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Akoy, Rebin Aswad Mirza

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By
Rebin Aswad Mirza Akoy

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Abstract

The microbial populations in the gastrointestinal tracts of poultry play an important role in normal digestive processes and in maintaining animal health. The purpose of this study was to evaluate the effects of probiotics, prebiotics and synbiotics on the growth parameters, gut ecosystem, histology and immune function. In this study, four experiments one *in vitro* and three *in vivo* were conducted using specific pathogen free (SPF) and Hubbard broiler chickens.

The first experiment was designed to determine the influence of inulin as an effective prebiotic on lactic acid bacteria (LAB) strains, and to screen LAB for selection as a source of chicken probiotic. Eight strains of LAB were isolated from chicken caeca and three strains from the Plymouth University culture collection were screened for potential probiotic properties for growth in inulin from Jerusalem artichoke (*Helianthus tuberosus*) and commercial inulin (Frutafit® HD, Netherlands). *Lactobacillus animalis* JCM 8692 strain isolated from chicken caeca showed the highest auto-aggregation and co-aggregation ability, resistance to acidity and bile salts, strong suppression of pathogens and ability to adhere to epithelial cells compared with other isolated strains.

The second experiment was conducted to investigate the influence of commercial inulin and Jerusalem artichoke tubers as prebiotic supplementation on the diversity of the caecal microflora, jejunum histology and immune organ of SPF chickens. This investigation has found that inulin which was extracted from JA had a similar result when compared with commercial inulin and could be a suitable candidate for an inulin source in broiler diets.

The third experiment was conducted to investigate the influence of Bactocell® (PRO1) and *Lb. animalis* (PRO2) as probiotic supplements on broiler chickens. EPEF was significantly increased in probiotic1 and probiotic2 compared with control (311.03, 309.87 and 260.06) respectively. Both types of probiotics supported the growth of chicks healthy and could be a suitable candidate as a source of probiotic in broiler diet.

The fourth experiment was conducted to investigate the influence of dietary supplementation of a probiotic (*Lb. animalis*), a prebiotic JA tuber and a combination of both (Synbiotic) in broiler chickens. Growth performance was improved in all additive supplementation compared with the control group. EPEF was increased in probiotic, prebiotic and synbiotic compared with control (290.8±11.8, 300.9±3.86, 322.1±7.09 and 262.3±5.94) respectively. Beneficial bacteria in the guts of chicks fed probiotic, prebiotic and synbiotic was increased compared with chicks fed control diet. The diversity of microbial population in the gastrointestinal tract of chickens improved due to additives. The intestinal villus lengths and microvilli density was improved in all additives supplementation in comparison with control. Overall, it was concluded that probiotic, prebiotic and synbiotics can positively affect production performance and can improve the gut health.

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List of abbreviations

ANOVA	Analysis of variance
AGP	Antibiotic growth promoter
BLAST	Basic local alignment search tool
BF	Bursa of Fabricius
CFU	Colony forming unit
CON	Control group
CL	Cooking loss
D	Day
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EPEF	European Production Efficiency Factor
FCR	Feed conversion ratio
FOS	Fructooligosaccharide
GE	Gross Energy
GI	Gastrointestinal
HPLC	High Performance Liquid Chromatography
JA	Jerusalem artichoke
IBDV	Infection bursa disease virus
LAB	Lactic acid bacteria
LBW	Live body weight
MOS	Mannanoligosaccharides
MRS	Man, Rogosa and Sharp
NCBI	National Center for Biotechnology Information
NCIMB	The National Collection of Industrial, Marine and food Bacteria
ng	Nanogram
nt	Nucleotide
OD	Optical density
OTU	Operative taxonomy unit

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rRNA	Ribosomal Ribonucleic Acid
SCAN	Scientific Committee for Animal Nutrition
SCFA	Short chain fatty acid
SD	Standard deviation
SEM	Scanning electron microscope
SF	Shearing force
TAE	Tris-acetate- Ethylene Diamine Tetra Acetic Acid
TE	Tris- Ethylene Diamine Tetra Acetic Acid
µg	Microgram
µl	Microlitre
XLD	Xylose Lysine Deoxycholate medium

All experimental work involving animals was carried out at the University of Plymouth, Animal housing unit. The studies were conducted according to UK Home Office regulations (Animal Scientific Procedure Act 1986) under the Home Office project license PPL 30/2640 and personal license PIL 30/10067.

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Rebin Aswad Mirza 2015

Dedication

I would like to express my love and gratitude to my wife (Lana), for her continued encouragement, support, and sacrifices throughout my educational pursuits and daily life. I would also like dedicate to my little son (Las).

This thesis is dedicated to my parents, whose love and support allowed me to complete this education. It is also dedicated to my brothers (Rekar, Rebaz and Rawen) who provided the support and encouragement that helped me make it through the tough times.

Conferences contributions and published papers

Oral presentations

Akoy, R., Merrifield D., Davies S. and Beal J. (2013) Effect of dietary inulin supplementation on microflora and intestinal histology of broiler chicks. *An international scientific conference on probiotics and prebiotics (IPC 2013), Kosice, Slovakia. 11-13 June 2013, Page 12.*

Akoy, R., Merrifield D., Davies S. and Beal J. (2013) Effects of inulin supplementation on structure of caecal microflora and intestinal Histomorphology of broiler chickens. Centre for Agricultural and Rural Sustainability (CARS) 4rd PG Symposium 2013 - *Duchy College, Cornwall- UK. 11/11/2013.*

Akoy, R., Merrifield D., Davies S. and Beal J. (2014) The Influence of Probiotics, Prebiotics and Synbiotics on Growth Performance, Intestinal Microflora and Histology of Broiler Chickens. Centre for Agricultural and Rural Sustainability (CARS) 5th PG Symposium 2014 - *BBSRC North Wyke Experimental Station – Rothamsted Research, Okehampton - UK. 06/06/2014.*

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Akoy, R., Merrifield D., Davies S. and Beal J. (2013) Effect of inulin on the growth of Lactic acid bacteria as prebiotic and screening of LAB toward their

selection for chicken probiotic. *Society for Applied Microbiology*. International conference, Cardiff-Wales, International Scientific Association for Probiotics and Prebiotics, 01/07 – 04/07/2013.

Akoy, R., Merrifield D., Davies S. and Beal J. (2014) The influence of feeding inulin supplementation on intestinal histology and immune functions of broiler chicks. Iraqi PG conference, Plymouth – UK, 01/03/2014.

Akoy, R., Merrifield D., Davies S. and Beal J. (2014) Effects of dietary inulin supplementation on caecal microflora and haematology of broiler chickens. Plymouth PG conference, Plymouth – UK, PG society 19/03/2014.

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Akoy, R., Merrifield D., Davies S. and Beal J. (2015) 'Effect of Probiotic, Prebiotic and Synbiotic Supplementation in the Diet on Performance, Intestinal length and Histology and Gut Microflora of Broiler Chickens'. In preparation.

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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CHAPTER ONE: Literature review

1.1 Introduction

The poultry industry has become an important economic activity in many countries, and has been due to developments in several areas such as nutrition, genetics and management strategies to maximize the efficiency of growth performance and meat production. The mortality of chickens due to intestinal pathogens such as *Escherichia coli*, *Salmonella*, *Campylobacter* and *Clostridium perfringens* continues to cause problems, especially with high stocking densities associated with intensive production systems. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines.

For the past four decades, antibiotics have been used as an additives in poultry feed to enhance the growth performance and protect birds from the negative consequences of pathogenic and non-pathogenic enteric microorganisms. Antibiotic feed additives were banned by the European Union in 2006 due to concerns over the rise of widespread antibiotic resistance in human pathogens. Consequently, poultry producers are seeking alternatives to maintain efficient poultry production.

Probiotic, prebiotic and synbiotic can be used as an attempt to reduce the chances of infection in poultry. There are various definitions of Probiotics for example, according to FAO/WHO (FAO/WHO, 2002), defined probiotics as mono or mixed cultures of “live microorganisms which, when administered in adequate amounts confer a health benefit on the host”. Prebiotics are defined as ‘a non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the

colon' (Gibson and Roberfroid, 1995). The combination of a probiotic and prebiotic is called synbiotic and includes both beneficial microorganisms and substrates, which may have synergistic effects on the intestinal tract of animals.

A number of probiotics are available commercially for use in poultry production, such as, *Lactobacillus* and *Bifidobacterium* (Ziggers, 2000), *Lactobacillus* strains (Lan *et al*., 2003), protexin® (multistrain probiotic) (Ayasan *et al*., 2006; Gunal *et al*., 2006), *Saccharomyces cerevisiae* (Zhang *et al*., 2005), Thepax® (Yousefi and Karkoodi, 2007). These bacteria are used alone or in combination. Prebiotics such as Mannanoligosaccharids (Flemming *et al*,. 2004), Fructooligosaccharides (Verdonk and Leeuwen, 2004) and inulin (Roberfroid, 2007; Sofia and Gibson, 2007; Rehman *et al*., 2008) enhance the growth of intestinal bacteria and may affect the intestinal histology. Synbiotics may work in one of two ways, they may promote the growth of the co-administered probiotic or they may promote the growth of other beneficial organisms in the gut that in turn benefit the co-administered probiotic.

Probiotic feed supplements have been used to modulate the composition of the gut microflora by successfully competing with pathogens through a competitive exclusion process (Mountzouris *et al*., 2007). Competitive exclusion by intestinal bacteria is based on bacteria-to-bacteria interaction mediated by competition for available nutrients and mucosal adhesion sites, it is one of the most important beneficial mechanisms of probiotic bacteria. The gastrointestinal tract (GIT) plays a fundamental role in the absorption of nutrients and protection against many kinds of pathogens that enter the body via the feed. The GIT also provides a suitable ecosystem for various populations of microorganisms that create a symbiotic relationship with the host. These microorganisms are found throughout

the tract and are most extensive in the cecum of chicken (Amit-Romach *et al*., 2004).

Inulin derived from some kind of plants such as Jerusalem artichoke, chicory, garlic, onion, asparagus; leak; banana, dandelion (Van Loo *et al.,* 1995). Jerusalem artichoke and chicory are natural sources of inulin those are rich in inulin (Kaur and Gupta, 2002; Stolzenburg, 2005). The plant that is most commonly used industrially for the extraction of inulin-type fructans are chicory (De Leenheer, 2007). Additions of inulin from chicory was found to affect positively on performance in monogastric animals (chicken, pig, rabbit, and rat), especially in young animals (Rehman *et al*., 2007a; Rehman *et al*., 2008; Liu, 2008; Rebole *et al*., 2010; Awad *et al*., 2011). However, in poultry, very few reports have focused on the effect of inulin from Jerusalem artichoke on the gut microflora of the chicken gastrointestinal tract at the present time.

1.2 The concept of probiotic

The concept of probiotic is relatively meaning “for life” and it is currently used to name bacteria associated with beneficial effects in humans and animals. The original observation of the positive role played by some selected bacteria is attributed to Metchnikoff, the Russian born Nobel Prize winner in Medicine in 1908, at the Pasteur Institute at the beginning of the last century, who proposed that the long and healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. He believed that when consumed, the fermenting bacillus (*Lactobacillus*) replaced the intestinal microflora (Metchinkoff, 1907).

Many definitions have been proposed for the term probiotics. But, Crawford (1979) was the first person to define probiotic as “a culture of specific living micro-organisms (primarily *Lactobacillus* spp.) which implant in the animal to ensure the effective establishment of intestinal populations of both beneficial and pathogenic organisms”. Fuller (1989) later gave an innovative definition of probiotics as “a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance”. The US National Food Ingredient Association presented, probiotic (direct fed microbial) as a source of live naturally occurring microorganisms and this includes bacteria, fungi and yeast (Miles and Bootwalla, 1991). According to the currently defined by FAO/WHO (FAO/WHO, 2002), probiotics were defined as mono or mixed cultures of “live microorganisms which, when administered in adequate amounts confer a health benefit on the host”.

A list of the probiotic species for studies or application are used in animal feeds that shown in (Table 1.1). These data were derived from extensive literature and internet search of commercial products. *Lactobacillus spp.*, *Enterococcus spp.*, *Bacillus spp.* and *Saccharomyces spp.* are actually the most used probiotics in livestock and poultry. Many studies indicate that the organisms cited on the labels of certain probiotic products.

Table 1.1: List of probiotics studied for application in animal feed.

Genus	Species
Bifidobacterium	*B. animalis*subsp. *animalis* (*B. animalis*) *B. lactis*subsp*. lactis (B. lactis)* *B. longum* subsp*. longum(B. longum)* *B. pseudolongum* subsp*. pseudolongum (B. pseudolongum)* *B. thermophilum*
Enterococcus	*E. faecalis* (*Streptococcus faecalis*) *E. faecium* (*Streptococcus faecium*)
Lactobacillus	*L. acidophilus, L. Amylovorus, L. brevis* *L. casei* subsp*. casei (L. casei), L. crispatus* *L. farmicinis, L. fermentum, L. murinus* *L. plantarum* subsp*. plantarum (L. plantarum)* *L. reuteri, L. rhamnosus, L. salivarius* *L. amylovorus (L. sobrius)* *Lactococcus L. lactis* subsp*. cremoris (Streptococcus cremoris)* *L. lactis*subsp*. lactis* *Leuconostoc L. citreum* *L. lactis* *L. mesenteroides*
Pediococcus	*P. acidilactici* *P. pentosaceus* subsp*. pentosaceous* *Propionibacterium P. Freudenreichii*
Streptococcus	*S. infantarius* *S. salivarius subsp. salivarius* *S. thermophilus*(*S. salivarius* subsp. *thermophilus*)
Bacillus	*B. cereus* (*B. cereus* var. *toyoi*) *B. licheniformis* *B. subtilus*
Saccharomyces	*S. cerevisiae* (*S. boulardii*) and *S. pastorianus* (*S. carlsbergensis*)
Kluyveromyces	*K. fragilis* and *K. marxianus*
Aspergillus	*A. orizae* and *A. niger*

(Gaggia *et al*., 2010).

There are several commercial probiotic products available in the market and some use in poultry diets are as follows (Table 1.2).

Table 1.2: A number of probiotic products are available commercially for use as bacteria supplements in poultry diets (SCAN, 2003).

Product name	Probiotic Types	Collection number	Chicken target
Bactocell®	*Pedicoccus acidolactici*	CNCM MA 18/5M	Broiler
Bioplus 2B®	*Bacillus licheniformis*	DSM 5749	Broiler & Turkey
	Bacillus subtilis	DSM 5750	
Cylactin LBC®	*Enterococcus faecium*	NCIMB 10415	Broiler
Lactobacillus acidophilus D2/CSL®	*Lactobacillus acidophilus*	CECT 4529	Broiler & Laying hens
Microferm®	*Enterococcus faecium*	DSM 5464	Broiler
Oralin®	*Enterococcus faecium*	NCIMB 10415	Broiler
Probios PDFM Granular®	*Enterococcus faecium*	DSM 4788/ ATCC 53519	Broiler

1.3 The concept of prebiotic

Prebiotics are defined 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’ (Gibson and Roberfroid, 1995). In other words, prebiotics are provided as a substrate for beneficial microorganisms in the gastrointestinal tract. Large amounts of beneficial bacteria are capable of consuming and digesting these types of carbohydrate sources for energy, where consequently cause increased activity of beneficial bacteria (Hillman, 2001).

There are some characteristics of prebiotics as an effective source to promote beneficial impact on poultry production and their health status, it should have the following properties: 1) it should be resisted in acidic in the GI tract, 2) is not hydrolyzed by GI tract enzymes, 3) is not absorbed in the upper part of GI tract, 4) is fermented by beneficial bacteria in the intestine, and 5) encourage selective stimulation of growth and/or activity of intestinal microorganisms, potentially associated with health and well-being (Gibson and Roberfroid, 1995).

Table 1.3: Major oligosaccharide candidates for prebiotics.

Oligosaccharides	Structure	Linkages	Process	Origin
Xylo-oligosaccharides	(Glu)n	β-1,4	Hydrolysis	Cereals
Lactulose	Gal-Fru	β-1,4	Isomerisation	lactose
Isomalto-oligosaccharides	(Glu)n	α-1,6	Hydrolysis	Algae
Gluco-oligosaccharides	(Glu)n	α-1,2 and α-1,6	Synthesis	Sucrose
Galacto-oligosaccharides	(Gal)n-Glu	β -1,4 and β -1,6	Synthesis	Lactose
Fructo-oligosaccharides	(Fru)n-Glu	(β-2,1)- α-1,2	Synthesis	Sucrose
Oligofructose	(Fru)n-(Fru)n-Glu	(β-2,1)	Hydrolysis	Inulin

(Shim, 2005).

1.4 The concept of synbiotic

Prebiotics and probiotics have been proven to promote gastrointestinal health and immune function. The concept behind probiotics is to enhance good bacteria and discourage bad bacteria in the animal gastrointestinal tract. Prebiotics, which enhance the growth of beneficial bacteria in the lower intestine, are primarily fibres naturally found in food. The food industry is in a position to recognize that prebiotics and probiotics may contribute to helping improve public health by promoting gastrointestinal health as well as immune function.

When probiotics and prebiotics are used in combination, they are known as 'synbiotics' (Collins and Gibson, 1999; Patterson and Burkholder, 2003; Buriti *et al*., 2007; Pool-Zobel and Sauer, 2007). This combination can improve the viability of probiotic microorganisms, since they are able to use prebiotics as a substrate for fermentation (Bengmark, 2001). This concept has been tested in poultry and it is shown that a prebiotic that is administered with a probiotic gives a greater response than when administered separately (Pelicano *et al*., 2005; Westhuizen, 2008). Many scientific studies are conducted in order to find the most effective probiotic and prebiotic supplements to achieve a synbiotic action by using them in combination (El-Banna *et al*., 2010).

The results on the efficacy of synbiotic products as feed additives in livestock and poultry needs further investigation. In the last few years, studies on synbiotics have started to elicit. Probiotic bacteria taken together with prebiotic that support their growth performance. Both probiotic and prebiotic work together in a synergistic way more efficiently promoting the probiotic and prebiotic benefits alone, and the coupling could also yield a synergistic effect in the reduction of pathogenic bacterial populations in the GIT.

Abdel-Raheem *et al.* (2012) found that the synbiotic product (Mannan-Oligosaccharide and *Saccharomyces cerevisiae*) had significantly higher ($p<0.05$) villus height in the duodenum, jejunum, ileum in comparison with the probiotic, prebiotic and control groups. On the other hand, there are numerical not statistical ($p>0.05$) decreases in the *E. coli* colony count in the different parts of the small intestine and the caecum as a response to dietary treatments and this decrease was more clear in synbiotic supplemented broilers compared other treatments. It seems that synergistic effects of prebiotics and probiotics can be useful in stimulating beneficial bacteria and improving the health of the gut. However, there is little information available to date on synbiotics and its possible mechanisms in broiler chickens.

1.5 Probiotics and prebiotics in poultry diet

In the short lifespan of broiler chickens any delay in microbial colonization of the intestinal tract can leave the bird's intestine open to disease. In the natural environment, the mother is always responsible for feeding their hatching chicks with a feed which stored in their crop. This feed was fermented in the mother's crop and mixed with beneficial microbes which transformed to the hatching chicks as a probiotic. On the other hand, the hatching chicks always eat some of mother feces and the beneficial microbes were transformed from mother to hatch chicks. These beneficial microbes from mother's feces were able to protect the hatching chicks from pathogenic microbes (Fuller, 2001). However, commercially reared chickens are hatched in incubators which are clean and do not usually contain organisms commonly found in the chicken gut. The young chickens lack contact with the natural environment so colonisation of the intestinal tract is often a more prolonged process taking around 21 days for broilers to develop a balanced intestinal flora (Barnes, 1979; Amit-Romach *et al*., 2004). This period represents around 50% of a broiler's lifespan and it has been found that the later intestinal colonisation occurs, the more vulnerable the intestinal ecosystem is to colonization by pathogenic microorganisms. After the first 21 days of life, other challenges such as stress, feed changes, antibiotic interventions, and disease can also upset the gastrointestinal flora and can lead to poor weight gain or considerable loss of stock (Gasson *et al*., 2004). Moreover, also hydrochloric acid (HCl) gastric secretion, which starts at 18 days of incubation, has a deep impact on microflora selection. Therefore, an immediate use of probiotics and prebiotics supplementation at hatch is more important and useful in avian species (Mirza, 2009).

1.6 Ecology of microflora in the chicken gastrointestinal tract

Generally, microflora of the digestive tract can be divided into two groups. The first, harmful bacteria, which is may be involved in the induction of infection, intestinal putrefaction and toxin production. The second, commensal bacteria, which may be involved the vitamin production, stimulation of the immune system and suppression of pathogen bacteria (Jeurissen *et al*., 2002).

On the other hand, there are two types of microorganisms as populations that are found within the GI tract of poultry. The first, established microflora or autochthonous bacteria, are colonized the gut by inoculation resulting from environmental exposure and normal feeding activities of the bird (Gusils *et al*., 1999a). The second, transitory microflora or allocthonous bacteria, are exogenous in nature and are introduced as a dietary supplement into the GI tract through the feed or drinking water as direct fed microbial (DFM) or probiotics (Patterson and Burkholder, 2003). Some data in the literature indicate that allocthonous bacteria introduced via probiotics may prevent infection and colonization of the GI tract by opportunistic pathogens (Fuller, 1989).

The GI tract consists of a diverse community of bacteria. The development of this community begins on hatching, and bacteria are raised from the environment, the feed, and the people handling the chicks post-hatch. Each of these three areas can, therefore, affect gut microbiota development. Microbes are found across the entire length of the GI tract, where they show locative variation in community composition biogeographically (Figure 1.1).

