamma (IFN-γ)

Interferon-γ (IFN-γ) is considered to be one of the pro-inflammatory cytokines (Dinarello 2000). It has a pivotal role in host defence, it is considered as a hallmark of innate and adaptive immunity as it is produced in response to infection (Mühl and Pfeilschifter 2003). Here, IFN-γ has been chosen as a marker for immunity response in inflammation in an early stage. (Kaiser, et al. 2000) found that the level of IFN-γ were increased in the chicken tissues that were infected with *Escherichia coli* or strains of *Salmonella* compared with uninfected tissue. The results of this study show that the levels of IFN-γ gene expression in the ileum tissue at day 21 were higher in control than in all treatments significantly ($p \leq 0.05$). Meanwhile, there was no significant difference at days 7 and 42. From these findings; there are two possible mechanisms leading to the observed effects. Firstly, the treatments may have had a direct biochemical effect on the immune system, or the treatments may have indirectly affected the gastrointestinal immune system by modulating the intestinal tract microbiome, which in turn produced metabolites that biochemically altered the immune system. The most likely of these two mechanisms is that IFN-γ has been induced in control and upregulated compared with treatments. This increasing might come as results of the response of immune system cells in the ileum against the pathogenic bacteria. As described in chapter 7, the percentage of *Escherichia/Shigella* was decreased in the treatments when using prebiotic, probiotic and symbiotic, resulting in the birds experiencing a lesser pathogenic challenge. This lower pathogenic challenge in the treatment-fed birds may have resulted in no requirement for the bird to activate the immune system to produce a high level of this cytokines. This findings was also observed in a previous study that studied a probiotic involving a pathogen challenge. Haghighi, et al. (2008) used treatments of *Salmonella* serovar Typhimurium only and *Salmonella* with probiotic of *Lactobacillus*

acidophilus, *Bifidobacterium bifidum*, and *Streptococcus faecalis,* they found that level of IFN-γ in the caeca of chicken was increased in the first treatment, while in the treatment of *Salmonella* with probiotic the level of this interferon was decreased.

8.3.3 Interleukin -10

IL-10 is a cytokine that has an anti-immune and anti-inflammatory activity (Mosser and Zhang 2008). The key role of this cytokine is inhibiting the production and function of pro-inflammatory cytokines, which in turn will regulate the inflammatory responses (Yamana, et al. 2004). It has a crucial role in modulating immune and inflammatory responses during infection with viruses, bacteria, fungi and protozoa (Couper, et al. 2008).

Results of this study have shown that there were significant ($p \leq 0.05$) differences in the levels of IL-10 expression in the ileum tissue, which was higher in the control in contrast with treatments in ileum tissue at days 7 and 21. While there were no significant ($p \leq 0.05$) differences at day 42. It seems that the level of IL-10 has increased at days 7 and 21 in the control compared to treatments. It could be suggested that the supplements have suppressed the pathogens in the gut (ileum) that can induce the production of IL-10 in the treatments. (Cyktor and Turner 2011) indicated that one of the most important roles of IL-10 is to regulate the immunity at the site of infection when it occurs, which means that it will be produced in the case of inflammation or when pathogen exist. Hence, the level of IL-10 was in normal level in the treatments meanwhile was in a high level in the control this is may be because of it was induced by pathogenies.

8.3.4 Interleukine-6 (IL-6)

IL-6 is considered to be multifunctional cytokines in both pro-inflammatory and anti-inflammatory role. It is a keystone cytokine in infection and inflammation, in which it can support the maintenance of reactions of immunity (Hunter and Jones 2015). IL-6 is an inflammatory cytokine, which provides protective role during a bacterial infection (Dube, et al. 2004). From the results showed in figure 8:3, it appears that the level of IL-6 in control was higher than in the treatments in the ileum

at day 7 significantly (p≤0.05). While it seems there were no significant (p≤0.05) differences at days 21 and 42. It seems that level of IL-6 has upregulated in the ileum tissue control of day 7.

The explanation for these findings could that because of all the birds were not challenge with pathogens and because from chapter 7 there was decreased in the *Escherichia*/*Shigella* in the ileum (gut) of treatments, and because of this cytokine will be induced and upregulated in the case of inflammation (Dube, et al. 2004). Therefore, it can argue that in the control this cytokine has induced (high expression in response to pathogen). As discussed previously for the other cytokines, it is likely that, as pathogens were suppressed by the supplements of prebiotic, probiotic and synbiotic in treatment-fed birds, there was no requirement for the treatment-fed birds to mount an immune response.

These findings are consistent with (Huang, et al. 2015) observations when they added the inulin to the diet of the broiler, they found that this supplement caused a decreasing in the level of IFN-γ and IL-6 at day 21 but there were no effects at day 42. These findings also agree with the findings reported by (Janardhana, et al. 2009), who found that there was no difference between control and treatments when they added a prebiotic (fructo-oligosaccharide) to chicken feed. Also, (Brisbin, et al. 2010b) found that *Lactobacillus reuteri* and *Lactobacillus salivarius* did not induce the production of IFN-γ and IL-10 in the caecal tonsil cells of chicken.

(Shang, et al. 2015) found that adding prebiotic (Fructooligosaccharide) to the chicken feed did not induce IL-10 in the ileum tissue compared with control. Meanwhile, these findings do not agree with findings of Yitbarek, et al. (2015) when they used a synbiotic in chicken feed, as they found that IFN-γ was upregulated in the synbiotic treatments compared with control.

The current finding is not consistent with the findings of Kareem, et al. (2017). When they examined the effects of different combinations of inulin and postbiotics (secretions of probiotic) on ileum cytokine expression in the broiler chickens, they found that IFN-γ was upregulated by the addition of the treatments, and IL-6 was downregulated in the tissue of ileum of the broiler. The administration of pre, pro or

synbiotic decreased the inflammation, damaged the tissue of the colon, and induced the secretion of IL-10 in this tissue as well, and downregulated the production of IFN-γ (Foye, et al. 2012).

No significant differences were observed at day 42 for all cytokines and that there was no difference in the percentage of *Escherichia/Shigella* (as a pathogenic indicator) between control and treatments (chapter 7), which suggest the level of immunity was similar in both control and treatments.

There were no significant differences observed in the tissue of bursa this may be as the variation in the levels of mRNA expression of the studied cytokines were high in some replicates, which led to non-significant ($p \leq 0.05$) differences between control and treatments. Also, it might be due to the numbers of replicates were not enough to reach the significance as they were just 6, and because the parameters are individual-related. Indeed, the SEM values for ileal tissue which are represented in the error bars in the graphs for individual genes and times points, and the SEM values for Bursa tissue which are represented in the error bars in the graphs for individual genes and timespoints, suggesting more replication would have increased statistical power, particularly for the Bursa measurements where little change was observed. On the other hand, it could be in relation to the previous chapter 7 as the microbiota was not consistent between the replicates of the same treatment, which might lead to these variances. Also, it is possible that there was no induction of cytokines by the supplements occurred in the tissue of bursa, as seen in the ileum there was no induction of the immune system. Therefore, these supplements did not affect the immunity in the bursa as well.

The immune system requires nutrients for normal development and function as does any other system in the body (Segerstrom 2007, Selvaraj 2012). When the immune system triggered by the infection with a pathogen to defend the body against this infection through production of cytokines and other products, these activities need energy (Segerstrom 2007) which in turn will alter the energy partitioning towards immune system, which will decrease the productivity of animal (Klasing 2007b). Findings of this study revealed that the supplements have downregulated the cytokines expression which in other word that the production of theses cytokines

was decreased in the treatments of supplements comparing with control in which can say the activity of immune system was less in the treatments, therefore the energy that vitalised in these birds was less than in the control.

8.4 Conclusion

The aim of this study was to investigate the effects of supplements of prebiotic, probiotic and synbiotic on the immunity in the tissue of ileum and bursa. It is clear that birds fed these supplements exhibited lower expression of cytokine INF, IL-10 and IL-6 genes via an indirect pathway through inhibition of pathogen colonisation. However, this may not be due to down-regulation: it is clear from chapter 7 that treatment-fed birds had decreases in the level of *Escherichia*/*Shigella* in the caeca (which is close to ileum) so there was no requirement to induce these cytokines to invoke as an inflammatory defence response (Dube, et al. 2004). Therefore, it can be argued that in the control-fed birds, gene expression for these cytokines has been necessarily induced, so they were at a higher level than treatment-fed birds. Also, it is possible to use these findings to support the hypothesis of prebiotic, probiotic and synbiotic can use to reduce/inhibit the pathogenic bacteria in the gut. Reducing the requirement for cytokine production is an important energy-sparing function associated with the use of these pre- pro- and symbiotic supplements. The implications of these findings and their relationship to previous investigations in this project are explored in the final chapter of this thesis.

Chapter nine:

Discussion and conclusion

9.1 Introduction

This chapter is split into four sections to discuss the potential of pre-, pro and synbiotic supplements as a feed ingredient in the broiler. Firstly, the success of the investigations undertaken will be discussed alongside their key findings. Secondly, the impact of these conclusions on global poultry production will be discussed alongside possible future directions for developing their application. Subsequently, key areas for future research and development are outlined and finally, key recommendations based on this work are given.

Concerns over the impact of antibiotic use on human and animal health have led to increased interest in the alternative methods of protecting humans and animals from gastro-intestinal infectious disease. Prebiotic, probiotic and synbiotic supplements have all been shown to provide some level of protection in both humans and animals via different mechanisms. One of the most important actions of all these supplements is capacity to advantageously modify the microflora of the gut.

In the animal production sector, commercial probiotic supplements often contain many genera and a range of different microbial species and even different strains of the same species. The cost of commercial probiotic products is usually justified in one of two ways; either use of the supplement creates a demonstrable improvement in a desirable feature, or it is used as a form of insurance policy against dysbacteriosis – a commonly used term for the poor performance and inflammatory response associated with sub-optimal microbial colonisation of the intestinal tract in the post-antibiotic era (Teirlynck, et al. 2011). In addition to these production-focussed features, there is also a strong desire for the action of probiotic bacteria to include minimisation of *Campylobactor* colonisation in the chicken as carcass contamination during processing of chicken is considered to be the most common cause of food-borne *Campylobacter* poisoning in humans (EFSA, 2014). Alongside probiotic products, plant-derived carbohydrate fractions such as fructo-oligosaccharide (FOS) have been used commercially as prebiotics to indirectly manipulate the gut microflora. Jerusalem artichoke (JA) plant has a relatively high content of this long chain oligosaccharide. JA already been used in chicken diet as a prebiotic and it has been shown that JA can increase the presence of beneficial bacteria in the gut. This

thesis included two studies to examine the bird performance and immunity effects of new isolates of *Lactobacillus* derived from chicken intestine, and further examination into JA plant as a source of prebiotic fed alone and in combination with previously isolated probiotic microbial strains.

9.2 Key findings and critique of investigations

A major challenge throughout this project was achieving baseline *Campylobacter* colonisation of birds. As no Home Office ASPA Licence was in place, it was not possible to inoculate the birds to ensure equal spread of *Campylobacte*r infection across all pens of birds or even all birds within a pen. The inconsistent initial colonisation among the birds made investigations into the impact of pre- pro- and synbiotic interventions on *Campylobacter* levels difficult to achieve. Another issue relating to the lack of ASPA Licence was the cleanliness of the experimental setting. The NTU poultry research unit was a challenging environment for studying colonisation of the poultry intestine due to the rigorous cleaning regime and disinfectants that are used in the unit. This approach to hygiene limits opportunity for the unit itself to harbour reservoirs of pathogens such as *Campylobacter* and the lack of Home Office ASPA licence permitting re-use of dirty litter did not allow any form of robust investigation into methods for reduction of pathogens in the chicken gut.

The first part of this thesis focussed on the strain *Lactobacillus johnsonii* FI9785 in collaboration with the Institute of Food Research (IFR). Previous work by the IFR had isolated and examined this strain as probiotic agent to be used in chicken diet (Mañes-Lázaro, et al. 2017). The initial IFR investigations worked on this strain to examine its ability to cope with environmental stress, and to measure intestinal colonisation of birds housed in individual laboratory incubators. Their findings prompted them to approach NTU in order to test FI9785 in birds housed in a more commercially relevant setting to investigate whether these bacteria can improve the GIT health of chicken and reduce the level of *Campylobacter*.

Three experiments were designed to determine the efficacy of feeding *Lactobacillus johnsonii* FI9785 as a probiotic to broiler chicks. The first study was in vitro, to investigate whether any of the environmental conditions associated with each

proposed delivery route for the probiotic would be detrimental to survivability of the microbes. Results found that water left standing overnight and distilled water are the best water-borne methods to deliver the bacteria while maintaining high viability of *Lactobacillus*. However, widespread use of sanitising agents such as CID2000^{TM} (CID Lines, Belgium) indicated feed should also be assessed as an alternative delivery route. The following in vivo studies, commenced with a pilot study, conducted to examine the most appropriate method of colonising the intestinal tract of the birds. Assessment of colonisation of several sections of the intestinal tract showed feed was the most appropriate method to deliver these bacteria into chicken gut. Subsequently, a larger scale chicken experiment was performed to monitor growth performance as well as the level of *Campylobacter* colonisation in birds fed LB for 7 days compared with a control group without probiotic over a 28 day trial period. Results of this third experiment revealed that FI9785 strain did not affect the performance of birds but there was no significant difference in the level of *Campylobacter*. From this study it was concluded that it is possible to produce a probiotic agent by isolating beneficial bacteria and feed was a good delivery route for colonisation of chicken gut. However the work was stopped by the sponsor (IFR) without further investigations into whether control and treated birds were colonised different strains of *Lactobacillus*.

Working with external collaborators in the early stages of the projects opened an interesting investigative opportunity but ultimately created a barrier to progressing logically through this programme of work. Collaboratively assessing a scientifically well-developed novel strain of *Lactobacillus johnsonii* bacteria (FI9785) give insight into the assessment stages of a candidate probiotic but waiting for the leading party to make decisions or provide information was difficult when time for this project was limited. Ultimately, a key piece of information (the PCR primers for FI9785 strain) was never provided so that the conclusions from these studies lack a definitive answer as to the degree of colonisation by strain FI9785 in comparison to wild type *Lactobacillus* strains.

The second part of this thesis focussed on in-house development of pre- pro and synbiotic supplements. The majority of the work was to isolate *Lactobacillus* strains from apparently healthy out door chickens at NTU and then assess them both in vitro

and in vivo as candidate probiotic agents in chicken feed. First, six strains of *Lactobacillus* were isolated from adults chicken and examined in vitro for potential as probiotic agents: morphological, biochemical and antiprogram tests confirmed that these isolates were all belonged to Lactic acid bacteria. In addition, genetic testing (16s) confirmed that the isolates were under the *Lactobacillus* genus. Also physiological tests examined these isolates for survivability in the conditions of gut, which confirmed that all isolates were able to retain their viability in conditions designed to mimic the gut. The isolates also showed in vitro antimicrobial activity against *Campylobacter*. Finally, physiological tests confirmed that all six isolates could survive and maintain their viability after processing, which is essential for commercial application of these strains as probiotic agents. It was concluded that the techniques used to process theses bacteria were successful and can be universally applied for production of probiotic bacteria. A weakness to this section of work was that the survivability in feed of each isolated strain not measured before bird feeding trials were conducted. Molecular assessment to confirm exactly which isolate had been produced would have then allowed for in-feed assessment of survivability, but the time and cost of undertaking this work was prohibitive, so in vivo trials were conducted without confirming in feed survivability.

Preparation of potential suitable supplements included the sourcing and preparation of Jerusalem artichoke (JA) plant as a prebiotic source material. The plant was prepared and dried, then the inulin content was measured. It was found that JA contains a relatively high concentration of Inulin as half of the dried plant was found to be inulin, suggesting this widely available plant could potentially be used as a prebiotic in chicken feed. In addition, all *Lactobacillus* isolates can use JA as a carbon sources as it was found that they all can grow in the media enriched with JA instead of glucose. This finding suggested that JA had additional potential to be mixed as a prebiotic with the *Lactobacillus* isolates to produce a synbiotic.

The second section of this study was a major in-vivo experiment conducted to examine the effects of the supplements prepared with different levels of prebiotic alone, or in mixed with probiotic (synbiotic) or probiotic alone in chicken feed. The parameters studied included their effects on the gut profile, immune function through cytokines and chicken growth performance. Limited expertise and time did

not allow more thorough investigations into immune response towards the chosen probiotic and prebiotic supplements.

The effects of dietary supplements of prebiotic, probiotic and synbiotic in chicken feed on the caeca microflora showed that there were effects on the microbial community of the gut. A major effect was on *faecalibacterium* genus, which was higher in treatments compared with control at day 7. This genus was previously shown to have some positive effects on performance of chicken (Stanley, et al. 2016, Fak and Backhed 2012). However, this study did not present a correlation between this genus and performance, as there were no significant differences between control and treatments in the body weight, body weight gain, feed intake and FCR. These non-effects of probiotic supplements on the performance of chicken are conflicted with the findings of (Stanley, et al. 2016) as they argued that this genus is directly related to improve FCR in meat chickens. The level of genus of *Erysipelotrichaceae* was decreased in all treatments which may be considered a positive response, as a previous study (Palm, et al. 2014) argued that there is a correlation between this genus and illness in humans. In addition, genera of *Escherichia/Shigella* were also in lower abundance in the supplements treatments than the control. This suggests that supplements of prebiotic, probiotic and synbiotic have affected the growth of this genera, possibly through similar mechanisms to those that inhibit pathogenic bacteria such as SCFA production, bacteriocin production or competitive exclusion. In addition, the cytokine data indicates that the inflammation in the chicken fed treated feeds was lower than in control-fed birds. Far more data is available from the 16s rRNA metagenomic screening than that covered in this thesis, as the relative new-ness of the technique and limited time available to gain expertise in bioinformatics. Future work that which could produce more data outputs relating to these supplements but would not require any further practical investigations would be wider bioinformatic analysis of the 16s data.

Results of this study showed that while prebiotic supplements did not affect the performance of chicken, the effects of probiotic were substantially negative. However, the effects of these supplements on the immune function were similar, suggesting therefore use of prebiotic alone is the best practical option to improve the health with no effects on the performance. The probiotic treatments are worthy

of further investigation, as they did positively affected the health of chicken, but they caused degradation in the performance. Key investigations are either using single strains to determine their individual effects or using a lower supplementation level of the mix, as may be the concentration used was too high or one or more of the strains in the mix had a negative effects on the production performance. Future bird trials could also address a major weakness in the current study: limitations to the size of the bird trial (number of available pens) that could be conducted at NTU prevented any benchmarking against a commercially available supplement. Another positive, health-related aspect of these supplements that can argued here is that the effect on the pathogen inhibition or cytokines regulation was local and not systemic, as the observed modulatory effects were only on the ileal cytokines while there was no significant difference in the level of cytokines in the Bursa tissue.

In summary, the prebiotic and probiotic supplements were equally effective at improving on gut health and immunity but prebiotic production is cheap, does not need extensive processing, and can be stored at room temperature. Therefore it can concluded that focusing on prebiotic development in the future may be the best way to improve the microbiota in the animal gut in a commercially viable way with minimum risk to health and safety.

9.3 Potential impact of this project

From this thesis, it has been shown that there is an economical viable route to implementing use of probiotic or prebiotic. However, there some limiting factors associated with production of probiotic supplements that must be considered. Firstly, the production of probiotics does require some investment in basic laboratory equipment. To produce a locally appropriate probiotic supplement for a given poultry production area of a developing country, it is necessary to buy the following: a large volume centrifuges (minimum 250ml buckets), a freeze drier, a bacterial fermenter and access to a basically equipped laboratory (such as clean benches, glassware, scales). A microaerobic cabinet was used in this study as it already available in the lab and because of *Lactobacillus* genera are facultative anaerobic and often they grow better under microaerobic conditions (Goldstein, Tyrrell and Citron 2015b).

A further consideration impacting on potential success in probiotic supplement manufacture is the availability of an appropriately qualified microbiologist to ensure all skilled procedures are carried out correctly, and also to perform screening of batches as a final quality control measure. If incubation is not carried out correctly and a contaminated product is made, this could cause a disease outbreak in the birds fed the supplement and the negative consequences would greatly outweigh any potential benefit, but implementing a simple screening procedure by culturing each batch produced would avoid this risk. Similarly, if freeze drying is not carried out correctly, the bacterial cells will die before delivery to the bird intestine, leading to poor product efficacy, which will have a negative economic impact.

Artichoke-derived prebiotic does not require major financial investment in its production, as the fresh plant cost about £0.75 per kg and because it only needs simple (low tech) processing such as washing and drying can be achieved cheaply in warm climates. Also artichoke-derived prebiotic does not require low temperature for storage, as room temperature (25°C) is sufficient to keep retain its bio-activity. However, exact guidelines for Jerusalem Artichoke preparation techniques that are viable in a field setting in Iraq need to be developed to ensure the drying temperature remains below levels (80°C) known to damage JA inulin levels (Kriukova, et al. 2018)

9.4 Recommendations for practical application of these findings:

1- It seem that prebiotic was more effective than probiotic and synbiotic from the results of this study in which can say that using it more cost effective compering with probiotic.

2- Throughout the rearing period, use of a prebiotic supplement such as Jerusalem artichoke provides a cost effective method of maintaining a healthy intestinal microbiome which in turn can maintain the health and reduce the antibiotic use in broiler feed.

3- Creating mixtures of probiotic microbial strains from local flocks of birds showing high health status which is seem to be an effective way of using probiotics in poultry. Use of locally ‘successful’ strains which can compete with the pathogenic bacteria that are common in the Iraqi farms which in turn can decreased the use of antibiotics and improve the health and performance of chicken.

4- In the immediate post hatch period, an appropriate mixture of probiotic microbial strains should be used to prevent any pathogenic colonisation during this period of high vulnerability of the chicks.

9.5 Future directions for the field of gut health in poultry

A major barrier to widespread acceptation of synbiotic use is occurrences where a farmer experience an apparent failure of their flock to respond to the supplements. This phenomenon is strongly linked to the unique and dynamic microbial populations associated with each poultry shed: prescribing use of a generic, unspecified strain of bacteria is less likely to aid the farm in ensuring good intestinal health than creating a bespoke solution for that farm. By understanding the existing microbial population (both pathogenic and benign species), of a geographical region, or even of a given shed, it may be possible to create a bespoke probiotic or synbiotic supplement exactly meeting the requirements of the situation.

Some potential routes to reducing use of antibiotics in poultry production are hampered by current legislation. It is well established that diversity in the microbiome reduces the risk of poor gut health (Human Microbiome Project Consortium 2012) and there is a route to colonising the intestine of chicks at placement in the shed by leaving in some litter from a previous, healthy batch of birds. However, current EU legislation requiring the removal of all litter and the implementation of a cleaning regime between batches of birds prevents this option being followed. While this EU legislation reduces risk of pathogenic bacteria being passed on following a batch of birds with poor gut health, it also prevents any benefits being conferred from one batch to the next. A screening programme at the end of the growth period to assess whether litter should be removed and a complete clean implemented However, use of probiotic feed additives such that those proposed in this thesis provide a more viable route to the same result: ensuring the intestinal is appropriately colonised as quickly as possible post hatch.

Even with the stringent cleaning regimes currently in place, the residual microorganisms in the shed impact on gut colonisation to a varying degree. Colonisation of the chick gut as soon as possible with benign microbes reduces the risk of colonisation by a pathogenic species. This mechanism is currently being explored in some commercial hatcheries where viability of adding probiotics via in *ovo* injection in the last three days of egg incubation (de Oliveira, et al. 2014). The uncertainty over whether a pathogenic species of microbe will colonise the intestine

introduces different views on the usefulness of pre- and probiotic supplements: as there is no guarantee that using a supplement will improve performance of the birds, many farmer chose not to do so, while others view their use as a form of insurance policy.

In summary, when comparing between cost of production of Jerusalem artichoke plant as prebiotic to improve the health as it does not need much preparation and because no side effects on the performance. Production of probiotic need developed facilities in addition to some negative effects on the performance therefore it can be conducted that using Jerusalem artichoke plant is a more effective way to manipulate the gut microflora, which can improve the health of chicken.

This study can recommended the use of JA plant as prebiotic in chicken feed as it improved the immunity and decreased the level of some pathogenic bacteria without effects on the performance.

9.6 Future research

1- More in-vitro investigations on the six isolated candidate probiotic need to be carried out to optimise their potential for use in chickens. In particular, measuring SCFA production by supplementing culture media with different prebiotics sources would give insight into which prebiotic sources would most efficaciously combine to form the best synbiotic.

2- The project examined only one common poultry gut pathogen; *Campylobacte*r. Understanding the inhibition activity of the six isolates against *Salmonella* and *E.coli* is also extremely important when considering the isolates as candidate probiotic strains.

3- Further experiments need to be done to study the effect of these isolates individually in the chicken feed to investigate which isolate showed the most positive activity in enhancing gut health and immune function, and which caused the negative effects on chicken performance so, these could be excluded.

4- Tracking the survivability of the six isolates throughout the chicken gut also should be undertaken to assess whether they all can survive in the gut conditions. This could be achieved by sequencing the whole genome of each isolates and then designing a unique primer for each isolate.

5- Determining the concentration of SCFA produced in the gut of chicken should be done to quantify the energy-related effects of these supplements of prebiotic, probiotic and synbiotic, as volatile acids such as butyric acid are the important end products of some microorganisms in the gut whose beneficial effects on health and performance of chicken are through providing an energy source for direct use by intestinal epithelial cells.

6- These supplements need to be assessed in the chicken diet using challenge studies where a dosages of pathogenic bacteria such as *Campylobacter* and *Salmonella* spp are used to get clear picture on the inhibition activity in vivo against each major pathogen, as all birds in the current studies were *Campylobacter* free throughout.

7- Finally, the supplements need to be studied in the layer and meat breeding flocks as other parameters than body weight are important in these settings, such as disease resistance throughout lay. Also, controlling the body weight of meat birds before they come into lay is important to maintain health during egg production.

REFERENCES

Abdel-Hafeez, H.M., Saleh, E.S.E., Tawfeek, S.S., Youssef, I.M.I. and Abdel-Daim, A.S.A., 2017. Effects of probiotic, prebiotic, and synbiotic with and without feed restriction on performance, hematological indices and carcass characteristics of broiler chickens. *Asian-Australasian Journal of Animal Sciences,* 30 (5), 672-682.

Abou-Arab, A., Talaat, H.A. and Abu-Salem, F., 2011. Physico-chemical properties of inulin produced from Jerusalem artichoke tubers on bench and pilot plant scale. *Aust.J.Basic Appl.Sci.,* 5 (5), 1297-1309.

Alemka, A., Clyne, M., Shanahan, F., Tompkins, T., Corcionivoschi, N. and Bourke, B., 2010. Probiotic colonization of the adherent mucus layer of HT29MTXE12 cells attenuates Campylobacter jejuni virulence properties. *Infection and Immunity,* 78 (6), 2812-2822.