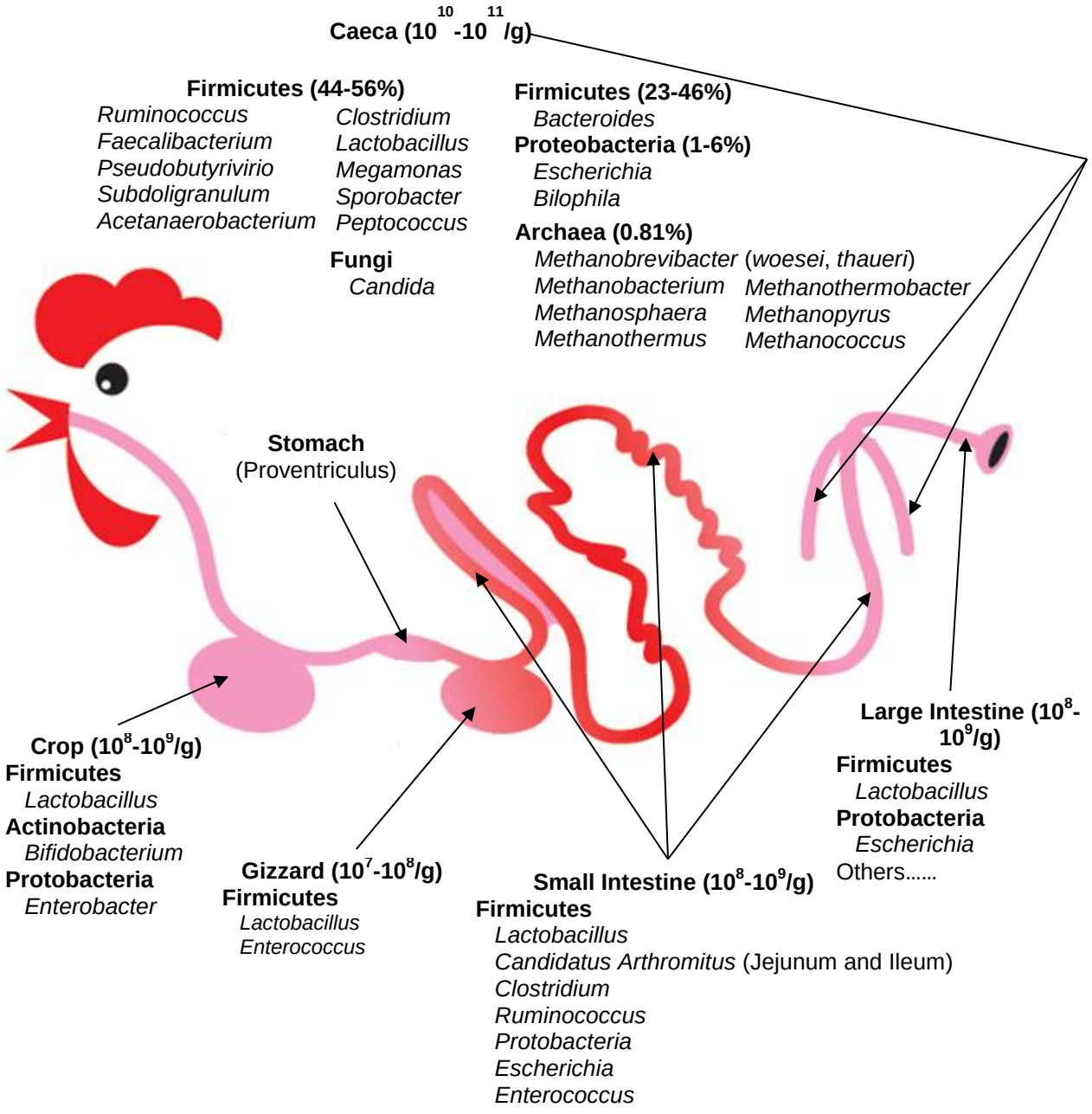


Figure 1.1: Major types of surveyed bacteria along the gastrointestinal tract of chicken. Adapted from (Yeoman *et al*., 2012).

1.7 Histology of the bird small intestine

The small intestine is differentiated into three main regions namely, the duodenum, jejunum and the ileum. The small intestine considered as the most important part in the GI tract, because majority of the enzymatic digestion occurs and that will remain the food mass for a long time and for more than eight hours in this part of GI tract. The small intestine is also the most important centre for the presence of microorganisms inside the digestive tract. The small intestine is histologically composed of four layers from inside to outside: mucosa, submucosa, muscularis, and serosa (Figure 1.2). The inner lining of the intestines (Mucosa) composed in the form of fingers-like form called villi. The role of these protrusions is to increase the surface area exposed to the absorption, and increasing the length of villi refers to the high efficiency of the process of digestion and absorption, and also protection against many kinds of pathogens that enter the body via the feed. Submucosa is a layer of dense irregular connective tissue that supports the mucosa. Muscularis is composed of several thin layers of smooth muscle fibres, keeping the mucosal surface and underlying glands in a constant state of gentle agitation to expel contents of glandular crypts and enhance contact between epithelium and the contents of the lumen. The serosa consists of a thin layer of loose connective tissue covered by mesothelium. Increased villus height indicates a greater surface area increasing absorption of available nutrients (Caspary, 1992). There are many columnar epithelial cells called enterocytes on the walls of villi, and contains all the enterocyte a large number of microvilli which are brush border-like (Figure 1.2). Positive effects of the use of probiotics and prebiotics on the intestinal mucosa have been reported, among which, Xu *et al*., (2003) found that feeding on FOS as prebiotic (0.4%) has been reported to

increase the ileal villus height and crypt depth in broilers. Pelicano *et al*. (2005) observed that beneficial effects were seen in histological indexes of the intestinal mucosa with the use of probiotics and prebiotics at 21 days of age.

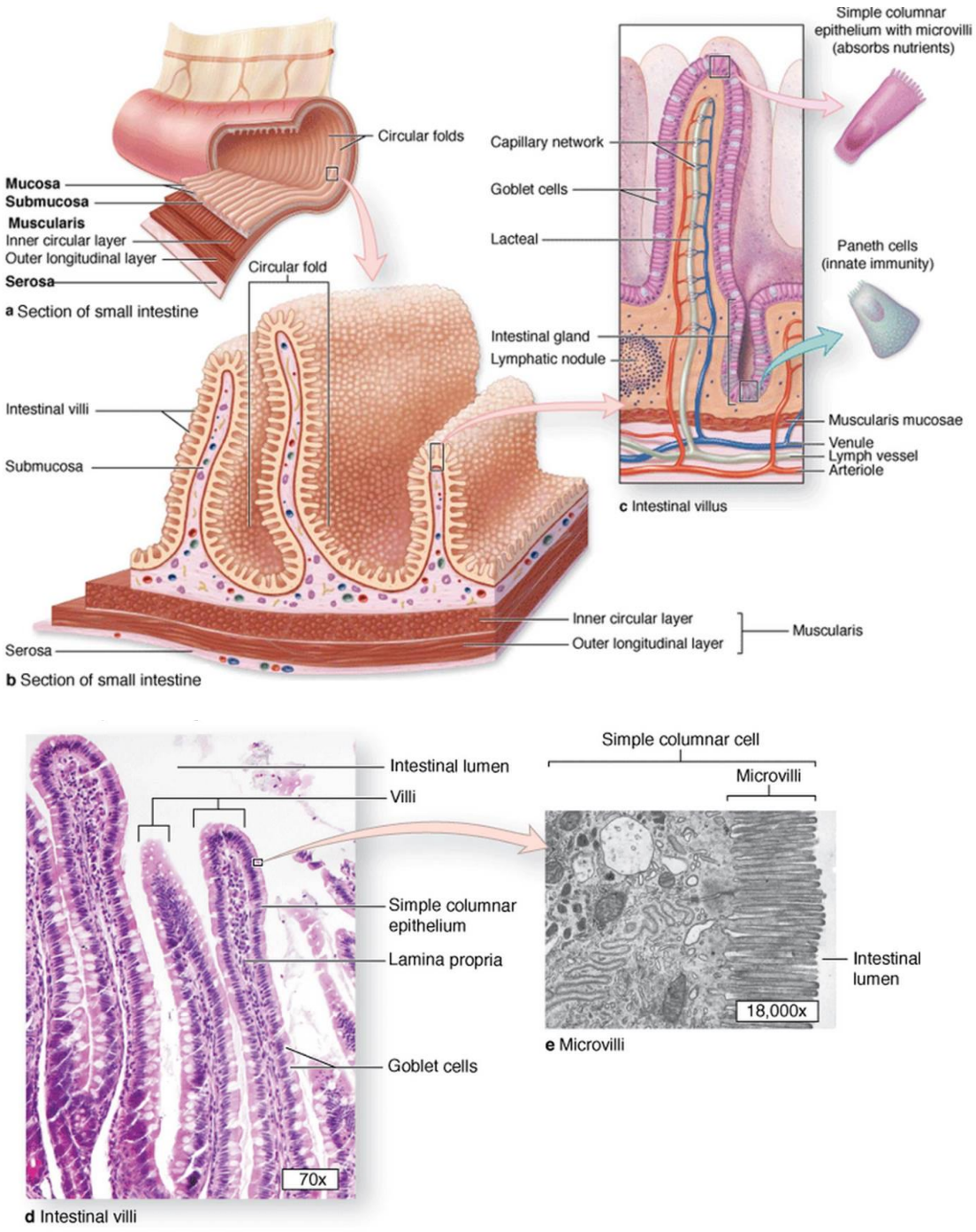


Figure 1.2: Histological structure of small intestine consist of four layers mucosa, submucosa, muscularis, and serosa (Mescher, 2013)

1.8 Action of probiotics in the gastrointestinal tract of chicken

The mode of action of probiotic feed additives in poultry is mainly based on four principles, (i) Maintaining normal intestinal microflora by competitive exclusion and antagonism (Kizerwetter - Swida and Binek, 2009), (ii) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production (Yoon *et al*., 2004), (iii) Improving feed consumption and digestion (Awad *et al*., 2006) and (iv) stimulating the immune system (Brisbin *et al*., 2008).

Also, Rolfe (1991) demonstrated that there are at least four major mechanisms involved in the development of a microenvironment that favours beneficial microorganisms. Beneficial microorganism's possess certain favourable characteristics that allow the expression of several mechanisms that prevent the pathogenic bacteria from colonising the gastrointestinal tract. These mechanisms are listed as follows; (i) creation of a microecology that is antagonistic to other bacterial species, (ii) elimination of available receptor sites, (iii) production and secretion of antimicrobial metabolites and (iv) competition for essential nutrition.

Enhancements of colonization resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms (Thomke and Elwinger, 1998).

The gastrointestinal tract is the largest immune organ in the body and is negatively affected by stress. Commercial poultry production will ultimately always have multiple stressors such as dietary changes, catching, transport, and feed

withdrawal. Stress will effectively and rapidly alter the intestinal population allowing for opportunistic pathogens to adhere to the gastrointestinal tract. *Lactobacillus* and *Bifidobacterium* species are examples of beneficial bacteria that populate the GIT and whose populations decrease when birds become stressed (Hong *et al*., 2005). A probiotic will work to repair or repopulate deficiencies within the intestinal microflora in turn stimulating the immune system against pathogenic infestation. To accomplish this probiotics work by indirectly and directly competing for nutrients and attachment sites in the intestine, enhancing the immune system, and producing antimicrobial compounds such as volatile fatty acids (Patterson and Burkholder, 2003; Ahmad, 2006; Callaway *et al*., 2008).

1.8.1 Competitive exclusion

The concept of competitive exclusion indicates that cultures of selected, beneficial microorganisms, supplemented to the feed, compete with potentially harmful bacteria in terms of adhesion sites and organic substrates, mainly carbon and energy sources (Schneitz, 2005). Probiotics may colonise and multiply in the gut, thereby blocking receptor sites and preventing the attachment of other bacteria including harmful species such as enteropathogenic *Salmonella* or *E. coli*. Certainly, probiotics have the potential to decrease the risk of infections and intestinal disorders. Wali (2012) showed that *Lactobacillus plantarum* (NCIMB 41607) significantly reduced the growth of *Salmonella Typhimurium* and *Salmonella Enteritidis* by 4 Log CFU/g in a chicken simulated digestive system *in vitro*. In piglets, attachment of enterotoxic *E. coli* to the small intestinal epithelium

was inhibited by dietary supplementation with *Enterococcus faecium* (Jin *et al.*, 2000).

Competitive exclusion of pathogens is thought to be one of the most important beneficial mechanisms of probiotic bacteria (Rolfe, 2000). Competitive exclusion by intestinal bacteria is based on bacteria-to-bacteria interaction mediated by competition for available nutrients and mucosal adhesion sites. In order to gain a competitive advantage, bacteria can also modify their environment to make it less suitable for their competitors (Gasson *et al.*, 2004). The production of antimicrobial substances, such as lactic and acetic acid, is one example of this kind of environmental modification (Liong and Shah, 2006). Competitive exclusion is a very effective measure to protect newly hatched chicks, turkey and possibly other game birds, to against *Salmonella* and other enteropathogens. Probiotics deliver many lactic acid bacteria into the gastrointestinal tract (Schneitz, 2005).

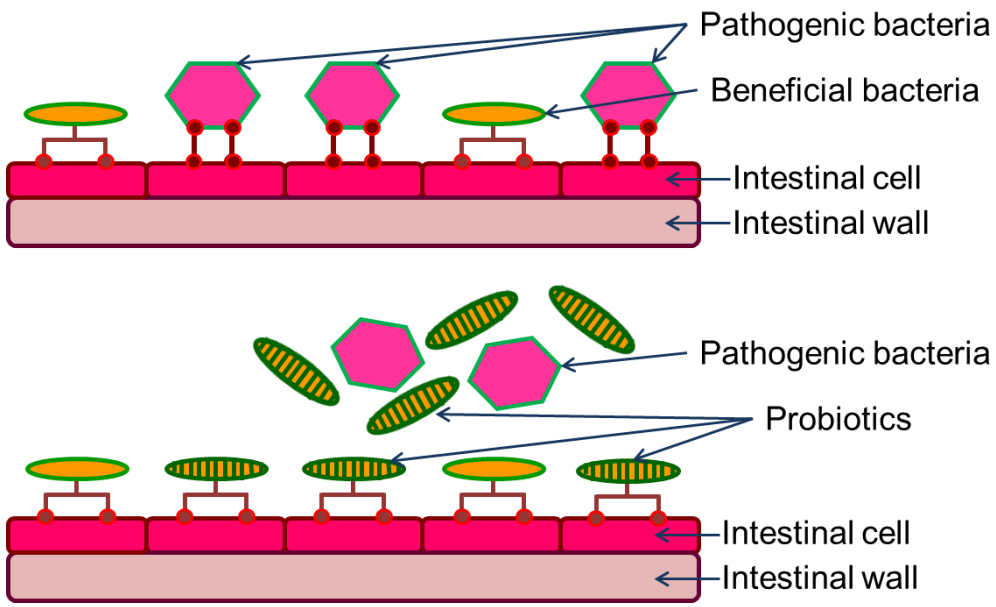


Figure 1.3: The beneficial bacteria when added to diet of poultry compete for binding sites on the intestinal epithelium.

1.8.2 Bacterial antagonism

Probiotic microorganisms, once established in the gut, may produce substances with bactericidal or bacteriostatic properties (bacteriocins) such as lactoferrin, lysozyme, and hydrogen peroxide as well as several organic acids. These substances have a detrimental impact on harmful bacteria, which is primarily due to a lowering of the gut pH (Fuller, 2001). In addition, competition for energy and nutrients between probiotic and other bacteria may result in a suppression of pathogenic species (Ewing and Cole, 1994).

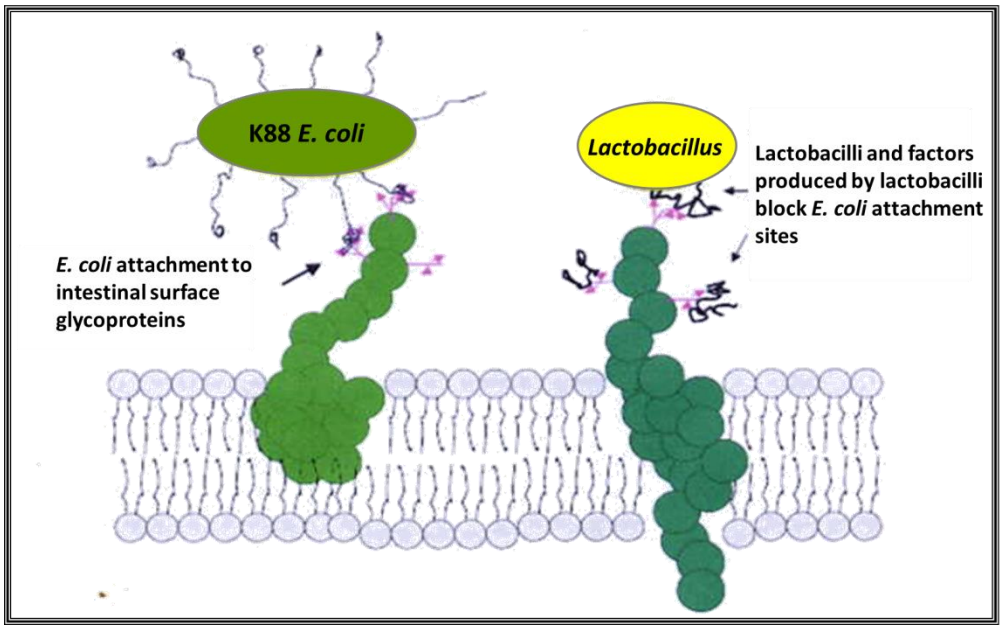


Figure 1.4: Inhibition of pathogenic bacteria by the antagonistic activity for Lactobacilli against *E.coli* through secrete some inhibitory growth to preventing adherence on receptors inside the gut (Patterson and Burkholder, 2003).

Savvidou (2009) resulted that all *Lactobacillus* strains isolated from healthy chickens were tested for their antagonistic activity against several pathogens showed able to inhibit the growth of *S.* Enteritidis (5188), *S.* Enteritidis of chicken origin, *S. typhimirium*, *E. coli* and *Cl. perfringens*.

1.8.3 Immune modulation

The other mode of probiotics action is to stimulate the efficiency of immune system. Chick is hatched with a sterile digestive system, and before its organism will be able to produce its own antibodies, microorganisms from the environment begin to colonize the digestive system. Therefore, the use of probiotics, due to their ability of adhesion to the intestinal mucosa, allows creating a natural barrier against potential pathogens, and thus enhances immunity. Probiotic stimulation of the immune system manifested by increased production of immunoglobulins, increased activity of macrophages and lymphocytes, and stimulates the production of γ-interferon (Yang and Choct, 2009).

The development and activation of the humeral and cellular gut-associated immune system is largely affected by the development of the gut microflora (Ouwerhand and Kirjavainen, 1999). According to Lan *et al*. (2005), microbial communities can support the animal's defence against invading pathogens by stimulating gastrointestinal immune response. Recent scientific investigation has supported the important role of probiotics as a part of healthy diet for human as well as animals and may be an avenue to provide a safe, cost effective and natural approach that sets up a barrier against microbial infection, thereby resulting in health maintenance and disease prevention (Parvez *et al.*, 2006). Consumption of LAB may have favourable effects on the immune system. Koenen *et al*. (2004) reported that the *Lactobacillus* strains have modulating effects on immune system of layer- and meat-type chickens. Furthermore, Nayebpor *et al*. (2007) reported that DFM enhanced humoral immune response in broiler chickens. Antibodies such as Immunoglobulin A (IgA) are produced by plasma cells of the immune system and are involved in protecting the body from

potentially harmful bacteria. Probiotic bacteria have been shown to alter host immune responses to infection by stimulating secretory IgA production (Fukushima *et al.*, 1998). While, Midilli *et al*. (2008) resulted that the dietary probiotic and prebiotic supplementation did not significantly effect on immunoglobulin concentration (IgG) in the serum of broilers.

1.9 Action of prebiotics in the gastrointestinal tract

Prebiotics are non-digestible in the upper part of intestinal tract. Prebiotic that has a beneficial effect through their selective metabolism in the intestinal tract (Gibson *et al*., 2004). The ability of a probiotic LAB strains to survive in the GI tract may be promoted by oligosaccharides facilitating the metabolism and growth of LAB in the lumen (Salminen *et al*., 1998a). Dietary fibre, mainly oligosaccharides and polysaccharides fermented in the colon may act as prebiotics (Ziemer and Gibson, 1998; Fooks *et al*., 1999). The importance of prebiotics as enhance of the growth and performance of probiotic bacteria has been documented in humans (Fooks *et al*., 1999; Crittenden *et al*., 2002). *Lactobacillus* sp. and *Bifidobacterium* sp. especially produce a positive effect on human health (Schaafsma *et al*., 1998; Gibson and Fuller, 2000).

Feeding prebiotic (fructans) from chicory to broilers may improve weight gain, feed conversion and carcass weight. Feeding chicory fructans may also have systemic effects like a decrease in serum cholesterol levels and deposit of fat tissue (Yusrizal and Chen, 2003). The selective interaction between prebiotics and the intestinal flora results in increased intestinal colonization resistance. This was demonstrated by Kleessen *et al.* (2003) who found lower numbers of total

aerobes, *Enterobacteriaceae*, and *C. perfringens* counts by supplement of fructan-rich from Jerusalem artichoke and increased significantly ($P < 0.01$) *B. bacteriovorus* counts in caecum, as well as reduced levels of endotoxins in the blood compared with control birds. Therefore, Jerusalem artichokes stimulate growth of broiler chickens and protect them against endotoxins and potential caecum pathogens. Mannanoligosaccharids (MOS) is another type of prebiotic that acts by binding and removing pathogens from the intestinal tract and stimulating the immune system (Patterson and Burkholder, 2003). Bacteria attach to the intestinal cells of the host with type 1 fimbriae and this attachment enables the bacteria to cause disease in the host (Figure 1.5). Mannose, the main component of MOS, is a unique sugar which also contains receptors for type 1 fimbriae. MOS functions as a competitive binding site to which the bacteria bind, after which they are carried out of the gut instead of binding to the intestine. *Salmonella typhimurium* colonisation of the intestine was decreased when 2.5% mannose was applied in the drinking water of broilers (Griggs and Jacob, 2005). Type 1 fimbriae are adhesion organelles expressed by many Gram-negative bacteria, and presence this kind of bacteria greatly enhances the bacteria's ability to attach to the host and cause disease (Connell et al., 1996).