Alkhalf, A., Alhaj, M. and Al-Homidan, I., 2010. Influence of probiotic supplementation on blood parameters and growth performance in broiler chickens. *Saudi Journal of Biological Sciences,* 17 (3), 219-225.

Allen, H.K., and Stanton, T.B., 2014. Altered egos: antibiotic effects on food animal microbiomes. *Annual Review of Microbiology,* 68, 297-315.

Alloui, N., Szczurek, W. and Swiatkiewicz, S., 2013. The Usefulness of Prebiotics and Probiotics in Modern Poultry Nutrition: a Review. *The Journal of National Research Institute of Animal Production,* 13, 17-32.

Amit-Romach, E., Sklan, D. and Uni, Z., 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Science,* 83 (7), 1093-1098.

Amsen, D., de Visser, K.E. and Town, T., 2009. Approaches to determine expression of inflammatory cytokines. *Methods in Molecular Biology (Clifton, N.J.),* 511, 107-142.

Apajalahti, J., 2005. Comparative Gut Microflora, Metabolic Challenges, and Potential Opportunities. *The Journal of Applied Poultry Research,* 14 (2), 444-453.

Apajalahti, J., Kettunen, A. and Graham, H., 2004a. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal,* 60 (2), 223-232.

Apajalahti, J., Kettunen, A. and Graham, H., 2004b. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal,* 60 (2), 223-232.

Apajalahti, J., and Vienola, K., 2016. Interaction between chicken intestinal microbiota and protein digestion. *Animal Feed Science and Technology; Protein*

Nutrition in Non-Ruminants and the Implications for Exogenous Protease Use, 221, 323-330.

Apolinário, A.C., de Lima Damasceno, Bolívar, Ponciano Goulart, de Macêdo Beltrão, Napoleão Esberard, Pessoa, A., Converti, A. and da Silva, J.A., 2014. Inulin-type fructans: A review on different aspects of biochemical and pharmaceutical technology. *Carbohydrate Polymers,* 101, 368-378.

Ashraf, R., and Smith, S.C., 2016. *Commercial lactic acid bacteria and probiotic strains- tolerance to bile, pepsin and antibiotics.*

Awad, W.A., Ghareeb, K., Abdel-Raheem, S. and Bohm, J., 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. *Poultry Science,* 88 (1), 49-56.

Bartholomew, J.W., and Mittwer, T., 1952. The Gram stain. *Bacteriological Reviews,* 16 (1), 1-29.

Beasley, D.E., Koltz, A.M., Lambert, J.E., Fierer, N. and Dunn, R.R., 2015. The Evolution of Stomach Acidity and Its Relevance to the Human Microbiome. *Plos One,* 10 (7), e0134116.

Bjerrum, L., Engberg, R.M., Leser, T.D., Jensen, B.B., Finster, K. and Pedersen, K., 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poultry Science,* 85 (7), 1151-1164.

Biotek, (2018) https://www.biotek.com/

Bless, P.J., Muela Ribera, J., Schmutz, C., Zeller, A. and Mausezahl, D., 2016. Acute Gastroenteritis and Campylobacteriosis in Swiss Primary Care: The Viewpoint of General Practitioners. *PloS One,* 11 (9), e0161650.

Brisbin, J.T., Gong, J., Parvizi, P. and Sharif, S., 2010a. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. *Clinical and Vaccine Immunology: CVI,* 17 (9), 1337-1343.

Brisbin, J.T., Gong, J., Parvizi, P. and Sharif, S., 2010b. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. *Clinical and Vaccine Immunology : CVI,* 17 (9), 1337-1343.

Brisbin, J.T., Gong, J. and Sharif, S., 2008a. Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews,* 9 (1), 101-110.

Brisbin, J.T., Gong, J. and Sharif, S., 2008b. Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews,* 9 (1), 101-110.

Buclaw, M., 2016. The use of inulin in poultry feeding: a review. *Journal of Animal Physiology and Animal Nutrition,* 100 (6), 1015-1022.

Burkholder, K.M., Thompson, K.L., Einstein, M.E., Applegate, T.J. and Patterson, J.A., 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers. *Poultry Science,* 87 (9), 1734-1741.

Callaway, T.R., Edrington, T.S., Anderson, R.C., Harvey, R.B., Genovese, K.J., Kennedy, C.N., Venn, D.W. and Nisbet, D.J., 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Animal Health Research Reviews,* 9 (2), 217-225.

Casteleyn, C., Doom, M., Lambrechts, E., Van, d.B., Simoens, P. and Cornillie, P., 2010. Locations of gut-associated lymphoid tissue in the 3-month-old chicken: a review. *Avian Pathology,* 39 (3), 143-150.

Caza, M., and Kronstad, J.W., 2013. Shared and distinct mechanisms of iron acquisition by bacterial and fungal pathogens of humans. *Frontiers in Cellular and Infection Microbiology,* 3, 80.

Celasco, G., Moro, L., Aiello, C., Mangano, K., Milasi, A., Quattrocchi, C. and DI Marco, R., 2014. Calcium butyrate: Anti-inflammatory effect on experimental colitis in rats and antitumor properties. *Biomedical Reports,* 2 (4), 559-563.

Celi, P., Cowieson, A.J., Fru-Nji, F., Steinert, R.E., Kluenter, A.-. and Verlhac, V., 2017. Gastrointestinal functionality in animal nutrition and health: New opportunities for sustainable animal production. *Animal Feed Science and Technology,* 234, 88-100.

Chaveerach, P., Keuzenkamp, D.A., Lipman, L.J. and Van Knapen, F., 2004. Effect of organic acids in drinking water for young broilers on Campylobacter infection, volatile fatty acid production, gut microflora and histological cell changes. *Poultry Science,* 83 (3), 330-334.

Chen, W., Liu, F., Ling, Z., Tong, X. and Xiang, C., 2012. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PloS One,* 7 (6), e39743.

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

Clavijo, V., and Florez, M.J.V., 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. *Poultry Science,* 97 (3), 1006-1021.

Clench, M.H., 2015. The avian cecum: Update and motility review. *Journal of Experimental Zoology,* 283 (4), 441-447.

Cohen, A., Klasing, K. and Ricklefs, R., 2007. Measuring circulating antioxidants in wild birds. *Comparative Biochemistry and Physiology.Part B, Biochemistry & Molecular Biology,* 147 (1), 110-121.

Collado, M.C., Gonzalez, A., Gonzalez, R., Hernandez, M., Ferrus, M.A. and Sanz, Y., 2005. Antimicrobial peptides are among the antagonistic metabolites produced by Bifidobacterium against Helicobacter pylori. *International Journal of Antimicrobial Agents,* 25 (5), 385-391.

Cook, M.T., Tzortzis, G., Charalampopoulos, D. and Khutoryanskiy, V.V., 2012. Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release : Official Journal of the Controlled Release Society,* 162 (1), 56-67.

Cortón, E., Piuri, M., Battaglini, F. and Ruzal, S.M., 2000. Characterization of Lactobacillus Carbohydrate Fermentation Activity Using Immobilized Cell Technique. *Biotechnology Progress,* 16 (1), 59-63.

Coudray, C., Rambeau, M., Feillet-Coudray, C., Tressol, J.C., Demigne, C., Gueux, E., Mazur, A. and Rayssiguier, Y., 2005. Dietary inulin intake and age can significantly affect intestinal absorption of calcium and magnesium in rats: a stable isotope approach. *Nutrition Journal,* 4, 29-2891-4-29.

Couper, K.N., Blount, D.G. and Riley, E.M., 2008. IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology,* 180 (9), 5771.

Cressman, M.D., Yu, Z., Nelson, M.C., Moeller, S.J., Lilburn, M.S. and Zerby, H.N., 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Applied and Environmental Microbiology,* 76 (19), 6572-6582.

Crhanova, M., Hradecka, H., Faldynova, M., Matulova, M., Havlickova, H., Sisak, F. and Rychlik, I., 2011. Immune response of chicken gut to natural colonization by gut microflora and to Salmonella enterica serovar enteritidis infection. *Infection and Immunity,* 79 (7), 2755-2763.

Cyktor, J.C., and Turner, J., 2011. Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens. *Infection and Immunity,* 79 (8), 2964-2973.

Daliri, E.B., and Lee, B.H., 2015. New perspectives on probiotics in health and disease. *Food Science and Human Wellness,* 4 (2), 56-65.

De Cesare, A., Sirri, F., Manfreda, G., Moniaci, P., Giardini, A., Zampiga, M. and Meluzzi, A., 2017. Effect of dietary supplementation with Lactobacillus acidophilus D2/CSL (CECT 4529) on caecum microbioma and productive performance in broiler chickens. *PloS One,* 12 (5), e0176309.

de Oliveira, J.E., van, d.H., van, d.L., Montijn, R.C. and van, d.V., 2014. In ovo inoculation of chicken embryos with probiotic bacteria and its effect on posthatch Salmonella susceptibility. *Poultry Science,* 93 (4), 818-829.

de Vrese, M., and Schrezenmeir, J., 2008. Probiotics, prebiotics, and synbiotics. *Advances in Biochemical Engineering/Biotechnology,* 111, 1-66.

Dec, M., Puchalski, A., Urban-Chmiel, R. and Wernicki, A., 2014. Screening of Lactobacillus strains of domestic goose origin against bacterial poultry pathogens for use as probiotics. *Poultry Science,* 93 (10), 2464-2472.

Delzenne, N.M., and Cani, P.D., 2011. Interaction between obesity and the gut microbiota: relevance in nutrition. *Annual Review of Nutrition,* 31, 15-31.

Deplancke, B., and Gaskins, H.R., 2001. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *The American Journal of Clinical Nutrition,* 73 (6), 1131S-1141S.

Deriu, E., Liu, J., Pezeshki, M., Edwards, R., Ochoa, R., Contreras, H., Libby, S., Fang, F. and Raffatellu, M., 2013. Probiotic Bacteria Reduce Salmonella Typhimurium Intestinal Colonization by Competing for Iron. *Cell Host & Microbe,* 14 (1), 26-37.

Dertli, E., Mayer, M.J. and Narbad, A., 2015. Impact of the exopolysaccharide layer on biofilms, adhesion and resistance to stress in Lactobacillus johnsonii FI9785. *BMC Microbiology,* 15 (1), 8.

Dibaji, S.M., Seidavi, A., Asadpour, L. and Moreira, d.S., 2014. Effect of a synbiotic on the intestinal microflora of chickens1. *The Journal of Applied Poultry Research,* 23 (1), 1-6.

Dinarello, C.A., 2000. Proinflammatory cytokines. *Chest,* 118 (2), 503-508.

Dinh, D.M., Volpe, G.E., Duffalo, C., Bhalchandra, S., Tai, A.K., Kane, A.V., Wanke, C.A. and Ward, H.D., 2015. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. *The Journal of Infectious Diseases,* 211 (1), 19-27.

Doleyres, Y., and Lacroix, C., 2005. Technologies with free and immobilised cells for probiotic bifidobacteria production and protection. *International Dairy Journal,* 15, 973-988.

Donaldson, E.E., Stanley, D., Hughes, R.J. and Moore, R.J., 2017. The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs. *PeerJ,* 5, e3587.

Donalson, L.M., McReynolds, J.L., Kim, W.K., Chalova, V.I., Woodward, C.L., Kubena, L.F., Nisbet, D.J. and Ricke, S.C., 2008. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation,

Salmonella enteritidis infection, and intestinal shedding in laying hens. *Poultry Science,* 87 (7), 1253-1262.

Dube, P.H., Handley, S.A., Lewis, J. and Miller, V.L., 2004. Protective role of interleukin-6 during Yersinia enterocolitica infection is mediated through the modulation of inflammatory cytokines. *Infection and Immunity,* 72 (6), 3561-3570.

Duke, G.E., 1986. Alimentary Canal: Anatomy, Regulation of Feeding, and Motility. *In:* P.D. Sturkie, ed., *Avian Physiology.* New York, NY: Springer New York, 1986, pp. 269-288.

Eckert, N.H., Lee, J.T., Hyatt, D., Stevens, S.M., Anderson, S., Anderson, P.N., Beltran, R., Schatzmayr, G., Mohnl, M. and Caldwell, D.J., 2010. Influence of probiotic administration by feed or water on growth parameters of broilers reared on medicated and nonmedicated diets. *The Journal of Applied Poultry Research,* 19 (1), 59-67.

Edens, F., 2003. *An alternative for antibiotic use in poultry: Probiotics.*

Egshatyan, L., Kashtanova, D., Popenko, A., Tkacheva, O., Tyakht, A., Alexeev, D., Karamnova, N., Kostryukova, E., Babenko, V., Vakhitova, M. and Boytsov, S., 2016. Gut microbiota and diet in patients with different glucose tolerance. *Endocrine Connections,* 5 (1), 1-9.

Fak, F., and Backhed, F., 2012. Lactobacillus reuteri prevents diet-induced obesity, but not atherosclerosis, in a strain dependent fashion in Apoe-/- mice. *PloS One,* 7 (10), e46837.

Ferens, W.A., and Hovde, C.J., 2011. Escherichia coli O157:H7: animal reservoir and sources of human infection. *Foodborne Pathogens and Disease,* 8 (4), 465-487.

Fijan, S., 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. *International Journal of Environmental Research and Public Health,* 11 (5), 4745-4767.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S. and Gil, A., 2013. Sources, isolation, characterisation and evaluation of probiotics. *The British Journal of Nutrition,* 109 Suppl 2, S35-50.

Forte, C., Manuali, E., Abbate, Y., Papa, P., Vieceli, L., Tentellini, M., Trabalza-Marinucci, M. and Moscati, L., 2018. Dietary Lactobacillus acidophilus positively influences growth performance, gut morphology, and gut microbiology in rurally reared chickens. *Poultry Science,* 97 (3), 930-936.

Foye, O.T., Huang, I.F., Chiou, C.C., Walker, W.A. and Shi, H.N., 2012. Early administration of probiotic Lactobacillus acidophilus and/or prebiotic inulin attenuates pathogen-mediated intestinal inflammation and Smad 7 cell signaling. *FEMS Immunology and Medical Microbiology,* 65 (3), 467-480.

Fukata, T., Sasai, K., Miyamoto, T. and Baba, E., 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on Salmonella colonization of chicks. *Journal of Food Protection,* 62 (3), 229-233.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., Suzuki, T., Taylor, T.D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M. and Ohno, H., 2011. Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature,* 469 (7331), 543-547.

Fuller, R., 1989. Probiotics in man and animals. *The Journal of Applied Bacteriology,* 66 (5), 365-378.

Gabriel, I., LESSIRE, M., MALLET, S. and GUILLOT, J.F., 2006. Microflora of the digestive tract: critical factors and consequences for poultry. *World's Poultry Science Journal,* 62 (3), 499-511.

Gaggia, F., Mattarelli, P. and Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology,* 141 Suppl 1, S15-28.

Ghareeb, K., Awad, W.A., Mohnl, M., Porta, R., Biarnes, M., Bohm, J. and Schatzmayr, G., 2012. Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of Campylobacter jejuni in broiler chickens. *Poultry Science,* 91 (8), 1825-1832.

Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A. and Roberfroid, M.B., 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews,* 17 (2), 259-275.

Gibson, G.R., and Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition,* 125 (6), 1401-1412.

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M. and Nelson, K.E., 2006. Metagenomic analysis of the human distal gut microbiome. *Science (New York, N.Y.),* 312 (5778), 1355-1359.

Giloh, M., Shinder, D. and Yahav, S., 2012. Skin surface temperature of broiler chickens is correlated to body core temperature and is indicative of their thermoregulatory status. *Poultry Science,* 91 (1), 175-188.

Gleeson, T.M., Stavric, S. and Blanchfield, B., 1989. Protection of chicks against Salmonella infection with a mixture of pure cultures of intestinal bacteria. *Avian Diseases,* 33 (4), 636-642.

Goldstein, E.J.C., Tyrrell, K.L. and Citron, D.M., 2015a. Lactobacillus Species: Taxonomic Complexity and Controversial Susceptibilities. *Clinical Infectious Diseases,* 60, S98-S107.

Goldstein, E.J.C., Tyrrell, K.L. and Citron, D.M., 2015b. Lactobacillus Species: Taxonomic Complexity and Controversial Susceptibilities. *Clinical Infectious Diseases,* 60, S98-S107.

Gong, e.a., 2007. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiol Ecol,* 59.

Guarner, F., and Malagelada, J.R., 2003. Gut flora in health and disease. *The Lancet,* 361 (9356), 512-519.

Haghighi, H.R., Gong, J., Gyles, C.L., Hayes, M.A., Sanei, B., Parvizi, P., Gisavi, H., Chambers, J.R. and Sharif, S., 2005. Modulation of antibody-mediated immune response by probiotics in chickens. *Clinical and Diagnostic Laboratory Immunology,* 12 (12), 1387-1392.

Haghighi, H.R., Gong, J., Gyles, C.L., Hayes, M.A., Zhou, H., Sanei, B., Chambers, J.R. and Sharif, S., 2006. Probiotics stimulate production of natural antibodies in chickens. *Clinical and Vaccine Immunology : CVI,* 13 (9), 975-980.

Haghighi, H.R., Abdul-Careem, M., Dara, R.A., Chambers, J.R. and Sharif, S., 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and Salmonella infection. *Veterinary Microbiology,* 126 (1), 225-233.

Hansson, G.C., and Johansson, M.E., 2010. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Gut Microbes,* 1 (1), 51-54.

Hardin, G., 1960. The competitive exclusion principle. *Science (New York, N.Y.),* 131 (3409), 1292-1297.

Hardy, H., Harris, J., Lyon, E., Beal, J. and Foey, A.D., 2013. Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. *Nutrients,* 5 (6), 1869-1912.

Harmsen, H.J.M., Gibson, G.R., Elfferich, P., Raangs, G.C., Wildeboer-Veloo, A.C.M., Argaiz, A., Roberfroid, M.B. and Welling, G.W., 2000. Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. *FEMS Microbiology Letters,* 183 (1), 125-129.

Hassanzadazar, H., Ehsani, A., Mardani, K. and Hesari, J., 2012. Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. *Veterinary Research Forum : An International Quarterly Journal,* 3 (3), 181-185.

Havenstein, G.B., Ferket, P.R. and Qureshi, M.A., 2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poultry Science,* 82 (10), 1500-1508.

Hegde, S.N., Rolls, B.A. and Coates, M.E., 1982. THe effects of the gut microflora and dietary fibre on energy utilization by the chick. *The British Journal of Nutrition,* 48 (1), 73-80.

Hemarajata, P., and Versalovic, J., 2013. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therapeutic Advances in Gastroenterology,* 6 (1), 39-51.

Henningsson, Å, Björck, I. and Nyman, M., 2001. Short-chain fatty acid formation at fermentation of indigestible carbohydrates. *Näringsforskning,* 45 (1), 165-168.

Hermans, D., Van Deun, K., Martel, A., Van Immerseel, F., Messens, W., Heyndrickx, M., Haesebrouck, F. and Pasmans, F., 2011. Colonization factors of Campylobacter jejuni in the chicken gut. *Veterinary Research,* 42, 82-9716-42-82.

Hird, S.M., Sanchez, C., Carstens, B.C. and Brumfield, R.T., 2015. Comparative Gut Microbiota of 59 Neotropical Bird Species. *Frontiers in Microbiology,* 6, 1403.

Hoffmann, C., Hill, D.A., Minkah, N., Kirn, T., Troy, A., Artis, D. and Bushman, F., 2009. Community-wide response of the gut microbiota to enteropathogenic Citrobacter rodentium infection revealed by deep sequencing. *Infection and Immunity,* 77 (10), 4668-4678.

Huang, Q., Wei, Y., Lv, Y., Wang, Y. and Hu, T., 2015. Effect of dietary inulin supplements on growth performance and intestinal immunological parameters of broiler chickens. *Livestock Science,* 180, 172-176.

Human Microbiome Project Consortium, 2012. Structure, function and diversity of the healthy human microbiome. *Nature,* 486 (7402), 207-214.

Hunter, C.A., and Jones, S.A., 2015. IL-6 as a keystone cytokine in health and disease. *Nature Immunology,* 16 (5), 448-457.

Huyghebaert, G., Ducatelle, R. and Immerseel, F.V., 2011. An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal,* 187 (2), 182-188.

Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., Mueller, O., Schroeder, A. and Auffray, C., 2005. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Research,* 33 (6), e56.

Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H. and Salminen, S., 2001. Probiotics: effects on immunity. *The American Journal of Clinical Nutrition,* 73 (2 Suppl), 444S-450S.

Jackson, S., and Duke, G.E., 1995. Intestine fullness influences feeding behaviour and crop filling in the domestic turkey. *Physiology & Behavior,* 58 (5), 1027-1034.

Janardhana, V., Broadway, M.M., Bruce, M.P., Lowenthal, J.W., Geier, M.S., Hughes, R.J. and Bean, A.G., 2009. Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. *The Journal of Nutrition,* 139 (7), 1404-1409.

Jeurissen, S.H., Lewis, F., van der Klis, J.D., Mroz, Z., Rebel, J.M. and ter Huurne, A.A., 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. *Current Issues in Intestinal Microbiology,* 3 (1), 1-14.

Jin, L.Z., Ho, Y.W., Abdullah, N. and Jalaludin, S., 1998. Acid and bile tolerance of Lactobacillus isolated from chicken intestine. *Letters in Applied Microbiology,* 27 (3), 183-185.

Jones, F.T., and Ricke, S.C., 2003. Observations on the history of the development of antimicrobials and their use in poultry feeds. *Poultry Science,* 82 (4), 613-617.

Jozefiak, D., Sip, A., Rawski, M., Rutkowski, A., Kaczmarek, S., Hojberg, O., Jensen, B.B. and Engberg, R.M., 2011. Dietary divercin modifies gastrointestinal microbiota and improves growth performance in broiler chickens. *British Poultry Science,* 52 (4), 492-499.

Juricova, H., Videnska, P., Lukac, M., Faldynova, M., Babak, V., Havlickova, H., Sisak, F. and Rychlik, I., 2013. Influence of Salmonella enterica serovar enteritidis infection on the development of the cecum microbiota in newly hatched chicks. *Applied and Environmental Microbiology,* 79 (2), 745-747.

Kaiser, M.G., Cheeseman, J.H., Kaiser, P. and Lamont, S.J., 2006. Cytokine Expression in Chicken Peripheral Blood Mononuclear Cells after In Vitro Exposure to Salmonella enterica serovar Enteritidis1. *Poultry Science,* 85 (11), 1907-1911.

Kaiser, P., Rothwell, L., Galyov, E.E., Barrow, P.A., Burnside, J. and Wigley, P., 2000. Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum. *Microbiology (Reading, England),* 146 Pt 12, 3217-3226.

Kamada, N., Chen, G.Y., Inohara, N. and Nunez, G., 2013. Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology,* 14 (7), 685-690.

Kaplan, H., and Hutkins, R.W., 2003. Metabolism of fructooligosaccharides by Lactobacillus paracasei 1195. *Applied and Environmental Microbiology,* 69 (4), 2217-2222.

Kaplan, M.L., and Kaplan, L., 1933. The Gram Stain and Differential Staining. *Journal of Bacteriology,* 25 (3), 309-321.

Kareem, K.Y., Loh, T.C., Foo, H.L., Asmara, S.A. and Akit, H., 2017. Influence of postbiotic RG14 and inulin combination on cecal microbiota, organic acid

concentration, and cytokine expression in broiler chickens. *Poultry Science,* 96 (4), 966-975.

Kasai, C., Sugimoto, K., Moritani, I., Tanaka, J., Oya, Y., Inoue, H., Tameda, M., Shiraki, K., Ito, M., Takei, Y. and Takase, K., 2015. Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. *BMC Gastroenterology,* 15, 100-015-0330-2.

Kaur, N., and Gupta, A.K., 2002. Applications of inulin and oligofructose in health and nutrition. *Journal of Biosciences,* 27 (7), 703-714.

Kelly, G., 2008. Inulin-type prebiotics--a review: part 1. *Alternative Medicine Review : A Journal of Clinical Therapeutic,* 13 (4), 315-329.

Kelsall, B.L., 2008. Innate and adaptive mechanisms to control [corrected] pathological intestinal inflammation. *The Journal of Pathology,* 214 (2), 242-259.

Kettle, H., Louis, P., Holtrop, G., Duncan, S.H. and Flint, H.J., 2015. Modelling the emergent dynamics and major metabolites of the human colonic microbiota. *Environmental Microbiology,* 17 (5), 1615-1630.

Kim, G.B., Seo, Y.M., Kim, C.H. and Paik, I.K., 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. *Poultry Science* [online], 90 (1), 75-82.

Kim, J., Muhammad, N., Jhun, B.H. and Yoo, J., 2016. Probiotic delivery systems: a brief overview. *Journal of Pharmaceutical Investigation,* 46 (4), 377-386.

Kizerwetter-Swida, M., and Binek, M., 2005. Selection of potentially probiotic Lactobacillus strains towards their inhibitory activity against poultry enteropathogenic bacteria. *Polish Journal of Microbiology,* 54 (4), 287-294.

Klasing, K.C., 2007a. Nutrition and the immune system. *British Poultry Science,* 48 (5), 525-537.

Klasing, K.C., 2007b. Nutrition and the immune system. *British Poultry Science,* 48 (5), 525-537.

Kleerebezem, M., and Hugenholtz, J., 2003. Metabolic pathway engineering in lactic acid bacteria. *Current Opinion in Biotechnology,* 14 (2), 232-237.

Kleessen, B., Elsayed, N.A., Loehren, U., Schroedl, W. and Krueger, M., 2003. Jerusalem artichokes stimulate growth of broiler chickens and protect them against endotoxins and potential cecal pathogens. *Journal of Food Protection,* 66 (11), 2171-2175.

Klipper, E., Sklan, D. and Friedman, A., 2001. Response, tolerance and ignorance following oral exposure to a single dietary protein antigen in Gallus domesticus. *Vaccine,* 19 (20), 2890-2897.

Knowles, T.G., Kestin, S.C., Haslam, S.M., Brown, S.N., Green, L.E., Butterworth, A., Pope, S.J., Pfeiffer, D. and Nicol, C.J., 2008. Leg disorders in broiler chickens: prevalence, risk factors and prevention. *PloS One,* 3 (2), e1545.

Koenen, M.E., van der Hulst, R., Leering, M., Jeurissen, S.H. and Boersma, W.J., 2004. Development and validation of a new in vitro assay for selection of probiotic bacteria that express immune-stimulating properties in chickens in vivo. *FEMS Immunology and Medical Microbiology,* 40 (2), 119-127.

Kogut, M.H., 2013. The gut microbiota and host innate immunity: Regulators of host metabolism and metabolic diseases in poultry? 1. *The Journal of Applied Poultry Research,* 22 (3), 637-646.

Konosonoka, I., Osmane, B., Cerina, S., Krastina, V., Vitina, I.I. and Valdovska, A., 2015. Feeding technology impact on broiler productivity and intestinal tract microflora. *Engineering for Rural Development,* 14, 126-132.