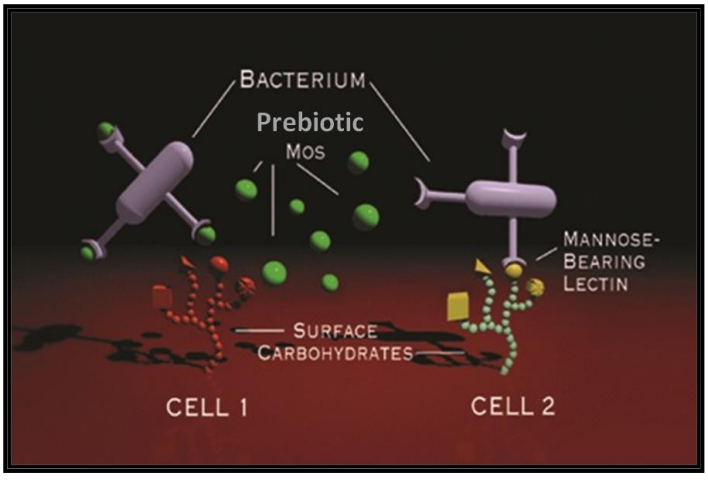


Figure 1.5: Blocking bacterial attachment and thus inhibiting host colonization by MOS as prebiotic (Wysong, 2003).

1.10 Jerusalem artichoke as prebiotic

1.10.1 General characteristics of Jerusalem artichoke

The common name for this plant in the world is Jerusalem artichoke. The scientific classification is as follows (Table 1.4):

Table 1.4: The scientific classification of Jerusalem artichoke.

Rank	Scientific name	Common name
Kingdom	Plantae	Plants
Subkingdom	Tracheobionta	Vascular plants
Superdivision	Spermatophyta	Seed plants
Division	Magnoliophyta	Flowering plants
Class	Magnoliopsida	Dicotyledons
Order	Asterales	
Family	Asteraceae / Compositae	Aster family
Genus	*Helianthus* L.	sunflower
Species	*Helianthus tuberosus* L.	Jerusalem artichoke

USDA (2006)

Figure 1.6: The tubers of Jerusalem artichoke.

Asterace as family (Compositae) that is grown as an annual crop. The tops die in the early winter and the tubers are harvested at which time in the winter. The

plant grows under different climatic conditions and shows a good frost and drought tolerance as well as resistance to pets and diseases (Slimestad *et al*., 2010). Jerusalem artichokes store carbohydrates in the form of inulin instead of starch. Inulin is a fructooligosaccharide, which has a range of healthy characteristics. Inulin can be regarded as a dietary fibre, a straight chain of fructan and it is not digested by enzymes in the digestive system by human. Inulin can be used as a bulking agent in foods when sugar is replaced with an artificial sweetener. The volume previously occupied by sugar is replaced by the low calorie inulin, allowing the total caloric content of the processed product to be greatly reduced. With little reformulation, inulin, though not sweet, functions similar to sugar, such as, browning reactions, aroma synthesis, textural properties, in many foods. Likewise, inulin, whether ingested as Jerusalem artichoke tubers or as a bulking agent, is a dietary fibre and confers a number of health advantages, such as, lowers blood cholesterol level (Kaur and Gupta, 2002), promotes *Bifidobacteria* in the large intestine (Hold *et al*., 2003; Bouhnik *et al.*, 2007).

1.10.2 Biological value of Jerusalem artichoke

Inulin is a naturally occurring storage polysaccharide present in numerous plants such as Jerusalem artichoke (Judprasong *et al.,* 2011) and chicory root (Mavumengwana, 2004). Jerusalem artichoke and Chicory are two plants rich in inulin in their underground parts. Naturally-occurring plant fructans are found as storage carbohydrates in a variety of vegetables including onions, garlic, asparagus and artichokes, in fruits such as bananas, and in cereals (Van Loo *et al*. 1995). It is not digested or absorbed in the small intestine, but is fermented in the colon by beneficial bacteria. Functioning as a prebiotic, inulin has been

associated with enhancing the gastrointestinal tract and the immune system. In addition, it has been shown to increase the absorption of calcium and magnesium, influence the formation of blood glucose, and reduce the levels of cholesterol and serum lipids (Coudray *et al*., 1997; Niness, 1999).

One of the interesting functions of inulin in human and animal nutrition is related to their prebiotic properties, i.e. the specific stimulation of growth and/or activity of a limited number of colonic bacteria beneficial to the host, as well as the growth inhibition of pathogens and harmful microorganisms (Roberfroid, 2007).

Chemically, inulin is a linear poly disperse fructan (degree of polymerization, DP, 2–60 or higher) consisting of fructose molecules (F) linked by β (2-1) glycosidic bonds with a terminal glucose molecule (G) connected to the last fructose with a α(1-2) bond (Figure 1.7). These linkages prevent inulin from being digested like a typical carbohydrate and are responsible for its reduced caloric value and dietary fibre effects. Further, these fructans are not hydrolyzed by the digestive enzymes in the small intestine; they reach the colon unabsorbed and are utilized selectively as a substrate for the growth of beneficial bacteria. Therefore, inulin is a potential candidate for development as a synbiotic along with a suitable probiotic. Several inulin types occur in nature and they differ in the degree of polymerization and molecular weight, depending on the source, the harvest time, and processing conditions (Chiavaro *et al*. 2007, Krivorotava and Jolanta, 2014).

The optimum storage conditions of tubers of Jerusalem artichoke can be stored for (6 - 12) months at (0 - 2°C) and (90 - 95%) rate of humidity. Some cultivars are much more susceptible to storage losses than others (Steinbauer, 1932). Tubers shrivel readily at low rate of humidity and are more likely to damaging.

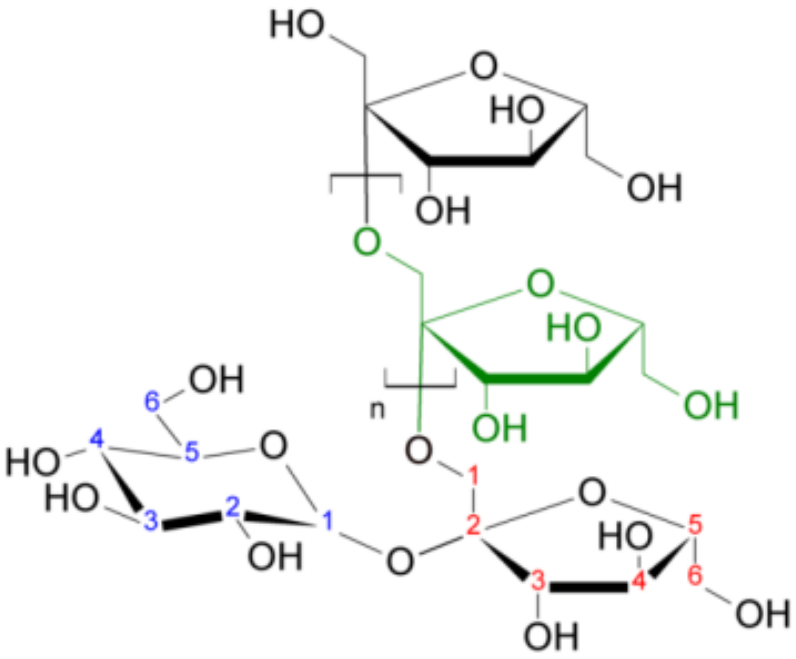


Figure 1.7: Chemical structure of Inulin. (Source: Florianfisch, 2006, From Wikimedia, Commonshttps://commons.wikimedia.org/wiki/File:Inulin_strukturformel.png)

The tubers of Jerusalem artichoke typically include about 80% water, 17% carbohydrate, and 1 to 2% protein (Kays and Stephen, 2008). The principal storage carbohydrate of Jerusalem artichoke is inulin. Jerusalem artichoke tubers have inulin contents of >15% on a fresh weight basis and >75% on a dry weight basis (Kays and Stephen, 2008). Gaafar *et al*. (2010) found that in their study the chemical composition of Jerusalem artichoke, moisture, total carbohydrate, inulin, crude protein, crude fibre and ash were 6.36, 78.03, 72.99, 7.55, 6.51 and 5.72 g / 100 g, respectively. The greater part of carbohydrate that is present in the Jerusalem artichoke present in inulin form.

The findings of determination of inulin in Jerusalem artichoke in the previous study corresponded with other results, but some degree of differences was observed in the levels of inulin. According to Lingyun *et al*. (2007) showed that the

valuable source of inulin can be 14-19% inulin in fresh weight of Jerusalem artichoke tubers, while Judprasong *et al.* (2011) showed that the tubers of JA contain 16-20% inulin in fresh weight.

Gaafar *et al.* (2010) showed that Jerusalem artichoke tubers contain 72.99% inulin in total carbohydrate content of Jerusalem artichoke were 78.03%, so that about 93% of total carbohydrate in inulin form. This results are in agreement with these of Sahar (2003) who reported that chemical composition of Jerusalem artichoke, Moisture, total carbohydrate, inulin, crude protein, crude fiber and ash were 6.50, 86.21, 71.78, 7.40, 7.52 and 5.30 g / 100 g, respectively. Also, these results are slightly agree with those of Fleming and Groot-Wassink (1979) and Rashwan (1996), who reported that, Jerusalem artichoke tubers contained 85.95% carbohydrates that were recovered mainly in the form of inulin. From the previous results, it could be concluded that, Jerusalem artichoke tubers have level of inulin high enough to be utilized commercially.

Meanwhile, Gaafar *et al*. (2010) showed that chemical composition of extracted inulin from Jerusalem artichoke tuber moisture, ether extract, crude protein, ash, inulin and crude fiber after chemical analysis of extracted inulin there were 4.57, 0.35, 0.49, 0.75, 96.87 and 1.54, respectively. The result of Gaafar *et al*. (2010) were in agreement with the findings of Shalaby (2000) who found that, inulin isolated from Jerusalem artichoke tubers was characterized by high value of inulin 96.25%.

The protein in Jerusalem artichoke tubers comprises around 1.6 to 2.4 g/100 g^{-1} of fresh weight, and tubers having a mean total crude protein of 5.9% of tuber dry matter. Crude protein content was found to vary among species, with a mean 5.9% crude protein recorded for the tubers of some species, and some others had 7.40%

crude protein (Gaafar *et al*., 2010). Ash content is around 1.2% of tuber dry weight, although some reports give an ash content as high as 5.30% (Gaafar *et al*., 2010).

1.10.3 The methods of determination of inulin

Several methods have been published for the determination of inulin using spectrophotometry (Ashwell, 1957; McCleary *et al*., 2000; Saengkanuk *et al*., 2011), ion-exchange chromatography (Hoebregs, 1997), an ion exchange chromatography equipped with pulsed amperometric detection HPAEC-PAD (Prosky and Hoebregs 1999; Van Waes *et al*. 1999; Katrin *et al*., 2006; Bach *et al*., 2012), high-performance liquid chromatography (Vendrell-Pascuas *et al*., 2000; Zuleta and Maria, 2011), Thin layer chromatography (Lingyun et al., 2007) and gas chromatography (Joye and Hoebregs, 2000). The general principles of the methods are extracted of inulin with hot water, follows by hydrolysis with inulinase enzyme, and determination of the released fructose and glucose. The difference between the content of each sugar with and without enzyme hydrolysis is the amount of fructan (most exclusively inulin) in the food sample as shown in figure 1.8. However, the gas chromatography shows accurate results for determination of inulin in foods (Joye and Hoebregs, 2000).

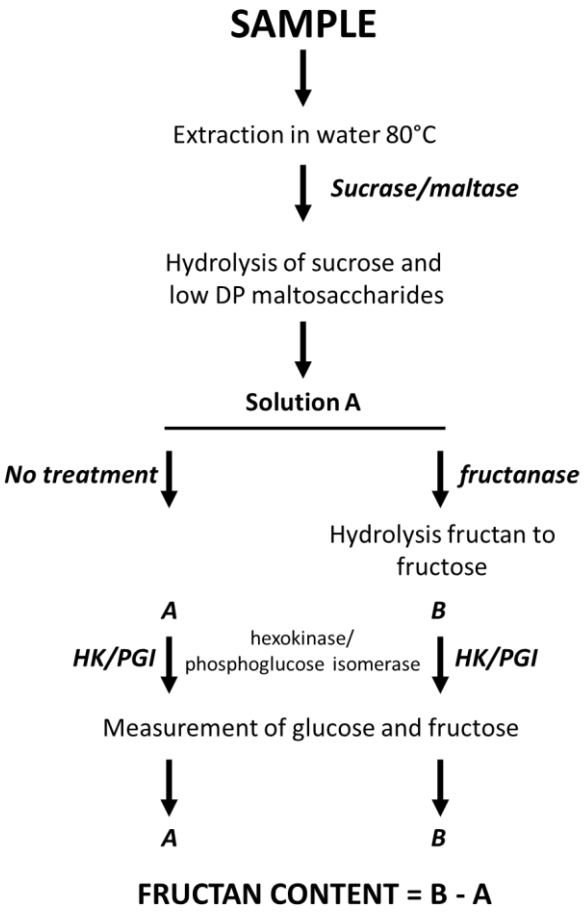


Figure 1.8: The principle of megazyme assay of enzymatic fructan method (Muir *et al*., 2007).

1.11 Selection probiotic properties

There are many criteria for selection of probiotics *in vitro* to be approved before applying *in vivo*. The microorganisms used in probiotic preparations should be (i) generally recognized as safe (GRAS). Non- toxic and non- pathogenic; (ii) exert a beneficial effect on the host; (iii) ability to adhere to the intestinal epithelium cell and colonize the lumen of the GI tract; (v) they are able to demonstrate antagonistic activity against pathogenic bacteria by itself or via bacterial by-products; (vi) They should be tolerant to acid and bile which ensures their viability and capability of being biological activated within the chicken GI tract; (vii) They should be able to adhere to the mucus and intestinal epithelium of the hosts, which ensures the bacterial maintenance in the GIT and thereby prevents their

rapid removal by contraction of the gut. They should be able to keep their viability during processing and storage. Also, they should be able auto-aggregation and co-aggregations with the pathogens (Ezema, 2013; Jin *et al*., 1998; Gaggia *et al*., 2010 ; Kos *et al*. 2003;). The most extensively studied and widely used probiotics are the lactic acid bacteria, particularly the *Lactobacillus* and *Bifidobacterium* strains.The expected health-promoting characteristics and safety criteria of probiotics are shown in (Table 1.5).

Table 1.5: Safety criteria and characteristics of probiotics as a health-promoting.

- Nontoxic and non-pathogenic
- Accurate taxonomic identification
- Normal inhabitant of the targeted species
- Survival, colonization and being metabolically active in the targeted site, which implies:
  - Resistance to gastric juice and bile
  - Persistence in the GIT
  - Adhesion to epithelium or mucus
- Competition with the resident microbiota
- Production of antimicrobial substances
- Antagonism towards pathogenic bacteria
- Modulation of immune responses
- Ability to exert at least one scientifically-supported health-promoting property
- Genetically stability
- Amenability of the strain and stability of the desired characteristics during processing, storage and delivery
- Viability of high populations
- Desirable organoleptic and technological properties when included in industrial processes

(Gaggia *et al*., 2010)

1.11.1 Aggregation ability

Auto-aggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells, and co-aggregation abilities may form a barrier that prevents colonisation by pathogenic microorganisms (Reid *et al*., 1988 and Del Re *et al*., 2000). In most cases, aggregation ability is related to cell adherence properties (Vandevoorde *et al*., 1992; and Del Re *et al*., 2000). The proteinaceous

nature of some surface components has been demonstrated, and surface layer (S-layer) proteins detected in some *Lactobacillus* strains which may contributed in adherence (Schneitz *et al*., 1993; Mukai and Arihara, 1994).

1.11.2 Antagonistic activity

The properties of antagonism between microorganisms are the common condition in the life. The secretion of beneficial microorganisms are the most important for antagonistic activity which carried out as acids production during the process of demolition carbohydrates which leads to increased Lag-phase for bacterial that sensitive to acidic (Baird, 1980). So, all bacteria have a mechanism to protect themselves against attack. For those reasons they produce and secrete some substances that are able to kill or inhibit the growth of related species; or even different strains of the same species of bacteria (Edens, 2003). Most of LAB is able to produce antibacterial materials, which has been shown to be inhibitory to poultry pathogens both Gram positive or negative bacteria (Jin *et al*., 1998). The production of hydrogen peroxide by LAB has a fatal effect on many pathogens (Jin *et al*., 1996).

1.11.3 Resistance to acidity and bile salts

Resistance to acidic pH and bile salts is of great importance in survival and growth of bacteria in the intestinal tract and thus is a prerequisite for choosing suitable probiotics (Kimoto *et al*., 1999).

The important things in the use of Lactic acid bacteria isolated from chickens caecum in the manufacture of probiotic are its ability to resist the low acidity through passing in the digestive tract, especially the proventriculus with low pH 4 when present the food in it and reduces to 2 in the absence of food to reach and the adhesion caecal area as a live cell (Spring, 1997). These things are a standard proposed by many researchers to identify the ability of microorganisms used in the probiotic to resist external conditions, both within the gastrointestinal tract or in the feed when mixed with it to be used in the poultry diets (Chang and Chen, 2000; Savvidou, 2009).

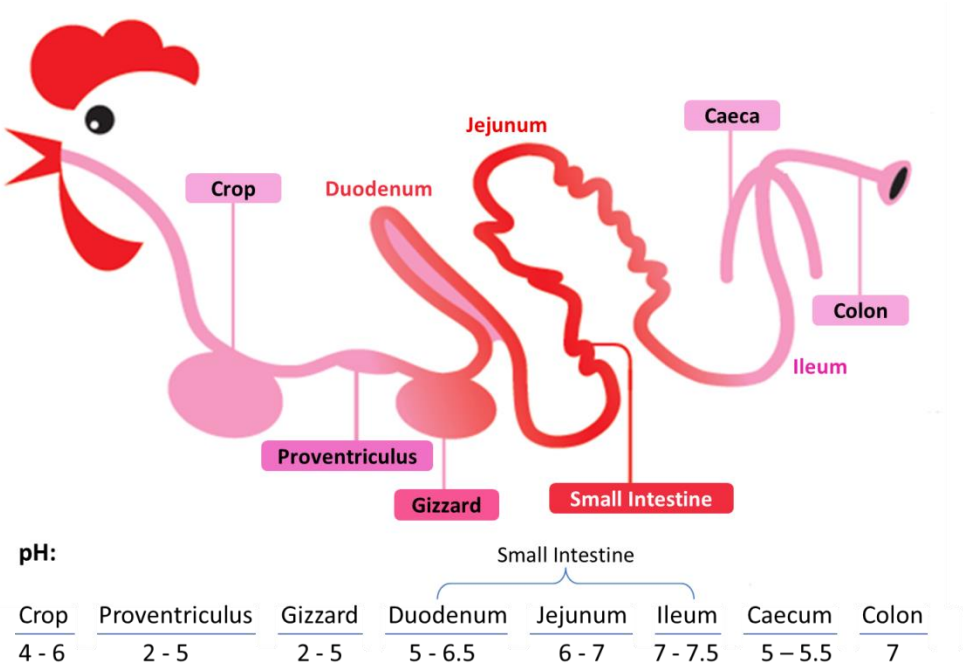


Figure 1.9: Sections of the digestive system of the chicken with the pH values. Adapted from (Westhuizen, 2008).

1.11.4 Adhesion of LAB strains to intestinal mucosa

The intestinal mucosa is densely populated with microorganisms (both commensal and pathogenic bacteria) capable of strong metabolic activities, such as the fermentation of complex carbohydrates contributing to host metabolism

(Macfarlane and Macfarlane, 2006). Enteric infections with pathogenic bacteria play an important role in animal health with the initiation and perpetuation of diseases (Wages and Opengart, 2003).

The intestinal mucus adhesion assay is a classical model to test *in vitro* adhesion ability (Tuomola *et al*. 1999). Several methods have been used to investigate the *in vitro* adhesion of probiotic bacteria. Samples of intestinal mucosa, epithelial cells and mucus can be used for adhesion assay (Tuomola *et al*., 2000; Rojas *et al*. 2002; Li *et al*., 2008).

The non-pathogenic indigenous microorganisms in the gut have received much less attention and the exact mechanisms by which LAB bind to intestinal mucosa has yet to be clarified. Adhesion to the intestinal mucosa, followed by at least transient colonisation is considered necessary for probiotic LAB to exert their favourable effects as it prolongs the contact period with the host, thus allowing more time for the probiotic to exert its beneficial health effects. Adhesion to mucosa is regarded as important for passing colonisation, modulation of the immune system (Salminen *et al*., 1998b) and antagonism against pathogens (Jin *et al.,* 2000).

Li *et al*., (2008) showed that the *Lactobacillus* strains with a higher adhesion ability displayed better competitive exclusion against pathogenic bacteria. *In vitro* model systems provide a very powerful and economic way to screen bacterial competitive exclusion ability. Potentially, adhesion test would provide the probiotic industry with one important step for the selection of candidate probiotic bacteria for the animal feed additives (Ehrmann *et al*., 2002 and Li *et al*., 2008).

The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. Several workers have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells (Green and Klaenhammer, 1994; Pelletier *et al*.,1997; Del Re *et al*., 2000).