Kristeen-Teo, Y.W., Yeap, S.K., Tan, S.W., Omar, A.R., Ideris, A., Tan, S.G. and Alitheen, N.B., 2017. The effects of different velogenic NDV infections on the chicken bursa of Fabricius. *BMC Veterinary Research,* 13 (1), 151-017-1071-y.

Kriukova, Y., Jakubiak-Augustyn, A., Ilyinska, N., Krotkiewski, H., Gontova, T., Evtifeyeva, O., Özcelik, T. and Matkowski, A., 2018. Chain length distribution of inulin from dahlia tubers as influenced by the extraction method. *International Journal of Food Properties,* , 1-11.

Krumbeck, J.A., Maldonado-Gomez, M.X., Martinez, I., Frese, S.A., Burkey, T.E., Rasineni, K., Ramer-Tait, A.E., Harris, E.N., Hutkins, R.W. and Walter, J., 2015. In vivo selection to identify bacterial strains with enhanced ecological performance in synbiotic applications. *Applied and Environmental Microbiology,* 81 (7), 2455-2465.

Kunová, G., Rada, V., Lisová, I., Ročková, Š and Vlková, E., 2011. In vitro fermentability of prebiotic oligosaccharides by lactobacilli. *Czech Journal of Food Sciences,* 29, S49-S54.

La Ragione, R.M., Narbad, A., Gasson, M.J. and Woodward, M.J., 2004. In vivo characterization of Lactobacillus johnsonii FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. *Letters in Applied Microbiology,* 38 (3), 197-205.

Lachman, J., 2008. *Kays, S.J., Nottingham, S.F.: Biology and Chemistry of Jerusalem Artichoke Helianthus tuberosus L.* USA: CRC Press ER.

Lan, P., Hayashi, H., Sakamoto, M. and Benno, Y., 2002. Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. *Microbiology and Immunology,* 46 (6), 371-382.

Lan, P.T.N., Sakamoto, M. and Benno, Y., 2004. Effects of Two Probiotic Lactobacillus Strains on Jejunal and Cecal Microbiota of Broiler Chicken under Acute Heat Stress Condition as Revealed by Molecular Analysis of 16S rRNA Genes. *Microbiology and Immunology,* 48 (12), 917-929.

Lan, Y., Verstegen, M.W.A., Tamminga, S. and Williams, B.A., 2005. The role of the commensal gut microbial community in broiler chickens. *World's Poultry Science Journal,* 61 (1), 95-104.

Lawley, T.D., and Walker, A.W., 2013. Intestinal colonization resistance. *Immunology,* 138 (1), 1-11.

LeBlanc, J.G., Chain, F., Martin, R., Bermudez-Humaran, L.G., Courau, S. and Langella, P., 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories,* 16 (1), 79-017-0691-z.

Lee, K.W., Li, G., Lillehoj, H.S., Lee, S.H., Jang, S.I., Babu, U.S., Lillehoj, E.P., Neumann, A.P. and Siragusa, G.R., 2011. Bacillus subtilis-based direct-fed microbials augment macrophage function in broiler chickens. *Research in Veterinary Science,* 91 (3), e87-91.

Lei, F., Yin, Y., Wang, Y., Deng, B., Yu, H.D., Li, L., Xiang, C., Wang, S., Zhu, B. and Wang, X., 2012. Higher-level production of volatile fatty acids in vitro by chicken gut microbiotas than by human gut microbiotas as determined by functional analyses. *Applied and Environmental Microbiology,* 78 (16), 5763-5772.

Li, Xu, Q.Q., Yang, C.J., Yang, X., Lv, L., Yin, C.H., Liu, X.L. and Yan, H., 2014. Effects of probiotics on the growth performance and intestinal micro flora of broiler chickens. *Pakistan Journal of Pharmaceutical Sciences,* 27 (3 Suppl), 713-717.

Li, G., Lillehoj, H.S., Lee, K.W., Lee, S.H., Park, M.S., Jang, S.I., Bauchan, G.R., Gay, C.G., Ritter, G.D., Bautista, D.A. and Siragusa, G.R., 2010. Immunopathology and cytokine responses in commercial broiler chickens with gangrenous dermatitis. *Avian Pathology : Journal of the W.V.P.A,* 39 (4), 255-264.

Lin, J., Hunkapiller, A.A., Layton, A.C., Chang, Y.J. and Robbins, K.R., 2013. Response of intestinal microbiota to antibiotic growth promoters in chickens. *Foodborne Pathogens and Disease,* 10 (4), 331-337.

Lingyun, W., Wang, J., Xiaodong, Z., Da, T., Yalin, Y., Chenggang, C., Tianhua, F. and Fan, Z., 2007. Studies on the extracting technical conditions of inulin from Jerusalem artichoke tubers. *Journal of Food Engineering,* 79, 1087-1093.

Liu, Y., Gibson, G.,R. and Walton, G., 2016. *Impact of high fat diets, prebiotics and probiotics on gut microbiota and immune function, with relevance to elderly populations.*

Livak, K.J., and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.),* 25 (4), 402-408.

Lourenço, M.C., de Souza, A.M., Hayashi, R.M., da Silva, A.B. and Santin, E., 2016. Immune response of broiler chickens supplemented with prebiotic from Sacharomyces cerevisiae challenged with Salmonella enteritidis or Minnesota. *The Journal of Applied Poultry Research,* 25 (2), 165-172.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J. and Lee, M.D., 2003a. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology,* 69 (11), 6816-6824.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J. and Lee, M.D., 2003b. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology,* 69 (11), 6816-6824.

Lutful Kabir, S.M., 2009. The role of probiotics in the poultry industry. *International Journal of Molecular Sciences,* 10 (8), 3531-3546.

Mackenzie, D.A., Jeffers, F., Parker, M.L., Vibert-Vallet, A., Bongaerts, R.J., Roos, S., Walter, J. and Juge, N., 2010. Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of Lactobacillus reuteri. *Microbiology (Reading, England),* 156 (Pt 11), 3368-3378.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H. and Zinkernagel, R.M., 2000. A Primitive T Cell-Independent Mechanism of Intestinal Mucosal IgA Responses to Commensal Bacteria. *Science,* 288 (5474), 2222.

Mañes-Lázaro, R., Van Diemen, P.M., Pin, C., Mayer, M.J., Stevens, M.P. and Narbad, A., 2017. Administration of Lactobacillus johnsonii FI9785 to chickens affects colonisation by Campylobacter jejuni and the intestinal microbiota. *British Poultry Science,* 58 (4), 373-381.

Mani-López, E., García, H.S. and López-Malo, A., 2012. Organic acids as antimicrobials to control Salmonella in meat and poultry products. *Food Research International.,* 45 (2), 713-721.

McBride, B.W., and Kelly, J.M., 1990. Energy cost of absorption and metabolism in the ruminant gastrointestinal tract and liver: a review. *Journal of Animal Science,* 68 (9), 2997-3010.

McCoy, S., and Gilliland, S.E., 2007. Isolation and characterization of Lactobacillus species having potential for use as probiotic cultures for dogs. *Journal of Food Science,* 72 (3), M94-7.

McLaurin, W.J., Somda, Z.C. and Kays, S.J., 1999. Jerusalem artichoke growth, development, and field storage. I. Numerical assessment of plant part development and dry matter acquisition and allocation. *Journal of Plant Nutrition,* 22 (8), 1303-1313.

McLelland, J., 1989. Anatomy of the avian cecum. *The Journal of Experimental Zoology.,* 3, 2-9.

Menconi, A., Kallapura, G., Latorre, J.D., Morgan, M.J., Pumford, N.R., Hargis, B.M. and Tellez, G., 2014. Identification and characterization of lactic Acid bacteria in a commercial probiotic culture. *Bioscience of Microbiota, Food and Health,* 33 (1), 25-30.

Mensink, M.A., Frijlink, H.W., van der Voort Maarschalk, K. and Hinrichs, W.L., 2015. Inulin, a flexible oligosaccharide I: Review of its physicochemical characteristics. *Carbohydrate Polymers,* 130, 405-419.

Methner, U., Barrow, P.A., Martin, G. and Meyer, H., 1997. Comparative study of the protective effect against Salmonella colonisation in newly hatched SPF chickens using live, attenuated Salmonella vaccine strains, wild-type Salmonella strains or a competitive exclusion product. *International Journal of Food Microbiology,* 35, 223-30.

Miremadi, F., and Shah, N.P., 2012. Applications of inulin and probiotics in health and nutrition. *International Food Research Journal,* 19, 1337-1350.

Mohan, V., 2015. The role of probiotics in the inhibition of Campylobacter jejuni colonization and virulence attenuation. *European Journal of Clinical Microbiology & Infectious Diseases,* 34 (8), 1503-1513.

Moncada, D.M., Kammanadiminti, S.J. and Chadee, K., 2003. Mucin and Toll-like receptors in host defense against intestinal parasites. *Trends in Parasitology,* 19 (7), 305-311.

Mookiah, S., Sieo, C.C., Ramasamy, K., Abdullah, N. and Ho, Y.W., 2014. Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. *Journal of the Science of Food and Agriculture,* 94 (2), 341-348.

Moreira, J.L., Mota, R.M., Horta, M.F., Teixeira, S.M., Neumann, E., Nicoli, J.R. and Nunes, A.C., 2005. Identification to the species level of Lactobacillus isolated in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA restriction profiling. *BMC Microbiology,* 5, 15-2180-5-15.

Mosser, D.M., and Zhang, X., 2008. Interleukin-10: new perspectives on an old cytokine. *Immunological Reviews,* 226, 205-218.

Mott, C.R., Siegel, P.B., Webb, J.,K.E. and Wong, E.A., 2008. Gene Expression of Nutrient Transporters in the Small Intestine of Chickens from Lines Divergently Selected for High or Low Juvenile Body Weight. *Poultry Science,* 87 (11), 2215-2224.

Moubareck, C., Gavini, F., Vaugien, L., Butel, M.J. and Doucet-Populaire, F., 2005. Antimicrobial susceptibility of bifidobacteria. *Journal of Antimicrobial Chemotherapy,* 55 (1), 38-44.

Mountzouris, K.C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G. and Fegeros, K., 2007. Evaluation of the Efficacy of a Probiotic Containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus Strains in Promoting Broiler Performance and Modulating Cecal Microflora Composition and Metabolic Activities1. *Poultry Science,* 86 (2), 309-317.

Mountzouris, K.C., Tsitrsikos, P., Palamidi, I., Arvaniti, A., Mohnl, M., Schatzmayr, G. and Fegeros, K., 2010. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. *Poultry Science,* 89 (1), 58-67.

Mühl, H., and Pfeilschifter, J., 2003. Anti-inflammatory properties of pro-inflammatory interferon-γ. *International Immunopharmacology,* 3 (9), 1247-1255.

Munyaka, P.M., Khafipour, E. and Ghia, J.E., 2014. External influence of early childhood establishment of gut microbiota and subsequent health implications. *Frontiers in Pediatrics,* 2, 109.

Muramatsu, T., Nakajima, S. and Okumura, J., 1994. Modification of energy metabolism by the presence of the gut microflora in the chicken. *The British Journal of Nutrition,* 71 (5), 709-717.

Murry, A., Hinton J, A., R and Buhr, R., 2006. Effect of Botanical Probiotic Containing Lactobacilli on Growth Performance and Populations of Bacteria in the Ceca, Cloaca, and Carcass Rinse of Broiler Chickens. *International Journal of Poultry Science,* 5.

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology,* 2 (3), 317-322.

Nabizadeh, A., 2012. The effect of inulin on broiler chicken intestinal microflora, gut morphology, and performance. *Journal of Animal and Feed Sciences,* 21 (4), 725-734.

Nagpal, R., and Kaur, A., 2011. Synbiotic effect of various prebiotics on in vitro activities of probiotic lactobacilli. *Ecology of Food and Nutrition,* 50 (1), 63-68.

Nakphaichit, M., Thanomwongwattana, S., Phraephaisarn, C., Sakamoto, N., Keawsompong, S., Nakayama, J. and Nitisinprasert, S., 2011. The effect of including

Lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. *Poultry Science,* 90 (12), 2753-2765.

Neveling, D.P., van Emmenes, L., Ahire, J.J., Pieterse, E., Smith, C. and Dicks, L.M.T., 2017. Safety assessment of antibiotic and probiotic feed additives for Gallus gallus domesticus. *Scientific Reports,* 7 (1), 12767.

Newell, D.G., 2002. The ecology of Campylobacter jejuni in avian and human hosts and in the environment. *International Journal of Infectious Diseases.,* 6, S16-S21.

Nhung, N.T., Chansiripornchai, N. and Carrique-Mas, J.J., 2017. Antimicrobial Resistance in Bacterial Poultry Pathogens: A Review. *Frontiers in Veterinary Science,* 4, 126.

Nisbet, D., 2002. Defined competitive exclusion cultures in the prevention of enteropathogen colonisation in poultry and swine. *Antonie Van Leeuwenhoek,* 81 (1-4), 481-486.

Nkukwana, T.T., Muchenje, V., Masika, P.J. and Mushonga, B., 2015. Intestinal morphology, digestive organ size and digesta pH of broiler chickens fed diets supplemented with or without Moringa oleifera leaf meal. *South African Journal of Animal Science,* 45 (4), 362-370.

Norris, D., and Ngambi, J.W., 2006. Genetic parameter estimates for body weight in local Venda chickens. *Tropical Animal Health and Production,* 38 (7-8), 605-609.

Nurmi, E., and Rantala, M., 1973. New aspects of Salmonella infection in broiler production. *Nature,* 241 (5386), 210-211.

O'Dea, E.E., Fasenko, G.M., Allison, G.E., Korver, D.R., Tannock, G.W. and Guan, L.L., 2006. Investigating the effects of commercial probiotics on broiler chick quality and production efficiency. *Poultry Science,* 85 (10), 1855-1863.

Olnood, C.G., Beski, S.S.M., Choct, M. and Iji, P.A., 2015. Novel probiotics: Their effects on growth performance, gut development, microbial community and activity of broiler chickens. *Animal Nutrition,* 1 (3), 184-191.

Olnood, C.G., Beski, S.S.M., Iji, P.A. and Choct, M., 2015. Delivery routes for probiotics: Effects on broiler performance, intestinal morphology and gut microflora. *Animal Nutrition,* 1 (3), 192-202.

Palm, N.W., de Zoete, M.R., Cullen, T.W., Barry, N.A., Stefanowski, J., Hao, L., Degnan, P.H., Hu, J., Peter, I., Zhang, W., Ruggiero, E., Cho, J.H., Goodman, A.L. and Flavell, R.A., 2014. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell,* 158 (5), 1000-1010.

Pan, D., and Yu, Z., 2014. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes,* 5 (1), 108-119.

Panda, A., Reddy, M.R., Rao, S., Raju, M.V.L.N. and Praharaj, N.K., 2000. Growth, carcass characteristics, immunocompetence and response to Escherichia coli of broilers fed diets with various levels of Probiotic. *Archiv Fur Geflugelkunde,* 64, 152-156.

Pandey, K.R., Naik, S.R. and Vakil, B.V., 2015. Probiotics, prebiotics and synbiotics- a review. *Journal of Food Science and Technology,* 52 (12), 7577-7587.

Park, Y.H., Hamidon, F., Rajangan, C., Soh, K.P., Gan, C.Y., Lim, T.S., Abdullah, W.N. and Liong, M.T., 2016. Application of Probiotics for the Production of Safe and High-quality Poultry Meat. *Korean Journal for Food Science of Animal Resources,* 36 (5), 567-576.

Parra, D., Takizawa, F. and Sunyer, J.O., 2013. Evolution of B cell immunity. *Annual Review of Animal Biosciences,* 1, 65-97.

Patterson, J.A., and Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. *Poultry Science,* 82 (4), 627-631.

Peralta, M.F., Magnoli, A., Alustiza, F., Nilson, A., Miazzo, R. and Vivas, A., 2017. Gut-Associated Lymphoid Tissue: A Key Tissue Inside the Mucosal Immune System of Hens Immunized with Escherichia coli F4. *Frontiers in Immunology,* 8, 568.

Perry, G.C., 2006. *Avian gut function in health and disease.* oxford,UK: CABI.

Pessione, E., 2012. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Frontiers in Cellular and Infection Microbiology,* 2, 86.

Peter, C.M., and Baker, D.H., 2001. Microbial Phytase Does Not Improve Protein–Amino Acid Utilization in Soybean Meal Fed to Young Chickens. *The Journal of Nutrition,* 131 (6), 1792-1797.

Pokusaeva, K., Fitzgerald, G.F. and van Sinderen, D., 2011. Carbohydrate metabolism in Bifidobacteria. *Genes & Nutrition,* 6 (3), 285-306.

Potturi-Venkata, L., Backert, S., Lastovica, A.J., Vieira, S.L., Norton, R.A., Miller, R.S., Pierce, S. and Oyarzabal, O.A., 2007. Evaluation of Different Plate Media for Direct Cultivation of Campylobacter Species from Live Broilers. *Poultry Science,* 86 (7), 1304-1311.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S. and Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. *FEMS Microbiology Letters,* 217 (2), 133-139.

Puangbut, D., Jogloy, S., Vorasoot, N., Srijaranai, S., Kesmala, T., C Holbrook,Corley and Patanothai, A., 2012. Influence of planting date and temperature on inulin content in Jerusalem artichoke (Helianthus tuberosus L.). *Australian Journal of Crop Science,* 6.

Purchiaroni, F., Tortora, A., Gabrielli, M., Bertucci, F., Gigante, G., Ianiro, G., Ojetti, V., Scarpellini, E. and Gasbarrini, A., 2013. The role of intestinal microbiota and the immune system. *European Review for Medical and Pharmacological Sciences,* 17 (3), 323-333.

Rafter, J., Bennett, M., Caderni, G., Clune, Y., Hughes, R., Karlsson, P.C., Klinder, A., O'Riordan, M., O'Sullivan, G.C., Pool-Zobel, B., Rechkemmer, G., Roller, M., Rowland, I., Salvadori, M., Thijs, H., Van Loo, J., Watzl, B. and Collins, J.K., 2007. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *The American Journal of Clinical Nutrition,* 85 (2), 488-496.

Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G. and Louis, P., 2009. Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. *The British Journal of Nutrition,* 101 (4), 541-550.

Ranjitkar, S., Lawley, B., Tannock, G. and Engberg, R.M., 2016. Bacterial Succession in the Broiler Gastrointestinal Tract. *Applied and Environmental Microbiology,* 82 (8), 2399-2410.

Ratcliffe, M.J.H., 2006. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. *Developmental & Comparative Immunology.,* 30 (1), 101-118.

Ratledge, C., and Dover, L.G., 2000. Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology,* 54, 881-941.

Rebole, A., Ortiz, L.T., Rodriguez, M.L., Alzueta, C., Trevino, J. and Velasco, S., 2010. Effects of inulin and enzyme complex, individually or in combination, on growth performance, intestinal microflora, cecal fermentation characteristics, and jejunal histomorphology in broiler chickens fed a wheat- and barley-based diet. *Poultry Science,* 89 (2), 276-286.

Rehman, H.U., Vahjen, W., Awad, W.A. and Zentek, J., 2007. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. *Archives of Animal Nutrition,* 61 (5), 319-335.

Ribet, D., and Cossart, P., 2015. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection,* 17 (3), 173-183.

Rios-Covian, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilan, C.G. and Salazar, N., 2016. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Frontiers in Microbiology,* 7, 185.

Roberfroid, M., 2007. Prebiotics: the concept revisited. *The Journal of Nutrition,* 137 (3 Suppl 2), 830S-7S.

Roggenbuck, M., Baerholm Schnell, I., Blom, N., Baelum, J., Bertelsen, M.F., Sicheritz-Ponten, T., Sorensen, S.J., Gilbert, M.T., Graves, G.R. and Hansen, L.H., 2014. The microbiome of New World vultures. *Nature Communications,* 5, 5498.

Rothwell, L., Young, J.R., Zoorob, R., Whittaker, C.A., Hesketh, P., Archer, A., Smith, A.L. and Kaiser, P., 2004. Cloning and Characterization of Chicken IL-10 and Its Role in the Immune Response to *Eimeria maxima*. *The Journal of Immunology,* 173 (4), 2675.

Roto, S.M., Rubinelli, P.M. and Ricke, S.C., 2015. An Introduction to the Avian Gut Microbiota and the Effects of Yeast-Based Prebiotic-Type Compounds as Potential Feed Additives. *Frontiers in Veterinary Science,* 2, 28.

Russell, J.B., and Rychlik, J.L., 2001. Factors That Alter Rumen Microbial Ecology. *Science,* 292 (5519), 1119.

Rutherfurd, S.M., Chung, T.K. and Moughan, P.J., 2007. The effect of a commercial enzyme preparation on apparent metabolizable energy, the true ileal amino acid digestibility, and endogenous ileal lysine losses in broiler chickens. *Poultry Science,* 86 (4), 665-672.

Saengkanuk, A., Nuchadomrong, S., Jogloy, S., Patanothai, A. and Srijaranai, S., 2011. A simplified spectrophotometric method for the determination of inulin in Jerusalem artichoke (Helianthus tuberosus L.) tubers. *European Food Research and Technology,* 233 (4), 609.

Saiyed, M.A., Joshi, R.S., Savaliya, F.P., Patel, A.B., Mishra, R.K. and Bhagora, N.J., 2015. Study on inclusion of probiotic, prebiotic and its combination in broiler diet and their effect on carcass characteristics and economics of commercial broilers. *Veterinary World,* 8 (2), 225-231.

Salehimanesh, A., Mohammadi, M. and Roostaei-Ali Mehr, M., 2016. Effect of dietary probiotic, prebiotic and synbiotic supplementation on performance, immune responses, intestinal morphology and bacterial populations in broilers. *Journal of Animal Physiology and Animal Nutrition,* 100 (4), 694-700.

Samal, L., Chaturvedi, V.B., Saikumar, G., Somvanshi, R. and Pattanaik, A.K., 2015. Prebiotic potential of Jerusalem artichoke (Helianthus tuberosus L.) in Wistar rats: effects of levels of supplementation on hindgut fermentation, intestinal morphology, blood metabolites and immune response. *Journal of the Science of Food and Agriculture,* 95 (8), 1689-1696.

Saminathan, M., Sieo, C.C., Kalavathy, R., Abdullah, N. and Ho, Y.W., 2011. Effect of prebiotic oligosaccharides on growth of Lactobacillus strains used as a probiotic for chickens. *African Journal of Microbiology Research,* 5 (1), 57-64.

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D. and Biavati, B., 2010. Characterization of probiotic strains: An application as feed additives in

poultry against Campylobacter jejuni. *International Journal of Food Microbiology.,* 141, S98-S108.

Sarangi, N.R., Babu, L.K., Kumar, A., Pradhan, C.R., Pati, P.K. and Mishra, J.P., 2016. Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on growth performance and carcass characteristics of broiler chickens. *Veterinary World,* 9 (3), 313-319.

Schoeni, J.L., and Doyle, M.P., 1992. Reduction of Campylobacter jejuni colonization of chicks by cecum-colonizing bacteria producing anti-C. jejuni metabolites. *Applied and Environmental Microbiology,* 58 (2), 664-670.

Schokker, D., Veninga, G., Vastenhouw, S.A., Bossers, A., de Bree, F.M., Kaal-Lansbergen, L.M., Rebel, J.M. and Smits, M.A., 2015. Early life microbial colonization of the gut and intestinal development differ between genetically divergent broiler lines. *BMC Genomics,* 16, 418-015-1646-6.

Segerstrom, S.C., 2007. Stress, Energy, and Immunity: An Ecological View. *Current Directions in Psychological Science,* 16 (6), 326-330.

Sekelja, M., Rud, I., Knutsen, S., Denstadli, V., Westereng, B. and Næs, T., 2012. Abrupt temporal fluctuations in the chicken fecal microbiota are explained by its gastrointestinal origin. *Appl Environ Microbiol,* 78.

Selvaraj, R.K., 2012. Maximum immunity effectors: Mechanisms and animal performance limitations1,2. *The Journal of Applied Poultry Research,* 21 (1), 185-192.

Semova, I., Carten, J., Stombaugh, J., Mackey, L., Knight, R., Farber, S. and Rawls, J., 2012. Microbiota Regulate Intestinal Absorption and Metabolism of Fatty Acids in the Zebrafish. *Cell Host & Microbe,* 12 (3), 277-288.

Servin, A.L., 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiology Reviews,* 28 (4), 405-440.

Shang, H.M., Hu, T.M., Lu, Y.J. and Wu, H.X., 2010. Effects of inulin on performance, egg quality, gut microflora and serum and yolk cholesterol in laying hens. *British Poultry Science,* 51 (6), 791-796.

Shang, Y., Regassa, A., Kim, J.H. and Kim, W.K., 2015. The effect of dietary fructooligosaccharide supplementation on growth performance, intestinal morphology, and immune responses in broiler chickens challenged with Salmonella Enteritidis lipopolysaccharides. *Poultry Science,* 94 (12), 2887-2897.

Shokryazdan, P., Sieo, C.C., Kalavathy, R., Liang, J.B., Alitheen, N.B., Faseleh Jahromi, M. and Ho, Y.W., 2014. Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains. *BioMed Research International,* 2014, 927268.

Shokryazdan, P., Faseleh Jahromi, M., Liang, J.B., Ramasamy, K., Sieo, C.C. and Ho, Y.W., 2017. Effects of a Lactobacillus salivarius mixture on performance, intestinal health and serum lipids of broiler chickens. *Plos One,* 12 (5), e0175959.

Siragusa, G.R., Haas, G.J., Matthews, P.D., Smith, R.J., Buhr, R.J., Dale, N.M. and Wise, M.G., 2008. Antimicrobial activity of lupulone against Clostridium perfringens in the chicken intestinal tract jejunum and caecum. *The Journal of Antimicrobial Chemotherapy,* 61 (4), 853-858.

Slavin, J., 2013a. Fiber and prebiotics: mechanisms and health benefits. *Nutrients,* 5 (4), 1417-1435.

Slavin, J., 2013b. Fiber and prebiotics: mechanisms and health benefits. *Nutrients,* 5 (4), 1417-1435.

Smirnov, A., Sklan, D. and Uni, Z., 2004. Mucin dynamics in the chick small intestine are altered by starvation. *The Journal of Nutrition,* 134 (4), 736-742.

Sohail, M.U., Hume, M.E., Byrd, J.A., Nisbet, D.J., Shabbir, M.Z., Ijaz, A. and Rehman, H., 2015. Molecular analysis of the caecal and tracheal microbiome of heat-stressed broilers supplemented with prebiotic and probiotic. *Avian Pathology : Journal of the W.V.P.A,* 44 (2), 67-74.