1.11.5 Cell surface hydrophobicity

In order to gain information on the structural properties of the cell surface of LAB, that is a relationship between auto-aggregation and adhesiveness of *Lactobacillus* strains on the cell surface. Physicochemical characteristics of the cell surface such as hydrophobicity may affect auto-aggregation and adhesion of bacteria to different surfaces (Del Re *et al*., 2000). Also, it is difficult to study bacterial adhesion *in vivo*, most experiments use *in vitro* models. Microbial adhesion to solvents (MATS) is one technique that has been used to investigate bacterial cell affinities for polar and non-polar solvents (Wadstrom *et al*., 1987). Non-polar solvents have been used to estimate their hydrophobic properties, while polar solvents have been used to help estimate Lewis acid/base properties (Gusils *et al*., 1999a). The low affinities of lactobacilli for non-polar solvents suggest that these bacteria possess a hydrophilic rather than a hydrophobic cellular surface (Huang and Adams, 2003). Kos *et al*. (2003) demonstrated that cell surface hydrophobicity was related to adhesion ability to intestinal cells. So, high values of hydrophobicity could indicate a greater ability of the bacteria to

adhere to epithelial cells (Rosenberg *et al*., 1980; Gusils *et al*., 1999b, Bomba *et al*., 2002). Many previous studies on the physic-chemistry of microbial cell surfaces have shown that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity (Slifkin and Doyle, 1999). For the assessment of the degree of surface hydrophobicity, the microbial adhesion to hydrocarbons method (MATH) described by Rosenberg *et al*. (1980), it was used with three different hydrophobic solvents: hexadecane, xylene and toluene.

1.12 Effects of probiotics, prebiotics and synbiotics on broiler chickens

1.12.1 Performance parameters

There are several reviews discussing the effect of probiotics, prebiotics and synbiotics on poultry performance. A growing body of scientific research supports the role of probiotics, prebiotics and synbiotics as effective alternatives to the use of AGP in animal nutrition (Patterson and Burkholder, 2003; Pelicano *et al*., 2004). The general level recommended for commercial probiotics in feed additives is around 10^8 CFU/g feed continuously not a single dose (Olnood, *et al*., 2007). LAB may enhance digestion by increasing enterocyte production (Banasaz *et al*., 2002). The gut microflora affects the digestion, absorption and the metabolism of dietary carbohydrates, protein, lipids and minerals and the synthesis of vitamins (Jin *et al*., 1997). Most of the volatile fatty acids formed by intestinal bacteria are absorbed and metabolized by the host, contributing to host energy requirements. Maintaining the balance of good gut health is a key aspect of ensuring the best bird performance and health. If an imbalance in gut microbiota occurs, nutrient digestion and absorption may be affected which, in turn, may affect bird health

and performance. The balance of the microbiota in the gut also can be significantly affected by bird management and environment.

Kalavathy *et al*. (2008) showed that the average live body weight of Hubbard broiler at 42 days of age that fed probiotic (*Lactobacillus* stain) at level (1g/kg feed) significantly ($P<0.05$) heavier than control were 1976.58 and 1700.33 g, respectively. Mountzouris *et al.* (2010) observed that diets containing 10^8 cfu probiotic/kg increased body weight of broilers significantly in compare of control group. Dizaji *et al*. (2013) showed that the dietary supplementation of Ross 308 broiler with prebiotic (1kg of ActiveMOS /ton), probiotic (150,100,50gm of Protexin /ton of the starter, grower and final diets respectively), synbiotic (1kg of Amax4x /ton) had a significant ($P<0.05$) increase on live body weight for prebiotic and synbiotic compared with the control group at 42 days of trial, and the higher performance was recorded for synbiotic group. Also, Feed Conversion Ratio decreased in synbiotic group compared with the control group at the end of experiment. Similarly, Mookiah *et al*. (2014) showed that use of prebiotic IMO (Wako, Osaka, Japan), probiotic 11 *Lactobacillus* strains (*Lb. reuteri* C 1, C 10 and C 16; *Lb. gallinarum* I 16 and I 26; *Lb. brevis* I 12, I 23, I 25, I 218 and I 211, and *Lb. salivarius* I 24) and combination of both (synbiotic) in poultry feed significantly ($P<0.05$) improved weight gain of broiler chickens at 22-42 and 1-42 days of age, and feed conversion ratio from 1 to 21, 22-42 and 1-42 days of age compared with control group. Addition of probiotic and prebiotic to the poultry diets have shown beneficial effects on growth *performance* of poultry as listed in Table 1.6.

Table 1.6: General effects of probiotics, prebiotics and synbiotics in poultry production.

Type of supplements used	Administration	General effect of performance	Reference
Bio-MOS	Feed 2g/kg	Improved the growth performance of birds compared to the control group	Hooge, 2004
MOS[1]	500g/ton	Improved daily weight gain, feed intake and feed conversion ratio	Flemming *et al*., 2004
Probiotic and Prebiotic (MOS)	1kg/ton from 1-42 days separately	Improved feed conversion ratio	Pelicano *et al*., 2004
Inulin	20g/kg diet, birds were fed during 35 days and orally challenged with *Salmonella* and *Campylobacter*	The performance of young broiler chickens increased	Verdonk and VanLeeuwen, 2004
Fermecto® (*Aspergillus mycelium*)	Feed	Weight of breast and thigh to body weight significantly increased	Piray *et al*., 2007
Lactobacillus - All-Lac XCL 5x™ (Challenged with *Salmonella enteritdis*)	Spray-mixing 5g/400ml/2000 chicks in distilled water	No significant effect on body weight, Weight gain, Feed intake, Feed conversion ratio and Livability	Riberio *et al*., 2007
LAB (FM-B11)	Drinking water 10^9cfu LAB/ml	No significant effect on body weight	Rodriguez *et al*., 2007
Bactocell	Feed 1.5kg/ton/ 42 days	Increased body weight significantly	Rowghani *et al*., 2007
LAB (FM-B11) + Lactose	Probiotic in drinking water and lactose in feed	Increase body weight significantly	Rodriguez *et al*., 2007
Synbiotic (BIOMIN/IMBO)[2]	Feed(1 kg /ton of the starter diets and 0.5 kg/ton of the grower diets)	increased the growth performance, improved intestinal morphology and nutrient absorption	Awad *et al*., 2008
Prebiotic (FOS)	Feed	Improved broiler's weight gain about 5-8% and Feed conversion ratio about 2-6%	Yang *et al*., 2009

[1]Mannanoligosaccharids.
[2] a combination of *Enterococcus faecium* and prebiotic derived from chicory.

1.12.2 Intestinal microflora

Probiotics have been demonstrated to improve microbial balance in the gastrointestinal tract through mode of action includes bacterial antagonisms, competitive exclusion and immune stimulation (Rolfe, 1991; Brisbin *et al*., 2008). Prebiotics which include non-digestible oligosaccharides may control or manipulate microbial composition and/or activity, there by assisting to maintain a beneficial microflora that suppresses through different regulatory mechanisms the growth of pathogens (Gibson *et al*., 2004). The combination of probiotics and prebiotics, also referred to as synbiotics, may improve the survival rate of probiotics during their passage through the digestive tract, thus contributing to the stabilisation and/or enhancement of the probiotic effects.

There are many studies that evidence probiotics and prebiotics inhibit some harmful bacteria via occupying cell wall spaces inside the intestinal mucosa. Mountzouris *et al*. (2007) and Higgins *et al*. (2007) demonstrated that some species of bacteria as a probiotic have a potential impact on pathogen inhibition and modulation on intestinal microflora, especially *Lactobacillus, streptococcus*. Recently, Mirza (2009) demonstrated that broilers fed with synbiotics had an ability to improve intestinal colonization via decrease *E. coli* and total aerobic bacteria count in the ileum than in the control group. Prebiotics in the intestinal tract causes the removal of pathogenic bacteria that might attach to the surface of the epithelium cells inside of the intestine (Newman, 1994). Oyofo *et al*. (1989) showed that dietary prebiotic was successful inhibition the intestine colonization of *S. typhimurium*. Studies on the effects of inulin prebiotic found that foods containing Jerusalem artichoke inulin at the level of 5 g/d significantly increased *Bifidobacterium* spp. (Ramnani *et al.,* 2010).

1.12.3 Histology of intestine

One of the roles of probiotics, prebiotics and synbiotics is the ability to change the morphology of the digestive tract, this, increases in villi length and crypt depth (Pelicano *et al*., 2005; Mirza, 2009). Samanya and Yamauchi (2002) demonstrated that birds treated dietary *Bacillus subtilis* var. natto for 28 days had an ability to display the higher villus extension than the control group.

An increase in villi length refers to high digestion and absorption efficiency with the presence of good microbial balance and healthy body. Enterocytes which present in the wall of the villi contain high numbers of microvilli to form brush boarder like shape. Awad *et al*. (2008) resulted that the addition of synbiotic BIOMIN IMBO (Combination between *Enterococcus faecium* and inulin prebiotic derived from chicory) with diet increased the villus height/crypt depth ratio and villus height in ileum. However, the ilium crypt depth was decreased by dietary supplementation of synbiotic compared with control in broiler chickens. The intestinal mucosal architecture can reveal useful information on the intestinal function. Increasing the villus height suggests an increased surface area capable of greater absorption of available nutrients (Caspary, 1992).

Also, Awad *et al*. (2009) showed that when the diet of broiler chicks was supplemented with the synbiotic (Biomin IMBO), there was a significant increase in ileum villus height compared to the control. Similarly, Xu *et al*. (2003) also reported that broilers fed Fructooligosaccharide 4g/kg diet had higher villi in the jejunum and ileum than control group. Rehman *et al*. (2007a) demonstrated that supplementation of dietary inulin increased the jejunal villus length and crypt depth in broilers, at 35 days old. Hassanpour *et al.* (2013) indicated that 0.1% synbiotic (Biomin IMBO) significantly increased villus height. Mirza (2009) also

reported that when the synbiotic was added to the diet of Cobb 500 broiler chicks, there was a significant increase in ileum villus height at 42 days compared to the control being 650.33 and 450.06 µm, respectively. Similarly, Zhang *et al*. (2005) showed that when 0.5% of *Saccharomyces cerevisiae* yeast was added to the diet of male broiler chicks, there was a significant increase in the villus height in the ileum at 21 days compared to the control being 430.67 and 396.87 µm, respectively. Also, Pelicano *et al*. (2007) reported that there was a significant increase in intestinal villus height of broiler chicks at 42 days when synbiotic was used compared to the control. Also Samli *et al*. (2007) reported that adding of probiotic containing *Enterococcus faecium* microorganism to broiler diets increased the ileal villus height. Similarly, Samanya and Yamauchi (2002) reported that villus height in duodenum and ileum increased significantly in 28-days old chicks fed *Bacillus subtilis*. Santin *et al*. (2001) showed that the broilers fed *Saccharomyces cerevisiae* had higher villus height than that of the control group during the first 7^{th} day. While, Rebole *et al*., (2010) showed that the effect of Inulin supplementation (10 and 20 gm/kg diet) on the male Cobb broiler, there was no significant differences on villus height, crypt depth and microvillus length and density in the jejunum at 35 days old.

1.12.4 Haematological parameters and cholesterol content

Haematological and biochemical parameters of animal are determined as an index of their health status. The colour of the blood in the birds is always red as a result of the presence of haemoglobin (Hb) in the erythrocytes (AL-Darajy *et al*. 2008). The normal range of Hb is about 7-13 g/dl (Jain, 1993). AL-Kassie *et al.*

(2008) reported that when the probiotic (*Aspergillus niger*) and the prebiotic (*Taraxacum officiale*) were added to the Arbor Acres broiler diet at a rate of 10 g/ kg there was a significant increase in Hb concentration at 42 days of age compared with the control group being 8.92, 8.85 and 7.20 g/100ml, respectively. Sarinee *et al*. (2008) also, showed that when the probiotic was added to the drinking water of male Cobb broiler chicks, there were a significant increase in the Hb concentration at 28 days compared with the control being 14.85 and 12.85 g/dl, respectively, but they found no significant effect in Hb concentration at 42 days compared with the control being 14.70 and 15.58 g/dl, respectively.

Haematocrit is used as an indicator of animal health and is the percentage of packed blood cells to plasma volume (Rao & Deshpande, 2005). The normal range of PCV (Hct%) is about 22-35% (Jian, 1993). AL-Kassie *et al*. (2008) found that when the probiotic (*Aspergillus niger*) and the prebiotic (*Taraxacum officiale*) were added to the Arbor Acres broiler diet at a rate of 10 g/ kg, only prebiotic significantly increased PCV% at 42 days compared with the control being 33.70, 34.53 and 33.55%, respectively. Also, Sarinee *et al*. (2008) reported that when the probiotic was added to the drinking water of male Cobb broiler chicks 28 and 42 days, there was no significant effect in the PCV % compared to the control being 25 and 25.75 % and 27.88 and 28.63 %, respectively.

Physiological and pathological stress in avian species affected neuro-endocrine system (glucocorticoids, catecholamins, epinephrine, norepinephrine, prolactin and growth hormones) and reduced the lymphocyte production (Marketon and Glaser, 2008). When birds are stressed, glucocorticoid hormones are secreted and the physiological stress is response (Dhabhar *et al*., 1996). Stress could cause an increase in the stimulation of the adrenal gland to produce hormones

which has a direct effect to analyses a lymphatic cell which causes an increase in H/L ratio (Gross and Siegel, 1983). Thus H/L ratio could be used as an indicator for the health of animals and any increase of H/L ratio refers to an increase in stress case (James and Stanley, 1989). Paryad and Mahmoudi, (2008) reported that when different levels 0, 0.5,1.5 and 2 % of *Saccharomyces cervisiae* were added to the diet of broiler chicks, there was a significant decrease in H/L ratio at 42 days being 0.820, 0.753, 0.708 and 0.691, respectively. While, Sarinee *et al*. (2008) assumed that when the probiotic was added to the drinking water of male Cobb broiler chicks, there was no significant effect in the H/L ratio at 28 and 42 days compared to the control being 0.45 and 0.37 and 1.01 and 0.95, respectively.

Cholesterol is a critical fatty substance necessary for the proper function of every cell in the body. Cholesterol is a structural component of cell membrane and plasma lipoproteins and is important in the synthesis of steroid hormones and bile acids. Mostly synthesized in the liver, some of it is absorbed through the diet, especially one high in saturated fats (Jaeger and Hedegaard, 2004). Panda *et al*. (2001) reported that supplementation of probiotics (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Aspergillus oryzae*) at a rate of 100 mg per kg in the diet of broiler chickens significantly reduced the serum cholesterol concentration. Mansoub (2010) found that when the diet of male Ross 308 broiler chicks was supplemented with 1% *Lactobacillus casei*, there was a significant decrease in serum cholesterol at 42 days compared with the control group being 151.23 and 199.76 mg/dl, respectively. Also, Ashayerizadeh *et al.* (2011) reported that probiotic, prebiotic and synbiotic supplementation to the Ross 308 broiler diet at 42 days, only synbiotic highly significantly decreased serum cholesterol compared with the control being 3.71, 3.77, 3.58 and 4.15 mmol/L, respectively. While,

Capcarova *et al*. (2010) reported that broilers was administrated with two type of the probiotics with concentration of 1×10^9 cfu of *Lb. fermentum* CCM 7158 and 2×10^9 cfu of *E. faecium* M 74 in 1 g of nutrient medium in drinking water for 42 days, there was no significant effect in serum cholesterol compared with the control being 4.813, 4.862 and 4.428 mmol/L, respectively. Yalcinkaya *et al*. (2008) reported that the use of MOS from *Saccharomyces cerevisiae* in broilers diet could not significantly reduce the serum cholesterol levels as compared with the control group.

1.12.5 Meat quality

In recent years, the high growth rate, and improvements in meat quality and properties of carcasses have been beneficial to the poultry industry, especially in broiler production. Currently, an important research area is the use of probiotics, prebiotics and synbiotics as feed additives as an alternative to antibiotics. There are many reports concerning the effect of using probiotics, prebiotics and synbiotics on feed performance (Abdel-Raheem *et al*., 2012; Banday and Risam, 2001; Gunal *et al*., 2006; Kumprechtová *et al.* 2000; Satbir and Sharma, 1999), but carcass and meat quality of broilers have not been studied. Broiler chickens have a rapid growth rate and have been genetically selected for high live body weight. Generally, probiotics are used to correct abnormalities caused by stress factors in the gastrointestinal tract, and thus normalize the chickens, which results in increased feed efficiency. The normalize by a microbial dietary associate that beneficially affects the host physiology by modulating mucosal and systemic

immunity, as well as improving nutritional and microbial balance in the intestinal tract (Fuller, 1989; Naidu *et al*., 1999).

Zhou *et al*. (2010) showed that the effect of *Bacillus coagulans* ZjU0616 with different concentrations (0 (Control), 1.0×10^6 cfu g^{-1} (T1), 2.0×10^6 cfu g^{-1} (T2) and 5.0×10^6 cfu g^{-1} (T3)) supplemented as probiotic to the diet on the breast chemical composition, and meat quality of Guangxi Yellow chicken, there was no significant differences for the breast chemical composition (Moisture%, Crude protein%, Crude fat% and Crude ash%) among the treatments and control group. while, Share force significantly decreased in T2 and T3 compared with control group.

Mahajan *et al.* (2000) reported that the supplementation of probiotic (Lacto-Sacc) composed of *Lactobacillus acidophilus* and *Streptococcus faecium* to broiler diets resulted meat from probiotic fed birds significantly higher ($P<0.001$) percentage of moisture, protein, ash, WHC, and lower fat percent, moisture, protein ratio, pH, shear press value as compared with the meat obtained from control birds at the end of the 6-week feeding trial. Endo and Nakano (1999) found that the use of probiotics (*Bacillus, Lactobacillus, Streptococcus, Clostridium, Saccharomyces* and *Candida* spp.) improved the characteristics of carcass and meat quality in male broilers, because using of probiotics in broiler diet affects on intestinal flora of broilers, improving the raising environment and decreasing the stress.

Colour is an important quality attribute that influences consumer acceptance of many food products, including poultry meat. Consumers will often reject products in which the colour varies from the expected normal appearance. Consequently, colour is often used to determine economic value of food (Qiao *et al*., 2001).

Broiler quality improvement may be depending on the selected feed ingredient. Appearance is the major criterion for purchase, selection and initial evaluation of meat quality (Fletcher, 2002). Other quality attributes, such as tenderness, colour, cooking loss and shelf-life are important to the consumer after purchasing the product (Jeremiah, 1982, Husak *et al*., 2008). The variations in colour of broiler breast meat fillets were significant correlated with muscle pH and extremes in colour variations. Breast meat may appear dark due to high muscle pH (Karaoglu *et al*., 2004).

1.13 The aims of this study:

The overall aim of this study programme was to investigate the effect of these additives supplementation of broiler chickens and the potential benefits of dietary feed additives {i.e. probiotic (*Lactobacillus animalis*), a prebiotic Jerusalem artichoke tuber (*Helianthus tuberosus*) and a combination of both (Synbiotic)} on the gut microflora, health and production performance of broilers. The specific objectives of this study were addressed by the following:

- To isolate and characterise of LAB strains from chicken caeca as well as to investigate their probiotic properties *in vitro*.
- To find a suitable candidate of LAB strain for an inulin synbiotic in broiler diet.
- To investigate the effects of probiotics, prebiotics and synbiotics on histological parameters.
- To investigate the effects of probiotics, prebiotics and synbiotics on intestinal microflora by culture based technique and molecular technique.

- To investigate the effects of probiotics, prebiotics and synbiotics on immune system.
- To investigate the effects of probiotics, prebiotics and synbiotics on blood characteristics (Haematocrit, Haemoglobin and H/L ratio).
- To investigate the effects of probiotics, prebiotics and synbiotics on cholesterol determination.
- To investigate the effects of probiotics, prebiotics and synbiotics on meat quality via the study of chemical analyses, colours and structure of meat.

CHAPTER TWO

Preparation of prebiotics, probiotics and its application *in vitro*

2.1 Introduction

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Fuller, 1989). Prebiotics are a more recent concept and are defined as chemical substances that act as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health" (Gibson and Roberfroid, 1995). This definition was revised in 2004 and prebiotics are now defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Gibson *et al*., 2004). A combination of probiotics and prebiotics, termed synbiotics has been used to improve various aspects of host health (Bengmark, 2001).

The development and use of probiotics for poultry is based on the knowledge that the microflora in the gut participates in the resistance to enteric infections and suppresses the growth of pathogenic bacteria. It has been shown to have a protective effect against a variety of pathogen bacteria including *Escherichia coli*, *Salmonella*, *Campylobacter*, *Clostridium* (Jin *et al*., 1997; Kalavathy *et al*., 2003, 2005, 2009; Murry *et al*., 2006).

The aim of this study was to investigate the potential use of inulin and a probiotic to produce a synbiotic for use in poultry diets. A selection of tests for probiotic efficacy was used to screen lactic acid bacteria (LAB) as suitable probiotics. The

tests included *in vitro* fermentation of bacteria strains with inulin, aggregation ability (auto-aggregation and co-aggregation), antagonistic activity against *S.* Enteritidis*, E. coli* and *Cl. Perfringens*, mucus binding test, and adhesion to the gut epithelial cell, tolerance to bile salts and acidic conditions and cell surface hydrophobicity.

2.2 Material and Methods and Results

2.2.1 Preparation of Jerusalem artichoke tubers and extraction of inulin

Jerusalem artichoke tubers (*Helianthus tuberosus* L.) were obtained from the local market in Erbil, Kurdistan-Iraq. The tubers of Jerusalem artichoke (JA) were kept in plastic bags and transferred to the laboratory. The tubers were cleaned with tap water to remove dust and other undesirable materials. The cleaned tubers were cut into small pieces and material was dried at 50 °C for 48 h and then ground to a powder (FOSS, Knifetec^{TM} 1095, Sweden) and sealed in polyethylene bags. The powdered of JA was stored at room temperature, in a dry container to avoid moisture absorption, for further use as recommended by (Modeler *et al*., 1993; Lingyun *et al*., 2007).

Figure 2.1: The process of prebiotic production from Jerusalem artichoke (*Helianthus tuberosus* L.)