Srinameb, B., Nuchadomrong, S., Jogloy, S., Patanothai, A. and Srijaranai, S., 2015. Preparation of Inulin Powder from Jerusalem Artichoke (Helianthus tuberosus L.) Tuber. *Plant Foods for Human Nutrition,* 70 (2), 221-226.

Stanley, D., Geier, M.S., Chen, H., Hughes, R.J. and Moore, R.J., 2015. Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiology,* 15, 51-015-0388-6.

Stanley, D., Geier, M.S., Hughes, R.J., Denman, S.E. and Moore, R.J., 2013a. Highly variable microbiota development in the chicken gastrointestinal tract. *PloS One,* 8 (12), e84290.

Stanley, D., Geier, M.S., Hughes, R.J., Denman, S.E. and Moore, R.J., 2013b. Highly variable microbiota development in the chicken gastrointestinal tract. *PloS One,* 8.

Stanley, D., Hughes, R.J., Geier, M.S. and Moore, R.J., 2016. Bacteria within the Gastrointestinal Tract Microbiota Correlated with Improved Growth and Feed Conversion: Challenges Presented for the Identification of Performance Enhancing Probiotic Bacteria. *Frontiers in Microbiology,* 7, 187.

Stanley, D., Keyburn, A.L., Denman, S.E. and Moore, R.J., 2012. Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. *Veterinary Microbiology,* 159 (1-2), 155-162.

Stavriac, S., and D'aoust, J.-., 1993. Undefined and Defined Bacterial Preparations for the Competitive Exclusion of Salmonella in Poultry - A Review. *Journal of Food Protection,* 56 (2), 173-180.

Stern, N.J., 2008. Salmonella species and Campylobacter jejuni cecal colonization model in broilers. *Poultry Science,* 87 (11), 2399-2403.

Sugiharto, S., 2016. Role of nutraceuticals in gut health and growth performance of poultry. *Journal of the Saudi Society of Agricultural Sciences,* 15 (2), 99-111.

Sule, J., Korosi, T., Hucker, A. and Varga, L., 2014. Evaluation of culture media for selective enumeration of bifidobacteria and lactic acid bacteria. *Brazilian Journal of Microbiology,* 45 (3), 1023-1030.

Sun, Y., and O'Riordan, M.X., 2013. Regulation of bacterial pathogenesis by intestinal short-chain Fatty acids. *Advances in Applied Microbiology,* 85, 93-118.

Sun, Z., Harris, H.,M.B., McCann, A., Guo, C., Argimón, S., Zhang, W., Yang, X., Jeffery, I.B., Cooney, J.C., Kagawa, T.F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M.C., O'Sullivan, O., Ritari, J., Douillard, F.P., Paul Ross, R., Yang, R., Briner, A.E., Felis, G.E., De Vos, W.M., Barrangou, R., Klaenhammer, T.R., Caufield, P.W., Cui, Y., Zhang, H. and O'Toole, P.W., 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications,* 6.

Surai, P., 2013. GUT IMMUNITY IN BIRDS: FACTS AND REFLECTIONS. *Agricultural Biology (Sel'skokhozyaistvennaya Biologiya),* 4, 3-25.

Svetoch, E.A., and Stern, N.J., 2010. Bacteriocins to control Campylobacter spp. in poultry--A review. *Poultry Science,* 89 (8), 1763-1768.

Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. *World's Poultry Science Journal,* 67 (2), 207-224.

Svihus, B., Choct, M. and Classen, H.L., 2013. Function and nutritional roles of the avian caeca: A review. *World's Poultry Science Journal,* 69.

Svihus, B., 2014a. Function of the digestive system1. *The Journal of Applied Poultry Research,* 23 (2), 306-314.

Svihus, B., 2014b. Function of the digestive system1. *The Journal of Applied Poultry Research,* 23 (2), 306-314.

Swiatkiewicz, S., Koreleski, J. and Arczewska-Wloek, A., 2011. Effect of inulin and oligofructose on performance and bone characteristics of broiler chickens fed on diets with different concentrations of calcium and phosphorus. *British Poultry Science,* 52 (4), 483-491.

Tahir, M., Shim, M.Y., Ward, N.E., Smith, C., Foster, E., Guney, A.C. and Pesti, G.M., 2012. Phytate and other nutrient components of feed ingredients for poultry. *Poultry Science,* 91 (4), 928-935.

Tanner, S.A., Chassard, C., Zihler Berner, A. and Lacroix, C., 2014. Synergistic effects of Bifidobacterium thermophilum RBL67 and selected prebiotics on inhibition of Salmonella colonization in the swine proximal colon PolyFermS model. *Gut Pathogens,* 6 (1), 44.

Teirlynck, E., Gussem, M.D., Dewulf, J., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F., 2011. Morphometric evaluation of "dysbacteriosis" in broilers. *Avian Pathology : Journal of the W.V.P.A,* 40 (2), 139-144.

Thibodeau, A., Fravalo, P., Yergeau, E., Arsenault, J., Lahaye, L. and Letellier, A., 2015. Chicken Caecal Microbiome Modifications Induced by Campylobacter jejuni Colonization and by a Non-Antibiotic Feed Additive. *PloS One,* 10 (7), e0131978.

Thompson, C.L., Vier, R., Mikaelyan, A., Wienemann, T. and Brune, A., 2012. 'Candidatus Arthromitus' revised: segmented filamentous bacteria in arthropod guts are members of Lachnospiraceae. *Environmental Microbiology,* 14 (6), 1454-1465.

Thursby, E., and Juge, N., 2017. Introduction to the human gut microbiota. *The Biochemical Journal,* 474 (11), 1823-1836.

Tsirtsikos, P., Fegeros, K., Balaskas, C., Kominakis, A. and Mountzouris, K.C., 2012. Dietary probiotic inclusion level modulates intestinal mucin composition and mucosal morphology in broilers. *Poultry Science,* 91 (8), 1860-1868.

Turroni, S., Rampelli, S., Centanni, M., Schnorr, S.L., Consolandi, C., Severgnini, M., Peano, C., Soverini, M., Falconi, M., Crittenden, A.N., Henry, A.G., Brigidi, P. and Candela, M., 2016. Enterocyte-Associated Microbiome of the Hadza Hunter-Gatherers. *Frontiers in Microbiology,* 7, 865.

van Asselt, M., Poortvliet, P.M., Ekkel, E.D., Kemp, B. and Stassen, E.N., 2018. Risk perceptions of public health and food safety hazards in poultry husbandry by citizens, poultry farmers and poultry veterinarians. *Poultry Science,* 97 (2), 607-619.

van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H. and Verstraete, W., 2007. Inulin-type fructans of longer degree of polymerization exert more pronounced in vitro prebiotic effects. *Journal of Applied Microbiology,* 102 (2), 452-460.

Van der Meulen, R., Avonts, L. and De Vuyst, L., 2004. Short fractions of oligofructose are preferentially metabolized by Bifidobacterium animalis DN-173 010. *Applied and Environmental Microbiology,* 70 (4), 1923-1930.

van Der Wielen, P.W., Biesterveld, S., Notermans, S., Hofstra, H., Urlings, B.A. and van Knapen, F., 2000. Role of volatile fatty acids in development of the cecal microflora

in broiler chickens during growth. *Applied and Environmental Microbiology,* 66 (6), 2536-2540.

van der Wielen, P.W., Keuzenkamp, D.A., Lipman, L.J., van Knapen, F. and Biesterveld, S., 2002. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology,* 44 (3), 286-293.

Van Immerseel, F., De Buck, J., De Smet, I., Pasmans, F., Haesebrouck, F. and Ducatelle, R., 2004. Interactions of butyric acid- and acetic acid-treated Salmonella with chicken primary cecal epithelial cells in vitro. *Avian Diseases,* 48 (2), 384-391.

Van Immerseel, F., Ducatelle, R., De Vos, M., Boon, N., Van De Wiele, T., Verbeke, K., Rutgeerts, P., Sas, B., Louis, P. and Flint, H.J., 2010. Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. *Journal of Medical Microbiology,* 59 (Pt 2), 141-143.

van Zanten, G.C., Krych, L., Röytiö, H., Forssten, S., Lahtinen, S.J., Al-Soud, W., Sørensen, S., Svensson, B., Jespersen, L. and Jakobsen, M., 2014. Synbiotic Lactobacillus acidophilus NCFM and cellobiose does not affect human gut bacterial diversity but increases abundance of lactobacilli, bifidobacteria and branched-chain fatty acids: a randomized, double-blinded cross-over trial. *FEMS Microbiology Ecology,* 90 (1), 225-236.

Varmuzova, K., Kubasova, T., Davidova-Gerzova, L., Sisak, F., Havlickova, H., Sebkova, A., Faldynova, M. and Rychlik, I., 2016a. Composition of Gut Microbiota Influences Resistance of Newly Hatched Chickens to Salmonella Enteritidis Infection. *Frontiers in Microbiology,* 7, 957.

Varmuzova, K., Kubasova, T., Davidova-Gerzova, L., Sisak, F., Havlickova, H., Sebkova, A., Faldynova, M. and Rychlik, I., 2016b. Composition of Gut Microbiota Influences Resistance of Newly Hatched Chickens to Salmonella Enteritidis Infection. *Frontiers in Microbiology,* 7, 957.

Vesterlund, S., Salminen, K. and Salminen, S., 2012. Water activity in dry foods containing live probiotic bacteria should be carefully considered: A case study with Lactobacillus rhamnosus GG in flaxseed. *International Journal of Food Microbiology,* 157 (2), 319-321.

Videnska, P., Sedlar, K., Lukac, M., Faldynova, M., Gerzova, L., Cejkova, D., Sisak, F. and Rychlik, I., 2014. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PloS One,* 9 (12), e115142.

Vieira, A.T., Teixeira, M.M. and Martins, F.S., 2013. The role of probiotics and prebiotics in inducing gut immunity. *Frontiers in Immunology,* 4, 445.

Waite, D.W., and Taylor, M.W., 2015. Exploring the avian gut microbiota: current trends and future directions. *Frontiers in Microbiology,* 6, 673.

Waititu, S.M., Yitbarek, A., Matini, E., Echeverry, H., Kiarie, E., Rodriguez-Lecompte, J. and Nyachoti, C.M., 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses1. *Poultry Science,* 93 (3), 625-635.

Walter, J., 2008. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Applied and Environmental Microbiology,* 74 (16), 4985-4996.

Wang, L., Lilburn, M. and Yu, Z., 2016. Intestinal Microbiota of Broiler Chickens As Affected by Litter Management Regimens. *Frontiers in Microbiology,* 7, 593.

Wang, M., Ahrne, S., Antonsson, M. and Molin, G., 2004. T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. *Journal of Microbiological Methods,* 59 (1), 53-69.

Wang, Y., Sun, J., Zhong, H., Li, N., Xu, H., Zhu, Q. and Liu, Y., 2017. Effect of probiotics on the meat flavour and gut microbiota of chicken. *Scientific Reports,* 7 (1), 6400.

Wasielewski, H., Alcock, J. and Aktipis, A., 2016. Resource conflict and cooperation between human host and gut microbiota: implications for nutrition and health. *Annals of the New York Academy of Sciences,* 1372 (1), 20-28.

Wei, S., Morrison, M. and Yu, Z., 2013. Bacterial census of poultry intestinal microbiome. *Poultry Science,* 92 (3), 671-683.

Wichienchot, S., Thammarutwasik, P., Jongjareonrak, A., Chansuwan, W., Hmadhlu, P., Hongpattarakere, T., Itharat, A. and Ooraikul, B., 2011. Extraction and analysis of prebiotics from selected plants from southern Thailand. *Songklanakarin Journal of Science and Technology,* 33.

Wigley, P., and Kaiser, P., 2003. Avian cytokines in Health and disease. *Brazilian Journal of Poultry Science,* 5, 1-14.

Woo, J., and Ahn, J., 2013. Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. *Letters in Applied Microbiology,* 56 (4), 307-313.

Woyengo, T.A., Kiarie, E. and Nyachoti, C.M., 2010. Metabolizable energy and standardized ileal digestible amino acid contents of expeller-extracted canola meal fed to broiler chicks. *Poultry Science,* 89 (6), 1182-1189.

Wu, Q.J., Zhou, Y.M., Wu, Y.N. and Wang, T., 2013. Intestinal development and function of broiler chickens on diets supplemented with clinoptilolite. *Asian-Australasian Journal of Animal Sciences,* 26 (7), 987-994.

Yamana, J., Yamamura, M., Okamoto, A., Aita, T., Iwahashi, M., Sunahori, K. and Makino, H., 2004. Resistance to IL-10 inhibition of interferon gamma production and expression of suppressor of cytokine signaling 1 in CD4+T cells from patients with rheumatoid arthritis. *Arthritis Research Therapy,* 6 (6), R567.

Yan, W., Sun, C., Yuan, J. and Yang, N., 2017. Gut metagenomic analysis reveals prominent roles of Lactobacillus and cecal microbiota in chicken feed efficiency. *Scientific Reports,* 7, 45308.

Yang, IJI, and Choct, 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. *World's Poultry Science Journal,* 65 (1), 97-114.

Yang, L., He, Q.S., Corscadden, K. and Udenigwe, C.C., 2015. The prospects of Jerusalem artichoke in functional food ingredients and bioenergy production. *Biotechnology Reports,* 5, 77-88.

Yeoman, C.J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N.D. and White, B.A., 2012. The microbiome of the chicken gastrointestinal tract. *Animal Health Research Reviews,* 13 (1), 89-99.

Yitbarek, A., Echeverry, H., Munyaka, P. and Rodriguez-Lecompte, J., 2015. Innate immune response of pullets fed diets supplemented with prebiotics and synbiotics. *Poultry Science,* 94 (8), 1802-1811.

Zaidel, O., and Lin, H.C., 2003. Uninvited guests: The impact of small intestinal bacterial overgrowth on nutritional status. *Practical Gastroenterology,* 27, 27-30+33.

Žaldarienė, S., Jurgita, K. and Judita, C., 2013. The quality comparison of different Jerusalem artichoke (Helianthus tuberosus L.) cultivars tubers. *Žemės Ūkio Mokslai,* 19.

Zarei, A., Lavvaf, A. and Motamedi Motlagh, M., 2018. Effects of probiotic and whey powder supplementation on growth performance, microflora population, and ileum morphology in broilers. *Journal of Applied Animal Research,* 46 (1), 840-844.

Zhang, H., DiBaise, J.K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., Parameswaran, P., Crowell, M.D., Wing, R., Rittmann, B.E. and Krajmalnik-Brown, R., 2009. Human gut microbiota in obesity and after gastric bypass. *Proceedings of the National Academy of Sciences of the United States of America,* 106 (7), 2365-2370.

Zhou, W., Wang, Y. and Lin, J., 2012. Functional cloning and characterization of antibiotic resistance genes from the chicken gut microbiome. *Applied and Environmental Microbiology,* 78 (8), 3028-3032.

Zhu, X.Y., Zhong, T., Pandya, Y. and Joerger, R.D., 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Applied and Environmental Microbiology,* 68 (1), 124-137.

Appendix A Bird trial LB01 diet specification and formulation

Diet	Control	LB
DM (g/kg)	890.76	900.69
Ash (g/kg)	48.43	51.48
Protein (g/kg DM)	22.21	22.19
GE (MJ/kg DM)	21.87	20.83
Fat (g/kg DM)	58.49	59.33

Wheat	61.40%
Soybean meal 48	29.44%
Soy oil	4.11%
Salt	0.25%
Sodium Bicarbonate	0.18%
DL Methionine	0.40%
Lysine HCl	0.46%
Threonine	0.19%
L-Tryptophan	0.018%
Limestone	0.95%
Dicalcium Phos	1.59%
Vitamin/mineral premix	0.50%
TiO	0.50%
Quantum Blue	0.01%
Econase XT	0.005%

Appendix B Bird trial LB02 diet specification and formulation

Ingredient	Starter	Grower
Wheat	54.4185	59.025
Rapemeal	5	5
Hipro Soya	29.712	29.151
Soya oil	2.689	3.682
Limestone	0.7905	0.753
Salt	0.0985	0.173
Sodium bicarbonate	0.218	0.206
MCP	0.94	0.936
Lysine HCl	0.2255	0.221
Dl methionine	0.335	0.291
Threonine	0.08	0.069
Phytase	0.015	0.015
Ronozyme	0.015	0.015
Maxiban	0.063	0.063
Vit min premix	0.4	0.4
Fishmeal	5	0
	100	100

Appendix C Bird trial LB03 diet specification and formulation

	Phase		
Ingredients (%)	Starter	Grower	Finisher
Barley	5.5	0.9	0
Rye	10	15	20
Wheat	45	47.5	47.2
Soybean meal, 48% CP	26	23	19
Full fat soybean meal	5	5	5
L lysine HCL	0.4	0.3	0.3
DL methionine	0.4	0.35	0.3
L threonine	0.15	0.15	0.15
Soya oil	4	4.5	4.75
Limestone	1.25	1.25	1.25
Monocalcium phosphate	1.5	1.25	1.25
Salt	0.25	0.25	0.25
Sodium bicarbonate	0.15	0.15	0.15
Premix*	0.4	0.4	0.4
Calculated analysis			
ME MJ/kg	12.75	12.99	13.11
CP%	21.75	20.49	18.91
Lys %	1.45	1.29	1.17
Met + Cys %	1.04	0.96	0.87
Ca	0.96	0.91	0.91
Available P	0.48	0.42	0.42
	100	100	100

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

Treatment	Week	Phase	% Dry matter	% Ash	% Fat	GE (MJ/kg)	N (%)	Protein (%)
A	1	Starter	87.70	5.46	5.12	16.85	3.53	22.06
B	1	Starter	87.76	4.48	5.19	17.05	3.44	21.50
C	1	Starter	88.04	5.12	4.85	16.89	3.29	20.56
D	1	Starter	88.21	5.62	4.85	16.98	3.33	20.81
E	1	Starter	87.51	4.61	5.65	16.86	3.39	21.19
F	1	Starter	87.73	4.66	5.32	16.93	3.43	21.44
A	2	Starter	87.71	5.91	5.34	16.85	3.3	20.63
B	2	Starter	87.41	3.56	5.28	17.17	3.04	19.00
C	2	Starter	88.05	6.41	5.29	16.80	3.34	20.88
D	2	Starter	87.65	5.58	5.33	17.00	3.54	22.13
E	2	Starter	87.54	3.67	5.39	16.96	3.29	20.56
F	2	Starter	87.54	3.11	5.40	17.11	3.19	19.94
A	3	Grower	87.86	5.16	5.59	17.05	3.13	19.56
B	3	Grower	87.60	3.81	5.26	17.02	3.19	19.94
C	3	Grower	88.16	5.00	5.31	16.88	3.03	18.94
D	3	Grower	88.26	5.24	5.28	17.06	3.25	20.31
E	3	Grower	87.38	3.54	5.28	16.98	3.01	18.81
F	3	Grower	87.43	4.06	5.29	17.18	3.33	20.81
A	4	Grower	88.29	5.66	5.35	17.10	3.19	19.94
B	4	Grower	89.24	5.02	5.32	16.96	3.09	19.31
C	4	Grower	86.55	5.22	5.31	17.03	3.28	20.50
D	4	Grower	86.80	4.69	5.30	17.15	3.05	19.06
E	4	Grower	88.16	4.59	5.35	17.15	3.01	18.81
F	4	Grower	88.05	4.50	5.39	17.01	3.18	19.88
A	5	Finisher	86.89	4.73	5.51	16.84	2.86	17.88
B	5	Finisher	87.87	4.44	5.27	17.10	3.03	18.94
C	5	Finisher	86.74	5.48	5.69	17.14	2.92	18.25
D	5	Finisher	87.30	5.19	5.42	16.99	3	18.75
E	5	Finisher	87.82	4.11	5.30	17.02	2.82	17.63
F	5	Finisher	87.81	4.08	5.40	17.05	2.92	18.25
A	6	Finisher	86.90	5.48	5.96	17.03	3.13	19.56
B	6	Finisher	87.89	4.95	5.38	17.39	2.81	17.56
C	6	Finisher	87.92	5.03	5.67	17.19	3.51	21.94
D	6	Finisher	87.88	5.16	5.63	17.51	2.77	17.31
E	6	Finisher	87.90	4.53	5.69	16.85	3.1	19.38
F	6	Finisher	87.95	4.50	5.32	16.86	3.08	19.25

Appendix D Bird trial LB03 Table Quality of extracted RNA from ileum tissue diet

Samples	D7 260/280	D7 260/230	D21 260/280	D21 260/230	D42 260/280	D42 260/230
T1R1	2.07	2.02	2.05	2.2	2.05	2.2
T1R2	2.07	1.83	2.04	2.18	2.04	2.18
T1R3	2.07	1.85	2.01	2.03	2.04	2.11
T1R4	2.06	2.23	2.01	2.07	2.03	2.08
T1R5	2.05	1.89	2.04	1.81	2.07	1.71
T1R6	2.01	2.06	2.05	2.23	2.07	1.73
T2R1	1.99	2.13	2.07	2.01	2.07	2.01
T2R2	2.04	2.13	2.07	2.09	2.04	1.87
T2R3	2.09	2.21	2.01	2.03	2.05	1.88
T2R4	2.04	2.22	2.05	1.85	2.04	1.76
T2R5	2.07	2.01	2.04	2.13	2.06	1.75
T2R6	1.98	1.9	2.06	1.92	2.05	1.67
T3R1	2.1	1.94	2.05	2.06	2.05	2.06
T3R2	1.91	1.77	2.0	2.0	2.05	2.17
T3R3	2.05	2.21	2.06	1.86	2.01	1.68
T3R4	2.07	2.03	2.07	2.17	2.07	2.28
T3R5	2.07	2.07	2.04	2.1	2.03	1.88
T3R6	1.94	1.85	2.06	1.85	2.1	1.73
T4R1	1.99	2.12	2.05	2.21	2.07	1.98
T4R2	2.06	2.14	2.05	2.23	2.06	2.27
T4R3	2.06	2.19	2.05	2.02	2.06	2.19
T4R4	2.06	1.8	2.03	1.95	2.04	1.88
T4R5	2.05	1.75	2.06	1.93	2.05	2.04
T4R6	2.07	2.13	2.06	2.23	2.03	2.09
T5R1	2.06	1.99	2.06	1.95	2.05	2.1
T5R2	2.06	2.12	2.05	2.17	2.03	2.07
T5R3	2.07	2.1	2.06	1.95	2.02	1.94
T5R4	1.92	1.75	2.06	2.28	2.06	1.77
T5R5	2.05	1.79	1.99	1.88	2.06	1.79
T5R6	2.07	2.01	2.07	1.87	2.06	2.11
T6R1	2.06	1.76	2.07	1.73	2.05	1.93
T6R2	2.03	1.99	2.01	1.81	2.02	2.16
T6R3	2.08	2.15	2.03	2.08	2.06	1.91
T6R4	1.95	1.79	2.03	1.93	2.04	2.18
T6R5	1.81	1.86	2	2.23	2.06	1.7
T6R6	2.06	2.1	2.08	2.24	2.06	2.16

*T: Treatments, R replicate for all samples Ratio 260/280 as an indicator for the pure RNA of DNA. Ratio 260/230 as an indicator for the purity of RNA of all treatments and control

Appendix E Bird trial LB03 Table Quality of extracted RNA from the Bursa of Fabricius tissue.

Samples	D7 260/280	D7 260/230	D21 260/280	D21 260/230	D42 260/280	D42 260/230
T1R1	1.84	1.59	2	1.96	1.98	2.31
T1R2	1.97	2.04	1.99	1.79	2.03	1.97
T1R3	2.07	1.86	2.02	1.56	2.01	1.92
T1R4	2.08	1.98	2.04	1.57	2.02	1.84
T1R5	2.03	1.93	1.94	1.72	1.99	2.13
T1R6	2.03	2.21	1.91	2	2	1.91
T2R1	1.98	1.74	1.99	2.7	2.01	2.27
T2R2	2	2.25	2	1.97	2.05	2.02
T2R3	2.06	1.87	2	2	2.05	1.65
T2R4	1.99	2.23	2	2.16	2.03	2.22
T2R5	1.94	1.67	2	1.98	2.03	2.08
T2R6	1.96	1.7	2	2	2.02	2.28
T3R1	2.05	2.01	2.01	1.96	1.99	2.1
T3R2	2	1.9	1.99	1.69	2.01	1.86
T3R3	2.04	1.96	2.02	1.66	2	2.08
T3R4	2.04	1.75	2.04	1.67	2.01	2.27
T3R5	2.01	1.8	1.94	1.72	2	2.01
T3R6	2.05	2.19	1.91	2.07	2	1.83
T4R1	2.05	2.13	1.99	2.17	1.99	2.3
T4R2	2.05	1.91	2.07	1.97	2.05	1.88
T4R3	1.99	1.92	2.02	2.1	2.03	2.27
T4R4	2.06	1.64	2.04	2	2.01	2.15
T4R5	2.02	1.96	2	2.26	2.02	2.24
T4R6	2.04	1.81	1.98	1.93	2.01	2.24
T5R1	2.02	2.02	2.01	2.07	2.03	2.24
T5R2	2.1	2.28	2.07	2.26	2.04	2.26
T5R3	2.06	2.13	2.06	2.31	2.05	2.25
T5R4	2.03	2.22	2.06	2.27	2.05	2.16
T5R5	2.03	2.22	2.07	2.25	2.04	2.22
T5R6	2.01	2.24	1.99	2	2.02	1.95
T6R1	1.95	1.79	2.08	2.2	2.04	2.4
T6R2	2.03	1.82	2.04	2.18	2.01	2.14
T6R3	2.03	1.56	2.06	1.72	1.92	2.36
T6R4	2.06	2.08	2.07	1.44	2.02	2.42
T6R5	2.03	1.99	2.05	2.05	2.04	2.28
T6R6	2	2	2	2	1.99	2.3

*T: Treatments, R replicate of all samples Ratio 260/280 as an indicator for the purity of RNA of DNA.

Ratio 260/230 as an indicator for the purity of RNA of all treatments and control

Appendix F Table Room Plan Diet Allocation bird trial LB03

36 T6			10 T1		9 T3
35 T5			11 T2		8 T2
34 T4			12 T3		7 T1
33 T6			13 T1		6 T3
32 T5			14 T2		5 T2
31 T4			15 T3		4 T1
30 T6			16 T1		3 T3
29 T5			17 T2		2 T2
28 T4			18 T3		1 T1
27 T6					
26 T5					
25 T4					
24 T6	23 T5	22 T4	21 T6	20 T5	19 T4

Appendix G lighting regimen for all bird studies

Day	Hours of light	Hours of dark
1	23	1
2	22	2
3	21	3
4	20	4
5	19	5
6	18	6
7 to end	18	6

Appendix I Performance of chicken (Ross 308) in Iraqi conditions

Age day	Body weight	Weight gain	feed intake	FCR
7	157	118	143	1.22
14	440	282	369	1.30
21	753	312	531	1.70
28	1149	396	755	1.92
35	1621	471	975	2.17
42	2504	882	1505	1.75