2.2.2 Determination of inulin content from Jerusalem artichoke

Inulin content of the Jerusalem artichoke sample was determined in triplicate using a protocol of the Fructan HK (K-FRUCHK 04/13) assay Kit (Megazyme International, Bray, Ireland). The sample was ground and extracted by hot water. One gm of sample was weighted into a beaker and 400 mL of hot distilled water (~ 80°C) was added, and then the beaker was placed on a hot-plate, magnetic-stirrer and stirred and heated (at ~ 80°C) for 15 min (i.e. until the sample is completely dispersed). The solution was allowed to cool to room temperature and then quantitatively transfer it to a 500 mL volumetric flask and adjusted the volume to the mark with distilled water, and the contents were mixed thoroughly. An aliquot of the solution was filtered through a Whatman No. 1 (9 cm) filter circle and the samples was analysed immediately. Accurately dispense 0.2 mL aliquots of solutions were analysed (containing approximately 0.1 to 2.0 mg/mL of fructan) 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 to the tube with vigorous stirring on a vortex mixer and this is called Solution A. Accurately and carefully dispense 0.2 mL aliquots of Solution A (in duplicate) was 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 (100 mM sodium acetate buffer, pH 4.5) 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 (in the cuvettes containing

the fructanase enzyme). The absorbance was read at 340 nm by spectrophotometer (Camlab, JENWAY, 7315 Spectrophotometer, Bibby Scientefic Ltd, UK) at 25°C. The amount of inulin was expressed in terms of fructan concentration (Simonovska, 2000; Muir *et al*., 2007; Saengkanuk *et al*., 2011). The procedure was shown in the table 2.1 to measure the fructan in the Jerusalem artichoke tubers.

Table 2.1: Procedure of fructan measurement in the Jerusalem artichoke tubers.

Pipette into cuvettes	**Sugars**	**Fructan + sugars**
sample	0.20 mL	0.20 mL
solution 4 (fructanase enzymes)	-	0.10 mL
buffer 2 (sodium acetate buffer)	0.10 mL	-
Ensure that all of the solutions are delivered to the bottom of the cuvette. Mix the contents by gentle swirling, cap the cuvettes and incubate them for 30 min at 40°C in a heated oven.		
Add:		
Distilled water (at ~ 25°C)	2.00 mL	2.00 mL
Solution 1 (buffer, pH 7.6)	0.20 mL	0.20 mL
Solution 2 (NADP+/ATP)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (**A1**) after approx. 3 min and start the reactions by addition of:		
Suspension 5 (HK/PGI/G-6-PDH)	0.02 mL	0.02 mL
Wait for the end of the reaction (approximate 10-12 min), and the absorbance was read of the solutions again (**A2**).		

The amount of fructan present in the sample was calculated according to the equations that mentioned in the appendix 1.

After applying the procedure of determination of inulin content from the Jerusalem artichoke tubers by spectrophotometry, the absorbance of unknown sample was plotted in final equation to measure the inulin as g/100 g of dry matter. The content of inulin in Jerusalem artichoke tubers was 74.48 g/100 g in dry matter bases. This result was in good agreement with other researchers by using different method to estimate the inulin in Jerusalem artichoke (Sahar, 2003; Gaafar *et al*., 2010; Saengkanuk *et al*., 2011).

2.2.3 Selection of bacteria strain as probiotics

The steps used in the bacterial identification and chosen as a probiotic that were followed are presented schematically in (figure 2.2)

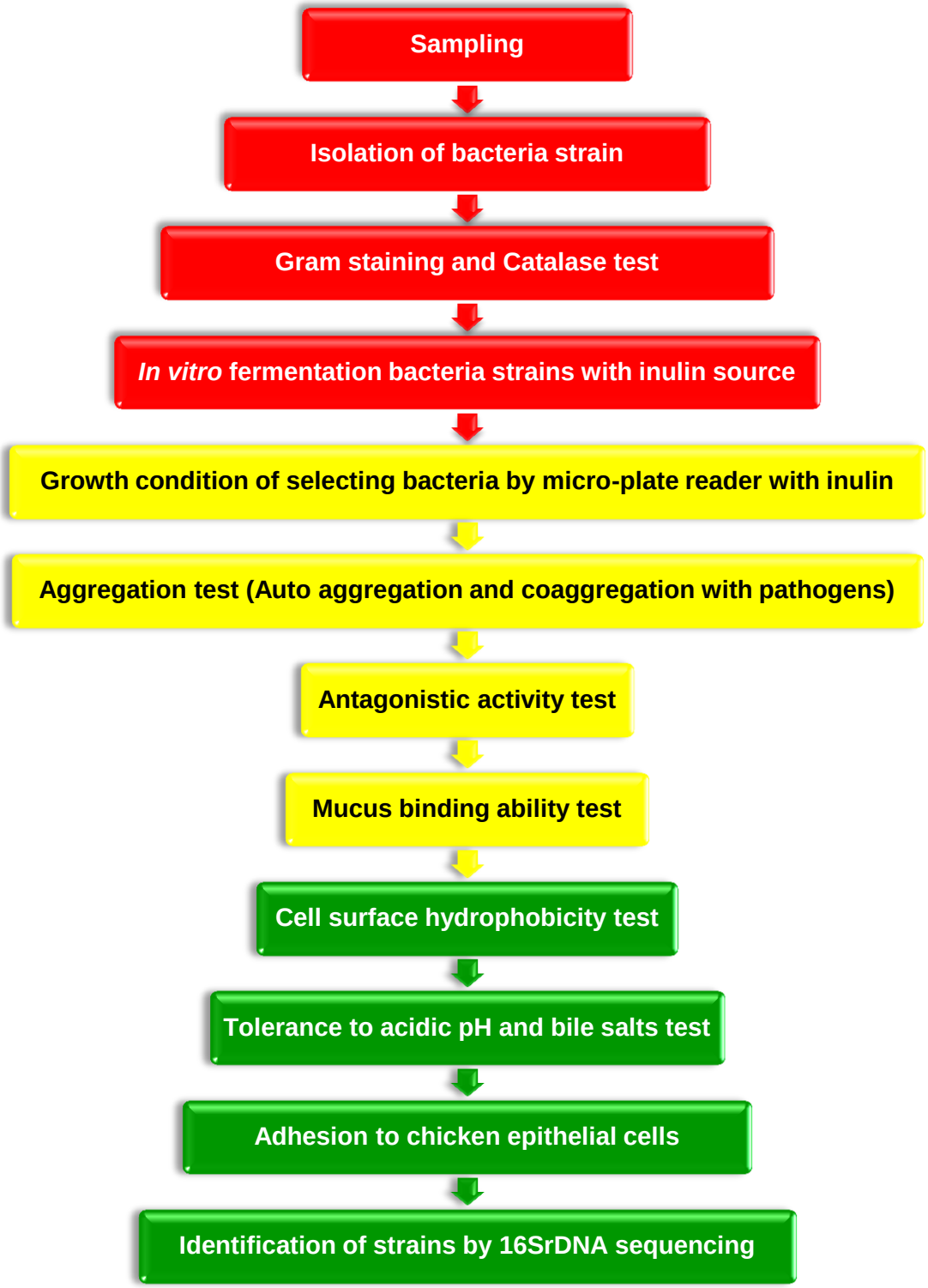


Figure 2.2: Layout of the screening and selection process.

2.2.4 Isolation of microorganisms

Eight strains of LAB (C1-C8) were isolated from chicken caeca. One four-week-old Leghorn chicken was killed by cervical dislocation. The caeca were removed from the carcass under sterile conditions, put on ice and transported to the laboratory. One gram of caecal content was added to 9 ml of PBS buffer solution (0.1 M, pH 7.0) and homogenized for 3 min in a stomacher (Bag mixer 100 MiniMix, Interscience 788860, Arpents, France). The homogenate was serially diluted to yield dilutions of 10^{-5}, 10^{-6} and 10^{-7}, and cultured onto MRS agar medium. The plates were incubated anaerobically at 37°C for 48 h. After incubation, eight isolates were randomly sampled and sub-cultured separately in MRS broth at 37°C under anaerobic conditions. Stock cultures were also prepared in 30% (vol/vol) glycerol and frozen at -80°C until further use. Stock cultures were reactivated by subculture in MRS broth and incubation at 37° C for 24 h, before their experimental use. The resulting colonies were first characterized morphologically and by gram staining and the detection of catalase activity. Gram-positive isolates devoid of catalase activity were considered as LAB, and then used in further studies.

Three other bacteria strains were used in this study, including a commercial probiotic *Pediococcus acidilactici* CNCM MA 18/5 M (Bactocell®), *Lactobacillus plantarum* and *Pediococcus pentosaceus* from microbiology lab, University of Plymouth. The cultures were maintained by routine sub-culturing in de Man-Rogosa-Sharpe (MRS) broth (Sigma-Aldrich, Fluka, Switzerland) using 1% (v/v) inocula from an overnight culture, and incubated under anaerobic conditions in an anaerobic jar (Oxoid Ltd) at 37° C with the gas generating kit (Fisher Scientific, Code, 10269582, UK)

Eight of the isolated lactic acid bacteria were found to be Gram positive, six of them rod shaped and the remaining were cocci. All the organisms showed negative results in the Catalase test and all the organisms were kept in MRS broth (Sigma-Aldrich, Fluka, Switzerland).

2.2.5 Screening of LAB strains

The following tests were done in sequence with each acting as a selection criteria for subsequent tests.

2.2.5.1 Growth on inulin at 37°C

The inulin was screened for its effects on the growth of the 11 Lactic acid bacteria strains. Basal MRS medium was used without glucose supplemented as shown in table 2.2, with 2% of the commercial inulin (Frutafit® HD, Netherlands) and inulin extracted from JA as the source of carbohydrate and glucose was used as a control treatment. Both types of inulin were sterilized by irradiation with 25 kGy from Co^{60} (Becton and Dickinson, Plymouth, UK) and the JA inulin compound extraction was performed by using the sterile hot water; sample to solvent ratio was 1:5 (w/v), at 80 ºC for 90 minutes according the method of Gaafar *et al*. (2010) and the obtained extract was added. MRS media were inoculated with 1% (v/v) inocula of an overnight culture of LAB strains. The inoculated media were incubated under anaerobic conditions using anaerobic jars with gas generating kits at 37°C. After 24 h of incubation, the cultures were vortexed for 30 s to disperse the bacterial cells, and the growth of each strain was determined by

measuring the optical density (OD) at 595 nm using a spectrophotometer (UNICAM, Thermo, USA). Three replications were made for the experiment.

Table 2.2: The preparation of MRS broth by diluting these amounts of compounds in 1 litre distillate water.

Ingredient	Amount (g)
Beef extract	10
Peptone	10
K_2HPO_4	2
Tri ammonium citrate	2
Glucose or Inulin	20
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Yeast extract	5
Sodium acetate hydrate	5
Tween-80	1 ml
pH at 25° C	6.5

2.2.5.2 Growth on inulin at 42°C

The 11 Lactic acid bacteria were screened for their ability to grow on inulin. A basal MRS medium was used without glucose supplemented with 2% of the commercial inulin (Frutafit® HD, Netherlands) and inulin extracted from JA as the source of carbohydrate and glucose was used as a control treatment. MRS media were inoculated with 1% (v/v) inocula of 10^5 CFU an overnight culture of each LAB strain. The inoculated media were transferred 48-well micro-plate and incubated in micro-plate reader (TECAN, Germany) at 41-42°C, which is the body temperature of chicken (Chang and Chen, 2000). The growth of each strain was determined every 3 h for 24 h by measuring the optical density (OD) at 595 nm. Three replications were made for the experiment.

The growth density (OD_{595}) of the eight LAB strains isolated from chicken caecum and three LAB strains in basal MRS media containing Frutafit and inulin extracted from Jerusalem artichoke were compared with glucose as a control (Table 2.3 and 2.4). Four of the isolated LAB (C2, C4, C6, and C7) and *Lb. plantarum* and *P. acidilactici* in anaerobic and aerobic conditions demonstrated that the best growth on Frutafit inulin and inulin extracted from JA. However, growth of most strains was significantly better in glucose ($P<0.05$) compared with Frutafit and Jerusalem artichoke extract.

The growth curves of the eleven LAB strains in MRS basal media supplemented with glucose, Frutafit inulin and inulin extracted from JA are shown in Figures 2.3. Generally, C4 strain which was isolated from a chicken caecum had a shorter lag-phase and much better growth than other strains in inulin extracted from JA.

Table 2.3: Growth (OD_{620} ± SD) of LAB strains on Frutafit inulin, Jerusalem artichoke inulin and glucose after 24h of incubation anaerobically at 37°C.

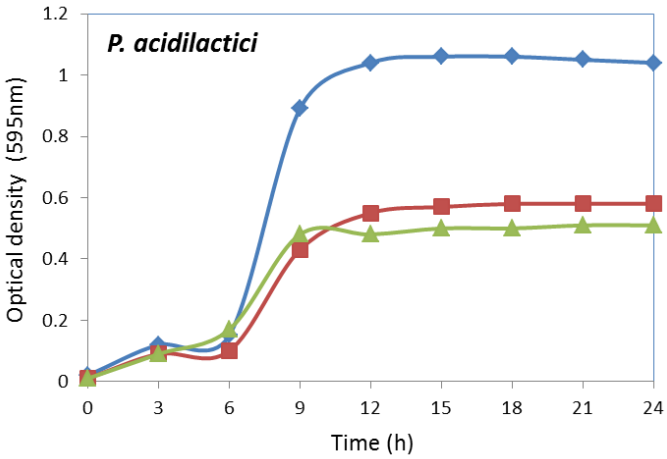
strains	Growth density[1]			p. value
	Glucose	Frutafit Inulin	JA Inulin	
Lactobacillus plantarum	0.81±0.05 ^{dA}	0.53±0.02 ^{fB}	0.34±0.05 ^{dC}	<0.001
Pediococcus acidilactici	0.73±0.04 ^{dA}	0.69± 0.02 ^{deA}	0.57±0.04 ^{cB}	0.006
Pediococcus pentosaceus	0.69±0.09 ^{dA}	0.58±0.07 ^{efA}	0.49±0.01 ^{cA}	0.116
C1	1.41±0.03 ^{abA}	0.68±0.05 ^{deB}	0.25±0.02 ^{deC}	<0.001
C2	1.20±0.19 ^{bcA}	0.96±0.06 ^{bAB}	0.83±0.06 ^{aB}	0.031
C3	1.29±0.12 ^{abcA}	0.55±0.04 ^{efB}	0.19±0.00 ^{eC}	<0.001
C4	1.53±0.03 ^{aA}	1.43±0.01 ^{aB}	0.92±0.02 ^{aC}	<0.001
C5	1.45±0.06 ^{abA}	0.63±0.07 ^{efB}	0.26±0.01 ^{deC}	<0.001
C6	1.43±0.12 ^{abA}	0.78±0.01 ^{cdB}	0.58±0.03 ^{cC}	<0.001
C7	1.12±0.01 ^{cA}	0.84±0.04 ^{bcB}	0.71±0.04 ^{bC}	<0.001
C8	1.46±0.05 ^{abA}	0.61±0.02 ^{efB}	0.30±0.02 ^{deC}	<0.001
P. value	<0.001	<0.001	<0.001	

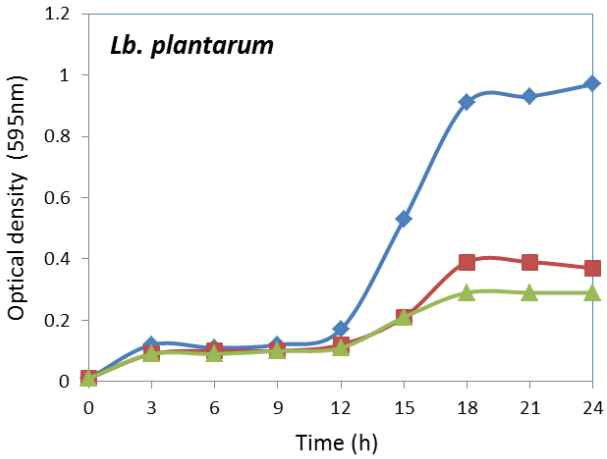
[1]Results are mean values from three replications ± standard deviations. $^{a-f}$ means in the same column with different superscripts are significantly different (P < 0.05). $^{A-C}$ means in the same row with different superscripts are significantly different (P < 0.05).

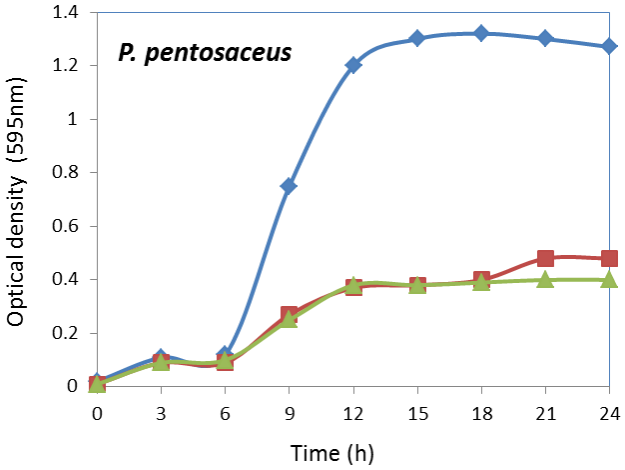
Table 2.4: Growth (OD_{595} ± SD) of LAB 10_5 CFU ml^{-1} on Frutafit inulin, Jerusalem artichoke inulin and glucose at 41-42 °C after 24h of incubation by (Tecan plate reader).

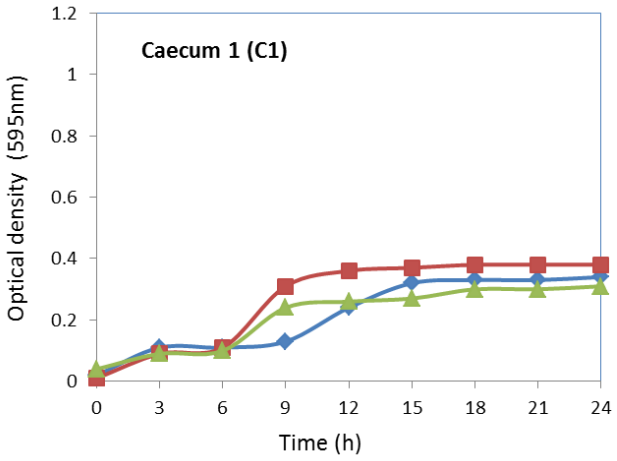
Strains	Growth density[1]			p. value
	Glucose	Frutafit Inulin	JA Inulin	
Lactobacillus plantarum	0.97±0.09 ^{bA}	0.37±0.01 ^{cdB}	0.29±0.01 ^{deB}	<0.001
Pediococcus acidilactici	1.04±0.05 ^{abA}	0.58±0.03 ^{bcB}	0.51±0.02 ^{bcB}	<0.001
Pediococcus pentosaceus	1.27±0.02 ^{dA}	0.48±0.03 ^{bcdB}	0.40±0.01 ^{cdC}	<0.001
C1	0.12±0.01 ^{dB}	0.38±0.13 ^{cdA}	0.31±0.03 ^{deAB}	0.021
C2	0.11±0.00 ^{dA}	0.10±0.01 ^{cA}	0.10±0.00 ^{fA}	0.377
C3	0.11±0.00 ^{dB}	0.32±0.09 ^{deA}	0.15±0.02 ^{fB}	0.01
C4	0.98±0.09 ^{bA}	0.92±0.02 ^{aAB}	0.80±0.05 ^{aB}	0.042
C5	0.59±0.09 ^{cA}	0.64±0.10 ^{bA}	0.19±0.05 ^{efB}	0.001
C6	0.65±0.06 ^{cA}	0.53±0.07 ^{bcdA}	0.50±0.09 ^{bcA}	0.111
C7	1.04±0.04 ^{abA}	0.66±0.02 ^{bB}	0.55±0.05 ^{bB}	<0.001
C8	0.66±0.2 ^{cA}	0.68±0.12 ^{abA}	0.28±0.04 ^{deB}	0.021
P. value	<0.001	<0.001	<0.001	

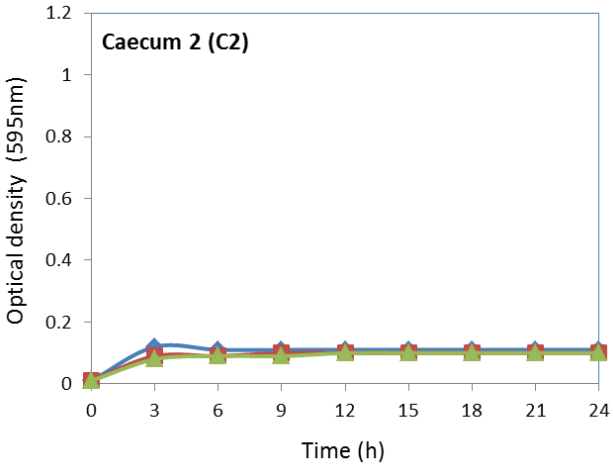
[1]Results are mean values from three replications ± standard deviations. $^{a-f}$ means in the same column with different superscripts are significantly different (P < 0.05). $^{A-C}$ means in the same row with different superscripts are significantly different (P < 0.05).


P. acidilactici
Optical density (595nm)
Time (h)


Lb. plantarum
Optical density (595nm)
Time (h)


P. pentosaceus
Optical density (595nm)
Time (h)


Caecum 1 (C1)
Optical density (595nm)
Time (h)


Caecum 2 (C2)
Optical density (595nm)
Time (h)

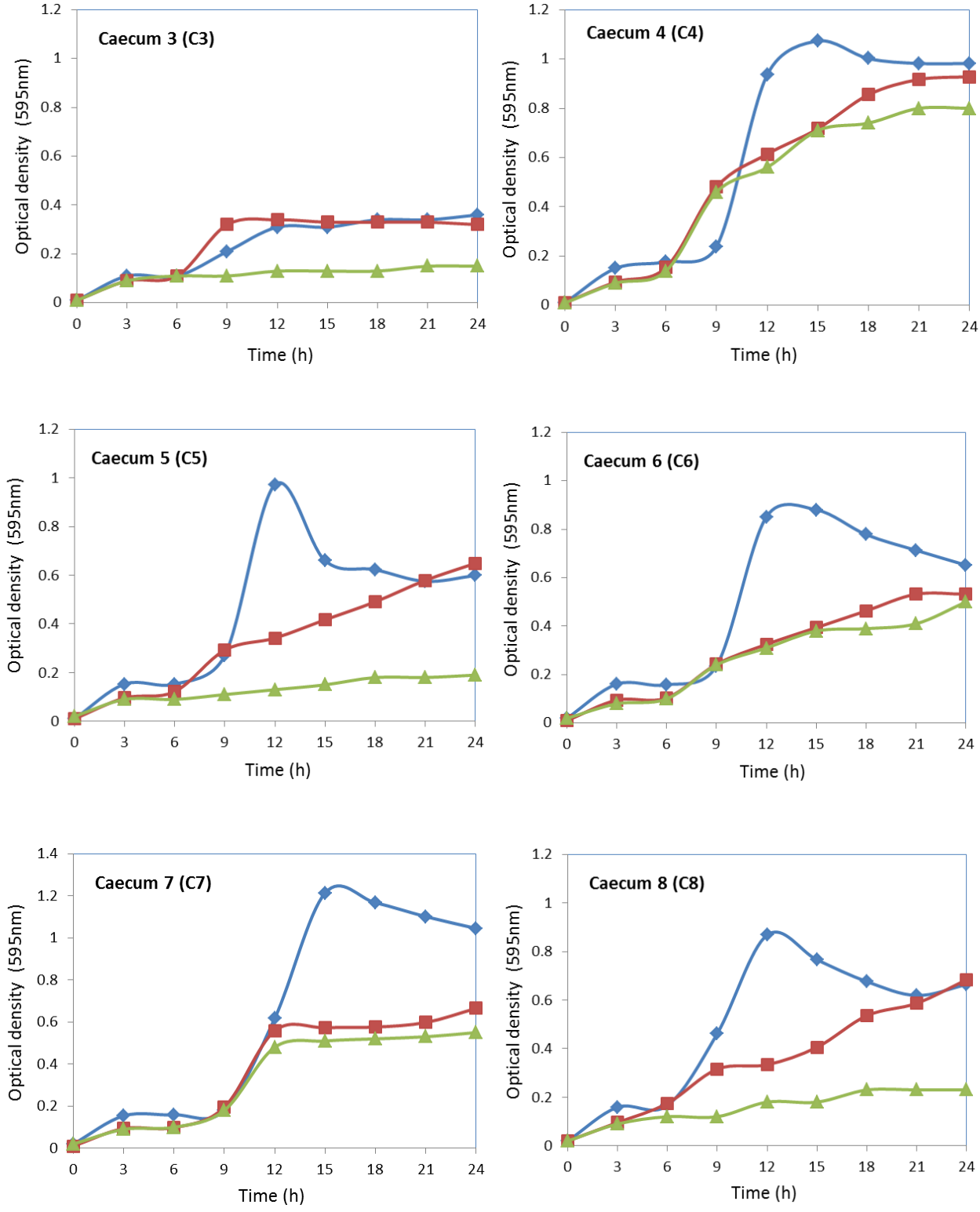


Figure 2.3: Growth curves of 11 probiotic LAB strains in basal MRS medium supplemented with glucose (control) (◆) commercial Inulin (■) and inulin extract from JA (▲) per every three hours by micro-plate reader. Results are the means from three replications.

2.2.5.3 Aggregation activity

2.2.5.3.1 Auto-aggregation test

Four LAB isolates (C2, C4, C6, and C7) and *Lactobacillus plantarum* and *Pediococcus acidilactici* were tested for aggregation ability. Lactic acid bacteria were grown overnight in MRS broth (Sigma-Aldrich, Fluka, Switzerland) at 37°C, in 5% CO_2 atmosphere. The next day the cultures were centrifuged (Harrier 18/80, MSE, UK) for 10 min at 10000 rpm and washed three times with sterile distilled water. Then, they were resuspended in the same initial volume of phosphate buffered saline (PBS) at a concentration of 10^9 CFU ml^{-1} (pH 6.0) and incubated, at room temperature in the presence of 10% (v/v) freshly prepared filter sterilised culture of their own LAB strain supernatant fluid. The total volume of the aggregation mixture was 1 ml. Auto-aggregation was considered as positive when clearly visible, sand-like particles (formed by the aggregated cells), gravitated to the bottom of the tubes, leaving a clear supernatant fluid , within 2 hours.

The results of six screened strains of LAB, C2, C4, C6, C7, *Lb. plantarum* and *P. acidilactici*, showed that all strains of bacteria showing significant auto-aggregation properties within 2 h, because the time needed for significant aggregation was between 0-120 min. One strain showed best auto-aggregation (within 45 min), and three strains had normal auto-aggregation within 90 min, and two remaining strains showed weak aggregation activity and they needed more than 90 min to aggregate (Table 2.5).

Table 2.5: Different auto-aggregation times of six LAB strains.

LAB strains	Source	Auto-aggregation time[1] (min)
C2	Caecum chicken	++
C4	Caecum chicken	+++
C6	Caecum chicken	++
C7	Caecum chicken	+
Pediococcus acidilactici	Plymouth University	++
Lactobacillus plantarum	*Plymouth* University	+

[1] Time needed to give a clear supernatant fluid, lower aggregation time indicates more aggregation of eachstrain.

+++= <45 min, ++= >45 min and += >90-120 min.

2.2.5.3.2 Co-aggregation test

The co-aggregation properties with three different indicator strains, *Salmonella enterica* Enteritidis NCTC 5188 (*S.* Enteritidis), *E. coli* K12, *Clostridium perfringens (*NCIBM 8693) were used for co-aggregation ability, obtained from laboratory microbiology Plymouth University stock cultures. Prior to use, the *S.* Enteritidis and *E. coli* were sub cultured twice in nutrient broth (Oxoid, UK) and *Cl. perfringens* in cooked meat broth (Oxoid, UK) at 37°C, for 24h in 5% CO_2 atmosphere, and under anaerobic conditions, respectively.

The co-aggregation test was performed according to (Kmet and Lucchni, 1997). Strains of LAB were grown in MRS broth (Sigma-Aldrich, Fluka, Switzerland) at 37°C for 24h, in 5% CO_2 atmosphere. Also, *S.* Enteritidis, E. *coli* and *Cl. perfringens* were grown as mentioned above. The next day the cultures were centrifuged for 10 min at 10000 rpm and washed three times with sterile distilled water. Pathogen cultures were resuspended in the same initial volume of phosphate buffered saline (pH 6.0) at a concentration of 10^9 CFU $ml^{-1}$and incubated at room temperature in the presence of 10^9 CFU ml^{-1} of LAB

resuspended in the same initial volume of freshly prepared, filter sterilised-supernatant fluid, of each LAB. The total volume of aggregation mixture was 1 ml. Co-aggregation was considered positive when clearly visible, the cells gravitated to the bottom of the tubes, leaving a clear supernatant fluid, within 2h, at room temperature.

Results of co-aggregation with three different indicator strains, *S.* Enteritidis, *E. coli* and *Cl. perfringens* are presented in the table 2.6. The best co-aggregation properties were obtained with C4 which has been isolated from chicken caeca with *S. enteritidis.* C7 and *Lactobacillus plantarum* showed no efficacy co-aggregation properties, because the cells needed more than 120 min to gravitate to the bottom of the tube.

Table 2.6: Co-aggregation activity of LAB strains isolated from chicken caeca and two pure LAB with pathogenic bacteria.

LAB strains	Coaggregation with[1]		
	S. Enteritidis	*E. coli*	*Cl. perfringens*
C2	+++	++	++
C4	+++	+++	+++
C6	+	+	+
C7	+	-	-
Pediococcus acidilactici	++	++	++
Lactobacillus plantarum	-	-	+

[1]Co-aggregation is given in time needed until clearly visible sand-like particles, formed by the aggregated cells, gravitated to the bottom of the tubes, leaving a clear supernatant fluid.

+++= <45 min, ++= >45 min, += >90-120 min and -=>120 min.

2.2.5.4 Detection of antagonistic activity

LAB C2, C4, C6 and C7, *Lactobacillus plantarum* and *Pediococcus acidilactici* were tested for antagonistic activity against *S.* Enteritidis*, E. coli* and *Cl. perfringens* using an agar spot test according to the method of (Santini *et al*., 2010). LABs were grown in MRS broth, at 37 °C for 24h under anaerobic conditions. Ten µl of the overnight cultures (A_{600} of about 0.1) were spotted on to the surface of MRS agar (Oxoid, England) plates and incubated anaerobically for 24 h at 37 °C, to allow colonies develop. Approximately 10^7 CFU/ml^{-1} of *Salmonella* Enteritidis, *E. coli* and *Cl. perfringens* were inoculated into 10 ml of soft nutrient agar (0.7%) and overlaid on the plate containing LAB colonies. These plates were incubated for 24 h at 37 °C for S. Enteritidis and *E. coli* and anaerobically at 37 °C *Cl. perfringens*. After incubation, they were examined for clear inhibition zones around each LAB strain. Each strain was performed in triplicate.

All six LAB strains were able to inhibit the growth of *S. enteritidis*, *E. coli* and *Cl. perfringens* to varying degrees, the results are shown in (Table 2.7). The radius of the inhibition zones were affected significantly ($P<0.05$) by LAB strains and the pathogens. Generally, all six LAB strains were more effective in inhibiting the growth of *Cl. perfringens* than other pathogen bacteria. *E. coli* was found more resistant to the LAB strains. C4 were found significantly ($P<0.05$) more effective in inhibiting the growth of pathogen bacteria compared the other LAB strains. These results are interesting, because LAB strains are not known to be strong inhibitors of gram-negative bacteria (Tag *et al*., 1976). Gilliland and Speck (1977) demonstrated that *Lactobacillus* strains showed stronger antibacterial properties against gram-positive bacteria than gram-negative bacteria.

Table 2.7: Antagonistic activity of LAB strains isolated from chicken caecal and two pure lactic acid bacteria against pathogenic bacteria.

LAB strains	Radius of inhibition zones (cm)[1]			Mean
	S. Enteritidis	*E. coli*	*Cl. Perfringens*	
C2	1.57±0.04 ^{b}	1.32±0.07 ^{b}	2.01±0.08 ^{b}	1.63 ^{ab}
C4	1.84±0.03 ^{a}	1.65±0.05 ^{a}	2.28±0.07 ^{a}	1.92 ^{a}
C6	1.19±0.03 ^{c}	1.17±0.04 ^{bc}	1.58±0.05 ^{c}	1.31 ^{abc}
C7	0.95±0.03 ^{d}	0.86±0.05 ^{de}	1.17±0.04d ^{e}	0.99 ^{bc}
Lactobacillus plantarum	0.99±0.09 ^{d}	0.73±0.07 ^{e}	1.01±0.07 ^{e}	0.91 ^{c}
Pediococcus acidilactici	1.23±0.04 ^{c}	0.98±0.09 ^{cd}	1.27±0.05 ^{d}	1.16 ^{bc}
P. value	<0.001	<0.001	<0.001	0.002

[1]Results are mean values from three replications ± standard deviations. Means in each column with different superscripts are significant different (P<0.05).

2.2.5.5 Tolerance Test to bile Salts

The sensitivity of LAB strains to bile salts was tested on MRS agar plates containing different levels of bile salts. Overnight cultures of the isolates were centrifuged (ROTOFIX 32 A, Hittich, Zentrifugen, Germany) at 7.500 ×g for 5 min at 4°C, resuspended pellets in phosphate buffer (pH 6) and serially diluted to 1 × 10^{-5}. Appropriate dilutions were plated onto MRS agar containing 0.075, 0.15, 0.3, and 1% (wt/vol) bile salts (Sigma-Aldrich, Buchs, Switzerland) and incubate aerobically at 37°C for 48 h (Garriga *et al*., 1998; Taheri *et al*., 2009).

All the tested cultures showed resistance against different concentrations of bile salts. Tolerance level was found significant differences (P<0.05) among all the test of LAB species (Table 2.8).

Table 2.8: Number of LAB strains (Log_{10} CFU ml^{-1}) after incubation at various levels of bile salts conditions.

LAB Strains	Bile salt %[1]			
	0.075	0.15	0.3	1
C2	7.367±0.04	7.355±0.03 [a]	7.340±0.01[a]	7.011±0.06 [a]
C4	7.368±0.04	7.368±0.02 [a]	7.329±0.02 [ab]	7.047±0.04 [a]
C6	7.340±0.03	7.339±0.03 [ab]	7.307±0.01 [ab]	6.905±0.02 [ab]
Lb. plantarum	7.279±0.00	7.264±0.03 [b]	7.204±0.02 [c]	6.832±0.04 [b]
P. acidilactici	7.334±0.03	7.333±0.02 [ab]	7.265±0.03 [bc]	6.891±0.08 [ab]
P. value	0.052	0.017	<0.001	0.006

[1]Results are presented as mean values from three replications ± standard deviations (ANOVA followed by Turkey's test). Means within a column with different superscripts differ significantly ($P < 0.05$).

2.2.5.6 Tolerance Test to acidic pH

Cell suspensions were prepared as above and diluted 1×10^{-5} in phosphate buffer at pH 2, 3, and 6. After incubating for 90 min at 37°C, 100 µl of different pH were spread over MRS agar and incubating anaerobically at 37°C for 48 h, then viable cells were enumerated (Garriga *et al*., 1998).

The effect of acid conditions (pH 2.0, 3.0 and 6.0) on the viability of LAB strains is showed in Table 2.9. The Results showed that all strains of LAB grew well at pH 2.0. The strains of LAB showed significant differences among the different pH values. C2 and C4 showed good resistance to low pH during 90 min at 37ºC. This result directly indicted that the tolerance of C2 and C4 to the low pH was strain-specific. Thus it determined that the final probiotic property was also strain specific.

Table 2.9: Number of LAB strains (Log_{10} CFU ml^{-1}) after incubation at different level of pH values for 90 min.

LAB Strains	pH values[1]		
	2.0	3.0	6.0
C2	7.217±0.03 [a]	7.259±0.01 [a]	7.408±0.02 [a]
C4	7.237±0.02 [a]	7.244±0.05 [a]	7.423±0.02 [a]
C6	6.868±0.07 [b]	7.075±0.06 [b]	7.273±0.03 [b]
Lb. plantarum	6.645±0.03 [c]	7.060±0.075 [b]	7.209±0.02 [b]
P. acidilactici	6.711±0.09 [bc]	7.094±0.025 [b]	7.281±0.03 [b]
P. value	<0.001	<0.001	<0.001

[1]Results are presented as mean values from three replications ± standard deviations (ANOVA followed by Turkey's test). Means within a column with different superscripts differ significantly ($P < 0.05$).

2.2.5.7 Mucus binding test

Five strains of LAB C2, C4, C6, *Lactobacillus plantarum* and *Pediococcus acidilactici* were tested for mucus binding using the method described by (Jonsson *et al*., 2001; Savvidou, 2009). In summary, Nunc-Immuno 96-well Micro well MaxiSorp flat bottom plates (Sigma, UK) were coated with 100µl mucin type II from porcine stomach (Sigma, UK) at a concentration of 1000 µg mucin proteins per ml of sodium carbonate buffer (pH 9.7) and incubated at 4 °C for 24 h. Mucin solutions were removed and the plates were washed three times with PBS (pH 7.3) supplemented with 0.05% Tween 20 (PBST). Each suspension of LAB was adjusted to an optical density of 0.5 at 600 nm, 100µl of individual LAB strains were added to each well and the plates were incubated on an orbital platform shaker (IKA vibrax-VXR S17, Staufen, Germany) at 40 rpm for 2 h at 37 °C. The unbound bacteria were removed by washing the wells twice with PBST. The absorbance values (OD_{405} nm) were determined in a VersaMax ELISA microplate reader (Molecular Devices, Wokingham, Berkshire, UK). Each batch of assays

also included blank wells (only mucin and PBST without bacteria) and *Lactobacillus reuteri* (NCIB 11951) used as a positive control (Alelijung *et al*., 1994; Savvidou, 2009). Generally, LAB were classified as strongly adherent (A_{405} nm > 0.3), weakly adherent (<0.1 <A_{405} nm >0.3), and non-adherent at <0.1<A_{405}nm (Jonsson *et al*., 2001; Savvidou, 2009).

Table 2.10: Adhesion of LAB strains to mucin from epithelial chicken intestinal.

Strains	Adherent to mucin
C2	0.55±0.05 [a]
C4	0.58±0.02 [a]
C6	0.56±0.03 [a]
P. acidilactici	0.57±0.11 [a]
Lb. plantarum	0.49±0.04 [a]
Lb. reuteri (Control)	0.45±0.10 [a]
P. value	0.268

[1]Results are presented as mean values from three replications ± standard deviations (ANOVA followed by Turkey's test). Means within a column with different superscripts differ significantly (P < 0.05).

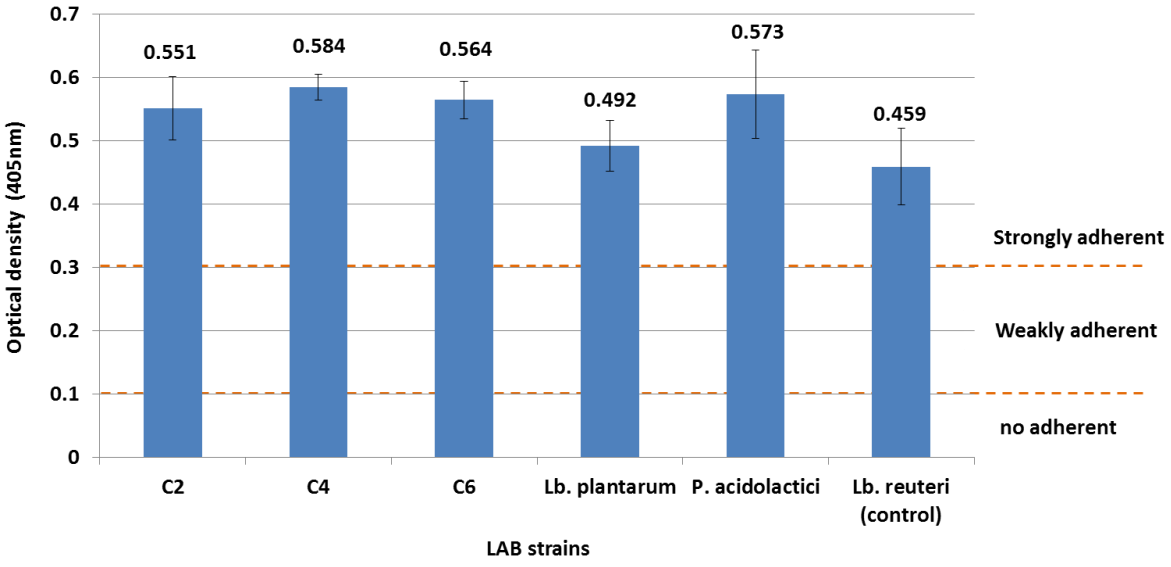


Figure 2.4: The level of mucus binding of five LAB strains.

2.2.5.8 Adhesion to chicken epithelial cells

Five strains of LAB C2, C4, C6, *Lactobacillus plantarum* and *Pediococcus acidolactici* were tested for adhesion to chicken epithelial cells using method described by Fuller (1975) and used later by (Garriga *et al*., 1998; Savvidou, 2009).

Organically farmed chickens were humanly slaughtered and the entire GI tract was removed and transferred to the laboratory on ice. Gut contents were removed aseptically and ileal segments were opened, washed with PBS and held in PBS (pH 7.2) at 4 °C for half an hour, to loosen the surface mucus. The epithelial cells were collected from the ileal part of the intestine (Figure 2.5), by gently scraping the epithelium with the edge of a microscope slide and scrapings were placed in sterile universal bottle samplers and suspended in PBS. The suspended scrapings were left on ice for 15 min to allow larger debris to settle. The debris that gravitated to the bottom was removed and the supernatant fluid centrifuged for 1 min at 500 rpm, to spin down the suspended cells. The pellet containing ileal cells was examined microscopically to ensure that they were free from any adherent bacteria. The number of cells present was determined using a haemocytometer. Cells were stained with Trypan Blue to identify dead cells. The number of live cells in the suspension was found to be 6.6×10^{6} ml^{-1}.

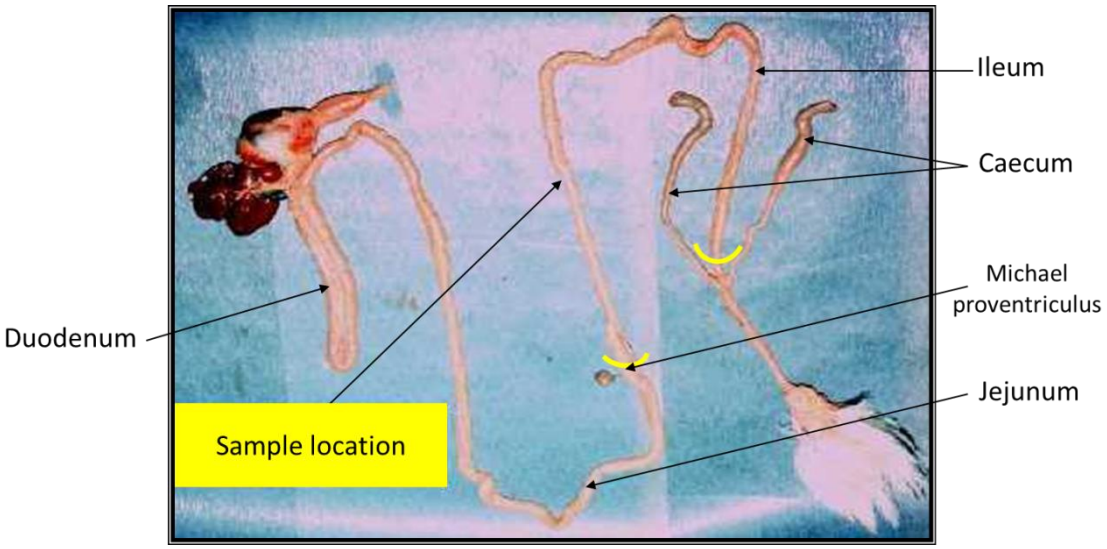


Figure 2.5: The location of sample of epithelial cells from ileum of chicken intestine.

Overnight cultures of the selected lactobacilli in MRS broth (10^9 CFU ml^{-1}) were resuspended in PBS to give a cell density of 10^8 CFU ml^{-1}. One hundred µl of each selected *Lactobacillus* suspension was added to 400 µl of the epithelial cell suspension and the mixture was incubated for 30 min at 37 °C in a shaking water bath (20rpm). The resuspended mixtures were fixed with methanol. When bacteria are fixed with methanol, are more resistant to discoloration. Then, the resuspended mixtures stained with Gram stain and the number of bacteria adhering to an epithelial cell was determined by phase contrast light microscopy. Ten epithelial cells were selected randomly and the mean number of bacteria attached per epithelial cell was calculated. Chains or pairs of bacterial cells were counted as one unit. The adhesion efficiency of the lactic acid bacteria to intestinal epithelial cells was observed microscopically by Olympus research Vanox-T microscope (Model AHBT, Japan) fitted with a digital camera (Olympus

E-620) and scored positive if at least 10 bacteria per epithelial cell could be observed (Ehrmann *et al*., 2002).

The adhesion of five LAB strains to intestinal epithelial cells was determined by light microscopy as summarising in Table 2.11, considerable differences were observed among the five strains. The adhesion ability of C2 and C4 were stronger compared to other strains, but, there was no difference between C2 and C4.

Table 2.11: Adhesion of LAB strains to chicken intestinal epithelial cells.

LAB Strains	Adhesion chicken intestinal epithelial cells
C2	Very good
C4	Very good
C6	Good
P. acidolactici	Good
L. plantarum	Good

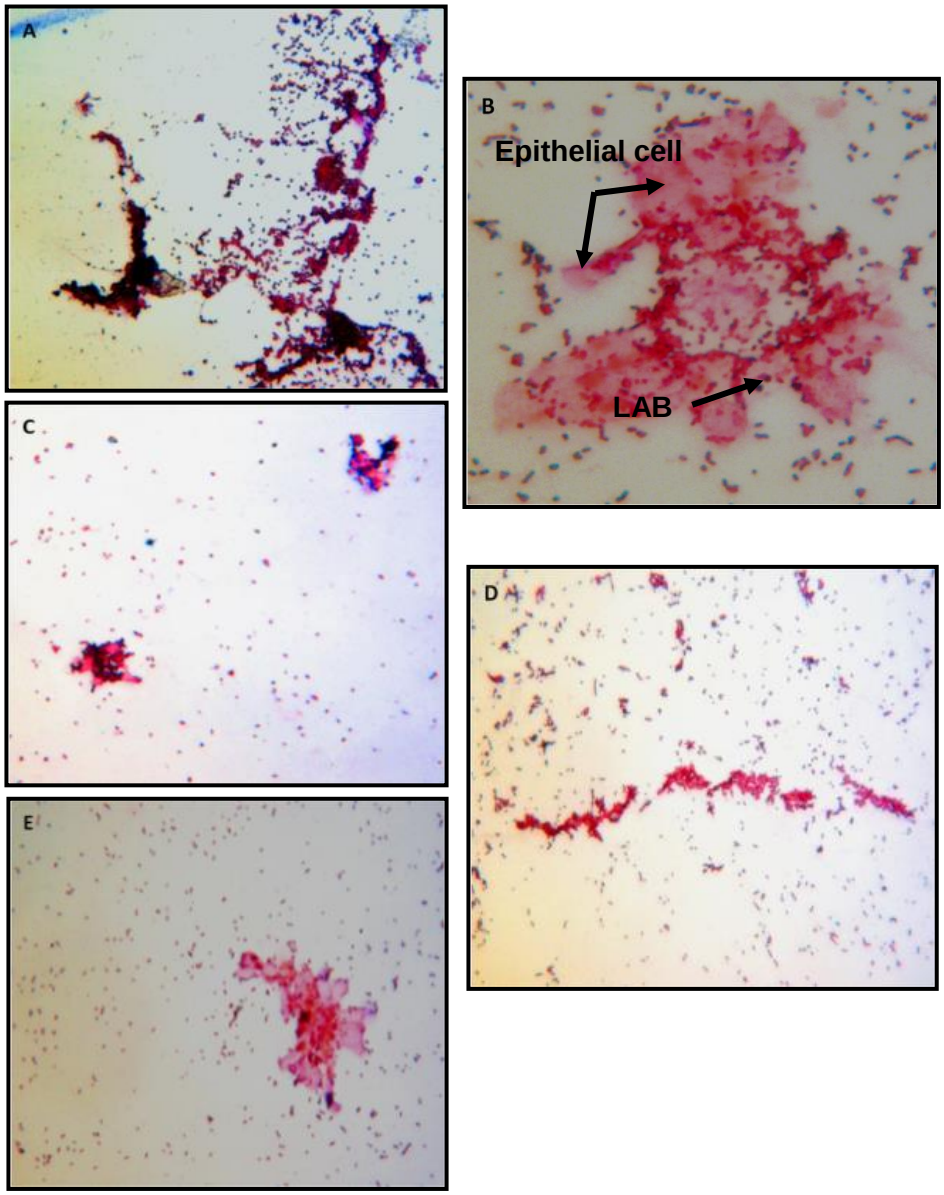


Figure 2.6: Adhesion of LAB strains to the intestinal epithelial cells of chicken GI tract observed using light microscopy after Gram-staining; A) C2, B) C4, C) C6, D) *Pediococcus acidilactici* and E) *Lactobacillus plantarum*. (100X magnification).

2.2.5.9 Cell surface hydrophobicity test

Four strains C2, C4, C6 and *Pediococcus acidilactici* that showed the greatest aggregation ability, antagonistic activity against pathogen bacteria and adherence to chicken intestine epithelial cells were tested in a cell surface hydrophobicity test as additional test for assessing their adhesion ability to epithelial cells. Hydrophobicity was expressed as the percentage of total cells removed from the aqueous phase.

Cell surface hydrophobicity was determined by the method of Rosenberg *et al*. (1980). Lactic acid bacteria were harvested after 18 h of growth, washed twice, and resuspended in physiological saline solution to an optical density of 0.5 at 600 nm (OD_{600}). One millilitre of toluene was added to test tubes containing 3mL of washed cells. The mixtures were blended on a vortex mixer for 90 s. The tubes were left to stand for 15 min for separation of the 2 phases, and the OD_{600} of the aqueous phase was then measured. Hydrophobicity was calculated as the percentage of decrease in the OD_{600} of the bacterial suspension due to the partitioning of cells into the hydrocarbon layer:

$$\text{Percentage of hydrophobicity} = \frac{OD_{600}\text{ before mixing} - OD_{600}\text{ after mixing}}{OD_{600}\text{ before mixing}} \times 100$$

The sample C4 showed a greater hydrophobic activity with toluene than the other sample C2, C6 and *Pediococcus acidilactici* (Table 2.12).

Table 2.12: Cell surface hydrophobicity of four selected LAB strains to toluene.

LAB Strains	Cell surface hydrophobicity[1] %
C2	91.33±0.49[a]
C4	92.39±0.99[a]
C6	89.52±1.51[a]
Pediococcus acidilactici	88.20±2.63[a]
P. value	0.052

[1]Resultsare presented as mean values from three replications ± standard deviations (ANOVA followed by Turkey's test). Means within a column with different superscripts differ significantly ($P < 0.05$).

2.2.5.10 Molecular Identification of strains by 16SrDNA sequencing

2.2.5.10.1 Bacterial DNA extraction

The lactobacilli strain C4 had good aggregation ability, resistance to acid and bile salts, high percentage of cell surface hydrophobicity and high antagonistic activity against several pathogens bacteria was identified by PCR-based methods. DNA was extracted by using a protocol of the GenElute^{TM} Bacterial Genomic DNA Kit (Sigma-Aldrich, Germany). The overnight culture was centrifuged for 2 minutes at 12000 xg and the culture medium completely removed. The pellet was resuspended thoroughly in 200 µl of Lysozyme solution which was prepared from chicken egg white Lysozyme (L4919) diluted by 50 mg/ml in TE buffer (10 mM Tris-HCl, pH 8.0), and the mixture incubated for 30 minutes at 37 °C. Twenty µl of the proteinase K solution was added to the sample followed by 200 µl of Lysis solution C (B8803) and vortexed thoroughly for about 15 seconds and then incubated at 55 °C for 10 minutes. Five hundred µl of the Column preparation solution was added to the pre-assembled Gen Elute Mini prep Binding Column

and seated in a 2 ml collection tube. The sample was centrifuged at 12000 x g (Sanyo, Micro Centaur, MSE, UK) for 1 minute and the eluate removed. Two hundred µl of ethanol (95-100%) was added to the sample in the lysate and mixed homogeneously for 5-10 seconds. The entire contents of the tube in the Load lysate were transferred into the binding column and then the sample was centrifuged at 8000 x g for 1 minute. The collection tube containing the eluate was discarded and placed the column in a new 2 ml collection tube. The first washing was added 500 µl Wash Solution 1 (W0263) to the column and centrifuged for 1 min at 8000 x g. The collection tube containing the eluate was discarded and placed the column in a new 2 ml collection tube again. The second washing was added 500 µl Wash Solution to the column and centrifuged for 3 min at 12000 x g to dry the column. The column was centrifuged for an additional 1 min at 12000 x g, because the column must be free from ethanol before eluating the DNA. Finally, the collection tube containing the eluate was discarded and placed the column in a new 2 ml collection tube. The Elute DNA was added 200 µl of the Elution Solution (B6803) directly onto the centre of the column and then incubated for 5 min at room temperature, to increase the elute efficiency, then the sample was centrifuged for 1 min at 8000 x g to elute the DNA. The eluate contains pure genomic DNA, then stored at 2-8 °C for short term storage.

2.2.5.10.2 Spectrophotometric test

The optical density of the DNA concentration was examined using 426 Nanodrop software. The concentration of DNA extract was determined using spectrophotometric at 260 nm (Thermo Scientific Nano Drop™ 1000, DE, USA)

and standardized. DNA in Elution Solution (B6803) was used as a blank to re zero the device. The DNA was measured and the average bacterial DNA has 50 ng/µl.

2.2.5.10.3 PCR amplification and DNA sequencing of 16S rRNA

Bacterial was amplified using PCR primers;

Forward primer **27(F)** 5'-AGAG TTTG ATCC TGGC TCAG-3' (20 bases)

Reverse primer **1492(R)** 5'-GGCT ACCT TGTT ACGA CTT-3' (19 bases)

The primers were obtained from lab microbiology, University of Plymouth (Eurofins MWG Operon, Germany).

A mixture of 1 µl of bacterial DNA extract, 24 µl of reaction mix, (12.5 µl Red Taq ready Mix (Sigma-Aldrich, USA), 1 µl forward primers, 1 µl reverse primers and 9.5 µl DNA grade water) was prepared and DNA was amplified in a PCR thermal cycler (TECHNE, Model TC-312) for a period of 4 h using the following program: denature at 95 °C for 1 min, anneal primers at 55 °C for 2 min and extension at 72 °C for 3 min. Each set of reactions included a negative and a positive control.

Eight µl of the PCR products were then analysed by electrophoresis on a 1.5%, agarose gel to check the size of amplicons. A mixture of 1.35 g of agarose powder and 90 ml of 1x TAE was dissolved in microwave with shaking, for 1 min. After cooling 4 µl of SYBR®safe stain was added to the gel. To prepare the sample for electrophoresis, 2 µl of DNA loading buffer (Blue Bioline) was added to 7 µl of PCR product, and the samples were added into wells. Nine µl of the 100bp

DNA ladder (Fisher, USA) was used to assess the size of DNA products. The gel was run at 90 volts for 45 h, and the bands were visualised and photographed using a camera on a UV transilluminator (Universal Hood ii, Bio-RAD Laboratories, Segrate, Milan, Italy).

2.2.5.10.4 DNA purification

Purification of PCR products were performed with the ChargeSwitch®-Pro PCR Clean-up Kit (invitrogen, Sigma-Aldrich, USA) by life technologies. All steps were performed at room temperature; three main steps were adjusted, based on the instructions of the manufacturer. Binding the DNA by added a 1:1 volume of ChargeSwitch®-Pro PCR Purification buffer to the PCR reaction, gently vortexed to mix well. The mixture was transferred onto the ChargeSwitch®-Pro PCR Clean-up column inserted in a collection tube. The column/tube was centrifuged at 10000 xg for 1 min. The column was removed from the tube and the flow-through discarded, and then the column was re-inserted in the same collection tube. The column was washed in 600 µL of ChargeSwitch®-Pro PCR wash buffer. The column/tube was centrifuged at 10000 xg for 1 min. The flow-through and the collection tube were discarded, and the column was inserted into a new sterile Elution tube. The final steps of purification were Eluting the DNA. 25 µL of ChargeSwitch®-Pro PCR Elution buffer was added onto the column, and incubated at room temperature for 2 minutes. The column/tube was centrifuged at 10000 xg for 1 min. The flow-through was contains the purified DNA. The Elution step was repeated one more time, and the flow-through was collected in the same tube. The quantity of DNA purified was determined by Electrophoresis assay,

after diluted to 1/10 by 1 µL of sample with 3 µL of loading buffer and 6 µL of DNA grade water, the DNA concentration was calculated by multiplying the bp of the sample with the 50 bp DNA ladder (21 ng/µL). Then DNA was sequenced by GATC Biotech (European Custom Sequencing Centre, Germany).

DNA concentration was determined by using gel electrophoresis. Only 5 µl of diluted to 20-80 ng/µl of PCR product of C4 strain and 5 µl of one of the primers (5 pmol/ µl) in Eppendorf tube together was sent for sequencing centre of GATC biotechnology in Germany and the sequencing results send via their website: http://www.gatc-biotech.com/en/index.html. Sequence was compared to those in available data-bases by use of the BLAST (Basic Local Alignment Search Tool) in Gene Bank network services at http://blast.ncbi.nlm.nih.gov/Blast.cgi to determine its approximate phylogenetic relationships. The strain identified (100%) as *Lactobacillus animalis* strain.

2.3 Statistical analysis

All data were subjected to one way analysis of variance (ANOVA) using Minitab statistics version 16.0 Statistical software (Minitab, Plymouth, UK). The one-way ANOVA test (Tukey's Multiple Comparison test) was used to determine significant differences at 0.05 levels among the different parameters. Data are presented as mean ± standard deviation (SD). All data were tested by a normality test.

2.4 Discussion

Lactic acid bacteria (LAB) especially lactobacilli are normal inhabitants of the intestinal tract of humans and animals (Mitsuoka, 1992). The use of LAB for their potential use as probiotics in animals is increasing (Denli *et al*., 2003). In the present study, LAB strains were isolated from caecal of a healthy broiler chicken and screened for probiotic characteristics.

Several oligosaccharides, such as inulin, isomaltooligosaccharides (IMO), fructooligosaccharides (FOS), galactooligosaccharides, (GOS), and lactulose have been used to significantly enhance the growth of desirable bacteria such as *Bifidobacteria* and *Lactobacillus* spp. (Kneifel *et al*., 2000; Saminathan *et al*., 2011). The results of the present study showed that all LAB strains were capable of utilizing both types of inulin examined but the growth varied among the strains. One of the reasons of growing LAB could be due to the reduction of pH value of media during fermentation (Westhuizen, 2008). Several researchers reported that inulin can support the growth of the intestinal bacterial community in broilers (Kleessen *et al*., 2003; Xu *et al*., 2003; Rehman *et al*., 2008, Park, 2008). However, other researchers reported that inulin supplementation did not affect the intestinal microflora (Yusrizal and Chen, 2003; Rehman *et al*., 2008). This inulin would have the potential to increase the population and activity of these LAB strains in the gastrointestinal tract of chickens when administered in the form of synbiotic, consequently enhance their beneficial effects on the host.

This investigation has found that strain C4 which was isolated from a chicken caecum had a shorter lag-phase and much better growth than other strains and could be a suitable candidate for an inulin synbiotic in broiler diet.

As *in vivo* studies investigating health benefits of potential probiotic properties are time consuming and often expensive, the consequent use of *in vitro* tests as selection criteria is inevitable to reduce the number of strains and, finally, to find the most effective organism (Nemcova, 1997). Bacteria must tolerate gastrointestinal stress conditions for their metabolic activity, as well as colonise the gastrointestinal tract. Therefore, it was necessary to evaluate the resistance ability of bacteria to gastrointestinal stress before their use as probiotics. The LAB strains isolated from chicken caecum were tested for resistance to bile salt, acidic pH, ability to inhibit pathogens, adhesion ability to the epithelial cells, antagonistic activities against pathogens and cell surface activity.

LAB Isolated from chicken caecum and some commercial and culture collection strains were grown with inulin from Jerusalem artichoke to select a suitable candidate for an inulin synbiotic for use in poultry diets. Tests used for screening should be simple and rapid to select one strain for probiotic efficacy from a large number of bacteria. Reports by Ehrmann *et al*. (2002); Taheri *et al*. (2009) and Bao *et al.* (2010) propose that the aggregation test is appropriate for the important step of screening because it is a simple method applicable to a large number of test strains, also the adhesion ability to the epithelial cells and antagonistic activities against pathogens seem to be accepted as being the most critical factors.

Before reaching the lower part of the gastrointestinal tract and exerting their probiotic properties, these bacteria must survive during transition through the stomach and the upper part of the gastrointestinal tract. The pH value in chicken GI tract ranges between 2-7.5 (Chang and Chen, 2000). So it is necessary to grow these bacteria at low pH 2.0 and tolerance to bile salts as selection criteria

as acid and bile tolerance strains are likely to survive in high numbers (Kimoto *et al*., 1999).

In the present study, light microscopy (LM) studies showed that *Lactobacillus* strains adhere to intestinal epithelial cells in pairs or in short chains, similar to those described by (Gopal *et al*., 2001; Li *et al*., 2008). The lectin-like proteins on the *Lactobacillus* cell surface may be the structural component in these aggregates (Gusils *et al*. 2002). Gusils *et al*. (1999c) previously found that *Lactobacillus animalis* have a lectin-like structure in the external layer and these molecules present in the cell surface would favour adhesion to epithelial cells. In this study, intestinal epithelial cells isolated from ileum part of chicken intestine for adhesion assay, because lactobacilli have higher adhesion ability in this part of intestine compared with duodenum and jejunum. On the other hand most of microorganisms remain in the later part of the gastrointestinal tract (Li *et al*., 2008). Gusils *et al*. (1999a) demonstrated that for adhesion assay with three different *Lactobacillus* strains (*Lactobacillus fermentum*, *Lactobacillus fermentum* subsp. *cellobiosus* and *Lactobacillus animalis*) were isolated from the gastrointestinal tract of chickens and intestinal fragments from chickens. *Lactobacillus animalis* and *Lactobacillus fermentum* were able to adhere to three kinds of epithelial cells (crop, small and large intestine) with predominance to small intestine. Among the strains considered *Lactobacillus fermentum* subsp. *cellobiosus* showed the lowest and *Lactobacillus animalis* the highest adhesion ability. Scanning electron microphotographs confirmed that the *Lactobacillus animalis* showing high adherent to intestinal cells compared to other strains.

Kos *et al*. (2003) suggested that the ability to adhere to epithelial cells and mucosal surfaces to be an important property of many bacterial strains used as

probiotics. Cell adhesion is a multi-step process involving contact of the bacterial cell membrane and interacting surfaces. Several workers have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells (Green and Klaenhammer, 1994; Pelletier *et al*., 1997; Del Re *et al*., 2000). In most cases, aggregation ability is related to cell adherence properties (Vandevoorde *et al*., 1992; Del Re *et al*., 2000).

It has been reported that the bacteria which shows a high aggregation (or in other words, low aggregation time), also have a high cell surface hydrophobicity and adhesion ability to the mucus (Del Re *et al*., 2000). Aggregation and cell surface hydrophobicity of the strains could be used instead of the examination of adhesion ability to the mucus because there is a strong relationship among these characteristics especially between aggregation time and adhesion ability to the epithelium of the gastrointestinal tract. In the present study showed that C4 strain needed less than 45 minute to aggregate properties and also had high attachment properties to the epithelial cells. The results are in agreement with Garriga *et al*. (1998) clearly showed that the strains with high aggregation had a better attachment to the epithelial cells. Taheri *et al*. (2009) showed in their results after screening 332 strains of LAB from the crop, ileum, and caecum, 62 bacteria (22, 22, and 18 bacteria from crop, ileum, and caecum, respectively) showed significant aggregation properties. Six strains needed only 15 minute to aggregate significantly.

The aggregation time and antibacterial activity demonstrate the ability of LAB to prevent the colonization of *E. coli*, *Salmonella*, and other enteric pathogens. Chaveerach *et al*. (2004) showed that five isolated strains of *Lactobacillus strains* have antibacterial effects against most strains of *Campylobacter jejuni*. Gilliland

and Speck (1977) and Kizerwetter-Swida and Binek (2009) found that lactobacilli have higher antibacterial effects against the gram-positive pathogenic bacteria (*Staphylococcus aureus* and *Clostridium perfringens*) than *E. coli* and *Salmonella*. Jin *et al*. (1996) reported that *Salmonella pullorum* is more sensitive to the antibacterial activity of LAB than *Salmonella Typhimurium* and *Salmonella enterica* Enteritidis. Gusils *et al*. (1999a) reported that *Lactobacillus fermentum* was effective in reducing the attachment of *Salmonella pullorum* by 77%, while *Lactobacillus animalis* was able to inhibit (90%, 88% and 78%) the adhesion of *Salmonella pullorum*, *S. enteritidis* and *S. gallinarum* to host-specific epithelial samples respectively. A strong tendency to auto-aggregation is not always combined with a strong co-aggregation property. Ehrmann *et al*., (2002) reported that *Lactobacillus agilis* TMW 1.964 and two strains of *Lactobacillus reuteri* (TMW 1.966 and TMW1.967) were isolated from duck caecum showed no significant co-aggregation with pathogenic bacteria but, in contrast, a strong auto-aggregation ability. But, all other strains were isolated from the crop and intestine of ducks with high co-aggregation activity showed high auto-aggregation as well.

Resistance to pH and bile salts is of great importance in survival and growth of bacteria in the intestinal tract and thus, is a prerequisite for probiotic properties (Havenaar *et al*., 1992). The effects of bile salts on the survival of LAB have been investigated by several authors (Floch *et al*. 1972; Gilliland *et al*. 1977; Tannock *et al*. 1989). Bile tolerance is considered as an important characteristic of the LAB strain which enables it to survive, grow and exert its action in gastrointestinal transit. LAB strains which could grow and metabolize in normal physical bile concentration could survive in gastrointestinal transit (Sanders *et al*., 1996). Furthermore, the effect of bile salts on the survivability of different LAB strains

depends on the concentration and the specific properties of the strains. It is well known that bile salt concentration in the gut is not static, ranging from 1.5% to 2% (w/v) in the first hour of digestion, and decrease afterwards to around 0.3% (Noriega *et al*., 2004). Lin *et al*. (2007) reported that *Lactobacillus fermentum* PG1, PGM1, PL1 and PLM1 strains, which were isolated from chicken, has a high tolerance to 0.3% bile salts. While, there is no tolerance found for *Lb. fermentum* PG3 and PGM3 to bile salt, which were isolated from poultry. On the other hand, Strompfova *et al*. (2006) demonstrated that *Lb. fermentum* AD1 strain was able to grow in the presence of 1% bile salts and 75.4% viable cells remained after 24 h of incubation. Recently, Raja *et al*. (2009) reported that *Lactobacillus fermentum* strain which was isolated from chicken gut showed tolerance to bile salts at 0.3 and 10%.

In order to gain information on the structural properties of the cell surface of LAB strains that are responsible for the aggregation and adhesion, its hydrophobicity assay was used to confirm the ability of aggregation and adhesion test. C2 and C4 showed more hydrophobic cell surface properties. Many previous studies on the physic-chemistry of microbial cell surfaces have shown that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Green and Klaenhammer 1994; Rojas and Conway 1996 and Pelletier *et al*., 1997). It is known that only pronase- and pepsin-sensitive surface molecules are responsible for cell surface hydrophobicity in bacteria (Kos *et al*., 2003). Bomba *et al*. (2002) also demonstrated that a higher percentage of hydrophobic bacteria adhere to intestinal epithelial cells than do hydrophilic strains.

2.5 Conclusion

The aim of this study was to investigate the potential use of inulin and to isolate and screen for the best lactic acid bacteria to produce a synbiotic for use in poultry diets. *Lactobacillus animalis* strain was the strain that had potential probiotic properties as ability to resistance to acidity and bile salts, strong suppression of pathogens as well as ability to adhere epithelial cells. Also, the results from this study showed that both types of Inulin supported good growth of this strain of LAB and could be a suitable candidate for an inulin synbiotic in broiler diet.

CHAPTER THREE

The effect of dietary inulin supplementation on intestinal microflora, immune functions and blood characteristics of SPF chicks

3.1 Introduction

This study dealt with the effects of inulin from commercial and Jerusalem artichoke (*Helianthus tuberosus*) supplementation on the microbial population and histology of the specific pathogen free chicks gut. The objectives were to examine any changes in the microflora of SPF chicks gut due to adding inulin. The use of prebiotics instead of antibiotics is going to be popular in birds as they have the potential to improve the useful microbial population of the GI tract (Park and Park, 2012)

The gastrointestinal tract (GIT) is important for absorption of nutrient and protection against many kinds of the pathogens that enter the body due to feeding (Mowat and Viney, 1997). The GIT also supports a micro-ecosystem that harbours a large and diverse population of bacteria (Drasar and Barrow, 1985; Franks *et al*., 1998) that create a symbiotic relationship with the host (Apajalahti, 2005). A diverse microbiota is found throughout the tract and is most extensive in the caecum (Amit-Romach *et al*., 2004). This microflora has a role in nutrition, detoxification of certain compounds, growth performance, and protection against pathogenic bacteria. The gut microflora influences health and well-being of host animals (Nurmi and Rantala, 1973; Van der Wielen *et al*., 2002).

The molecular method has been used to determine the variation in bacterial population in the chicken caecum (Gong *et al*., 2002; Zhu *et al*., 2002). The advances of molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation (Harmsen *et al*., 2000).

No information is available regarding the effect of inulin from Jerusalem artichoke on the intestinal microflora changes, the intestinal histological changes and immune organs in SPF chicks, as well as measurement of pH from the ileum and caecum digesta.

Therefore, the objective of this experiment was to investigate the potential influences of inulin from Jerusalem artichoke tubers on the jejunum histology and intestinal microflora.

3.2 Material and Methods

3.2.1 Ethical approval

The study was carried out at the University of Plymouth, Animal housing unit. The study was conducted according to UK Home Office regulations (Animal Scientific Procedure Act 1986) under the Home Office project license PPL 30/2640 and personal license PIL 30/10067 (Appendix 5).

3.2.2 Experimental design and treatments

One hundred clean eggs from a specific pathogen free (SPF) white Leghorn flock (VALO BIOMEDIA GMBH, Germany) were obtained as fertilized eggs. The eggs were incubated in animal facility at Plymouth University for 21 days in an egg incubator (Cuvatutto, Italy). The temperature and humidity were controlled. The experiment was conducted a completely randomised design with three treatments. The house and equipment were thoroughly washed and disinfected with virkon spray. It was prepared to insure proper temperature, ventilation, light and humidity

for 24 hours before chick access to the room. A total of twenty seven newly hatched chicks were randomly divided into three treatments, nine chicks per treatment with three replications. The chicks were weighed and housed in floor pens (100 × 80 cm), on wood shavings fitted with electrical lamp heaters per pen during the 21 days experimental period. The temperature started approximately at 35°C (from d 0 to 3) and was gradually reduced according to normal management practice by 5 ºC every week until 22-24 ºC and measured by temperature logger (Tiny tag, tv-4050, UK). Chicks were maintained on a 24 h constant light schedule until the end of the experiment. Chicks were fed and water was provided *ad libitum* throughout the experimental period (21 days). Circular plastic drinkers of (1.3 L) and long plastic feeders were used during the whole experiment (Figure 3.1).

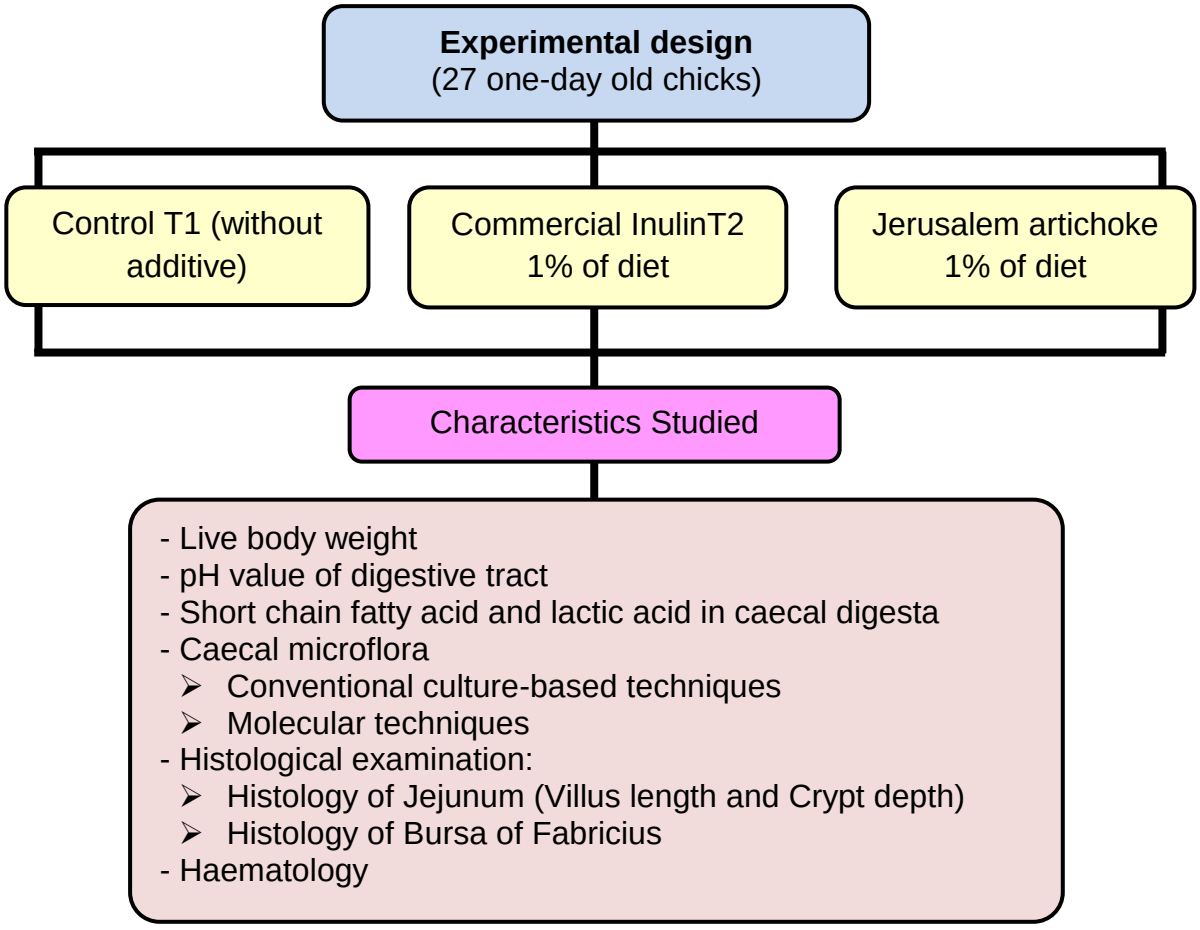


Figure 3.1: Layout of the feed trial.

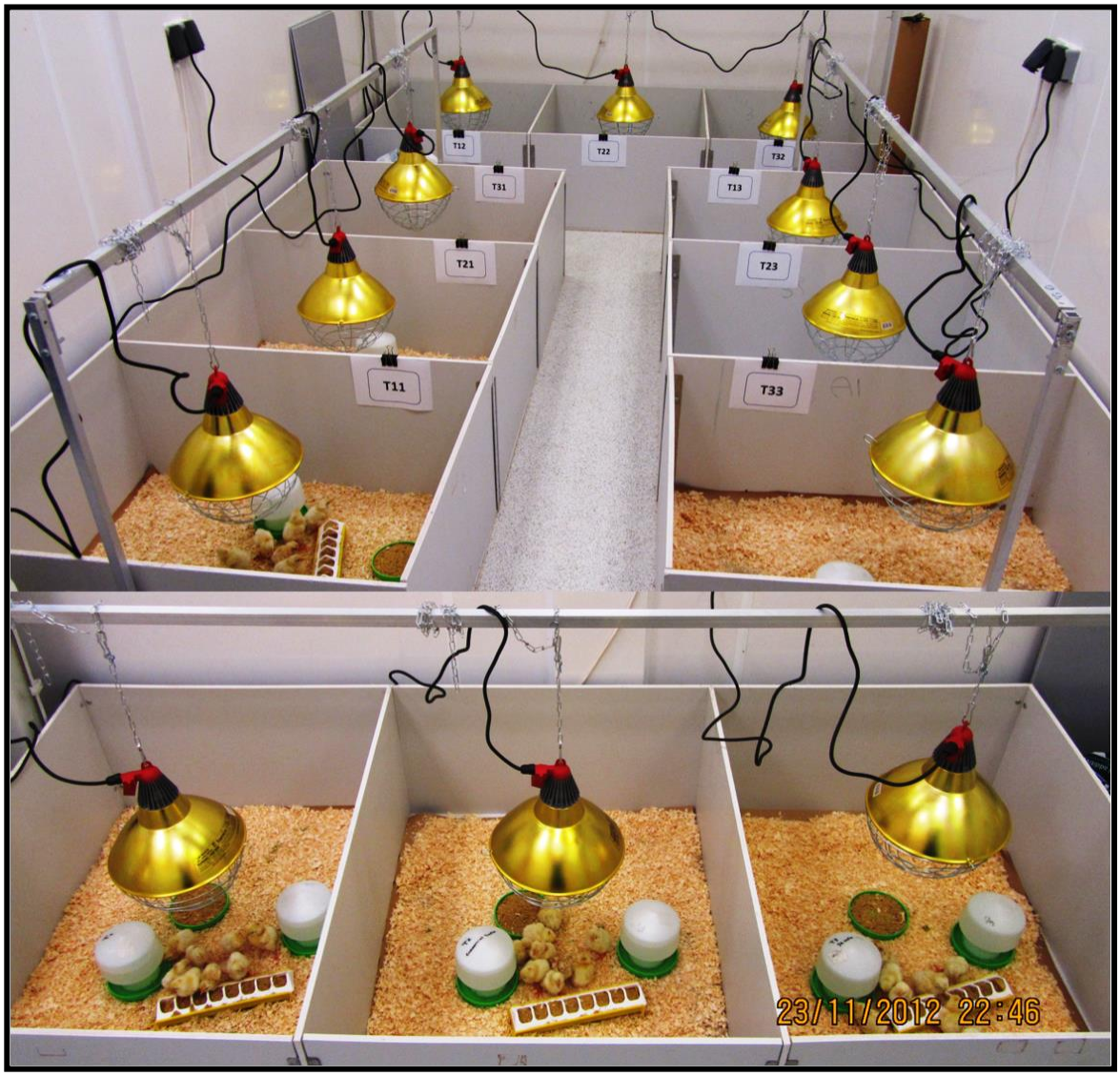


Figure 3.2: Chicks House located at University of Plymouth animal housing research unit, which used in this study.

3.2.3 Diets

A basal chick ground feed obtained from ISCA agriculture Ltd, (Venn Ottery, Devon, EX11 1RY, UK) with the composition shown in Table 3.1 was used. Chicks in the control group (T1) were fed basal diet which was formulated according to NRC standard (1994). The commercial inulin group (T2) was fed the basal diet with 1.0% inulin (Frutafit® HD, Highly Dispersible native inulin, Sensus, Roosendaal, Netherlands). The JA powder group (T3) was fed basal diet with 1% JA inulin. The inulin in powder form was mixed thoroughly in mentioned quantities to a small amount of feed (100 gm). The resultant mixture was then mixed with the rest of the feed in a covered pail until a thorough and consistent mixture was obtained. The chemical composition of Frutafit® HD commercial inulin and dried Jerusalem artichoke tubers were shown in table 3.2.

Therefore, chicks were assigned to the following treatments:

T1= control group feed standard diets (no additives).

T2= standard diets + 1% commercial inulin (Frutafit® HD)

T3= standard diets + 1% Jerusalem artichoke inulin

Because JA contained 74.48% inulin, 13.42 g of product/kg of diet were added to the basal diet to include 10 g of inulin/kg of diet. The feed was devoid of any coccidiostats or antibiotics. Diet's samples after adding inulin from the feeding trial were analysed according to AOAC (2002) standard methods for proximate composition. All samples were analysed in triplicate (except GE in duplicate). Moisture content (dry matter) was determined using drying oven (105 ºC for 24 h).

Crude protein was calculated from sample nitrogen content was determined using Kjeldahl apparatus (Gerhardt Kjeldatherm method, N % x 6.25) and crude lipid using ether extraction in multi-unit extraction Soxtec apparatus (dichloromethane extraction by Soxlhet method). Ash Content was analysed using a muffle furnace (incineration at 550 ºC for 12 h). Gross energy analysed using (Parr bomb calorimeter).

Table 3.1: The composition of standard diet of starter (1-21 days) which was used for the all trials

Ingredients	Composition (g/100 g)
Maize meal	54.75
Soybean meal	27.38
Fish meal	11.41
Soya oil	4.57
Limestone	0.20
Di calcium phosphate	0.11
Salt	0.17
Lysine	0.11
DL-Methionine	0.05
Chick premix [1]	1.25
Calculated values[2]	
ME (kcal/kg)	3178.00
CP %	22.61
Lysine %	1.40
Methionine %	0.43
Calcium %	1.24
Available phosphate %	0.56

[1]The chick premix (MINSAL P330 Chick, Derbyshire, England) provided the following per kilogram of diet: 800000 IU of vitamin A, 240000 IU of vitamin D3, 2581 mg of Iron, 126 mg of Iodine, 40 mg of Cobalt, 1600 mg of Copper, 10322 mg of Manganese, 6667 mg of Zinc and 44.5 mg of Selenium.

[2]Food requirements were estimated according to (NRC, 1994).

Table 3.2: The nutritional information of Frutafit® HD commercial inulin and Jerusalem artichoke inulin which was used as a prebiotic sources.

Items	Frutafit® HD/100 g[1]	Jerusalem Artichoke /100 g[2]
Carbohydrates (g)	97	81.02
Digestible (Sugars) (g)	7	6.54
Non-digestible (Inulin) (g)	90	74.48
Proteins (g)	0	7.43
Fats (g)	0	0.40
Dietary fibres (g)	90	74.48
Moisture (g)	3	5.56
Gross energy (kcal/g)	2	0.4
Minerals - Ash (g)	--	5.59
Sodium (mg)	40	--
Calcium (mg)	11.5	--
Potassium (mg)	7.5	--
Iron (mg)	0.4	--

[1] The chemical composition recommended by the company.

[2] The chemical composition were analysed in the lab nutrition-Plymouth University.

3.2.4 Measurement of pH value of the digestive tract

Digesta from the ileum and caeca were tested according to the method of (Baurhoo *et al*., 2007). The samples were diluted with distilled water (1:10), and the pH was measured using a glass electrode pH meter (European Instrument, Oxford, Germany).

3.2.5 Short-Chain fatty acids and lactic acid analysis

Frozen samples from caecal digesta were measured for SCFA and lactic acid concentration by High Performance Liquid Chromatography (HPLC) according to the method of Niven *et al*. (2004) with some modifications in sample preparation. About 0.5±0.01 g of the digesta of caeca samples was dispersed in 1 mL of Milli-Q water. Then, the Samples were mixed for 30 seconds using a vortex mixer and then centrifuged at 17000 xg for 20 min (VWR MICRO STAR 17, Laboratory centrifuge, Germany). Concentration of acetic, propionic, butyric and lactic acid in the samples was determined following analysis of external calibration standards. A Dionex Ultimate 3000 with UV detector (220 nm) and agilent PL Hi-Plex H, 300 mm × 7.7 mm was utilized for the separation with a PL Hi-Plex H Guard Column 50 × 7.8 mm of the same phase. The column (Agilent Technology, USA) was maintained at 25°C. The eluent, 5mmol sulphuric acid, was pumped through the column at a flow rate of 0.5 mL/min.

To each sample 20µL of 7% (v/v) sulphuric acid was added to denature dissolved proteins and shift the acid dissociation equilibrium towards complete protonation of fatty acids. Samples were mixed for 30 seconds using a vortex mixer and centrifuged at 13000 rpm for 10 minutes. The supernatant was extracted using 1ml polypropylene disposable syringe (Fisher Scientific, BD A-Line, UK) and filtered through 0.2µm syringe filters (SMI-LabHut Ltd, Gloucester, UK) to remove any particulate material still present into vials and sealed with crimp cap (11mm, Ruber/PTFE, Fisher Scientific, Loughborough, UK) and stored at −80°C until needed for analysis.

All data obtained were processed using Chromeleon® 7.1 Chromatography Data System Software (Dionex Softron GmbH, Germering, Germany). A calibration

curve for each (lactic acid, propionic acid, butyric acid and acetic acid) was obtained from six different concentrations (0.2 µmol, 2 µmol, 20 µmol, 200 µmol, 2 mmol and 20 mmol) of the standards' stock solutions.

3.2.6 Conventional culture-based techniques

At 14, 18 and 21 d of age, three broilers was selected from each treatment and caecal digesta were aseptically collected to investigate the intestinal microorganisms. The caecum was removed from the carcass under sterile conditions, and immediately transported to the laboratory, Plymouth University, Microbiology lab. One hundred milligram of each caecum contents was mixed with 0.9 ml of sterile PBS (pH 7.0) and vortexed for 1 min to homogenize. The homogenate was diluted serially from an initial 10^{-1} dilution to 10^{-9}. For each dilution 0.1 ml was subsequently plated onto sterile selective medium agar for enumeration of target bacteria groups as following Columbia agar (Sigma-Aldrich, UK) with 5% sheep's blood (Oxoid, England) for total anaerobic and aerobic bacteria, MRS agar for *Lactobacillus* spp., Liver veal agar (DIFCO, USA) for *Bifidobacterium* spp., MacConkey agar (Sigma-Aldrich, UK) for total coliform and XLD agar (Oxoid, England) for *Salmonella* spp.

All anaerobic media were incubated in an anaerobic jar with Anarogen (Fisher, England). Columbia agar was incubated in anaerobic cabinet for total anaerobic bacteria and incubated aerobically for total aerobic bacteria at 37 °C for 48 hours. MRS and Liver veal agar medium were anaerobically at 37 °C for 48 hours. MacConkey agar and XLD agar medium were incubated aerobically at 37 °C for 72 hours. The numbers of colonies were then counted to determine the colony

forming units (CFU) using a Colony Counter (Gallenkamp, UK). CFU per gram of fresh caecal digesta were then expressed as logarithms.

3.2.7 Molecular microbial techniques

Three chicks per treatment at 14 and 21 d of age were selected and killed by cervical dislocation. The intestine and cecum were removed and treated as described by Zhu *et al*., (2002). The contents of caecum digesta were put into a sterile 1.5 mL Eppendorf tube. The samples were stored at −20°C until DNA extraction. All molecular work and protocols were carried out in a Labcaire PCR workstation (Labcaire System Ltd, Clevedon, UK).

3.2.7.1 Bacterial DNA extraction and PCR

A combination of the QIAamp stool mini kit (QUIAGEN, West Sussex, UK) and phenol-chloroform method was used for DNA extraction with some modification to the manufacturer's instruction. Two hundred milligram of sample was prepared in a sterilized Eppendorf tube, and DNA extracted by the following five stages:

1- **Lysis stage:** 200 mg of samples were mixed with 500 µl of fresh lysozyme solution (50 mg/ml TE buffer). Then, the samples were incubated at 37 ºC for 30 minutes. 700 µl of buffer ASL was added and mixed for 1 minute. The mixture was placed on a hot plate at 90 ºC for 10 minutes and vortexed for 5 seconds with centrifugation for 1 min at 14000 xg.
2- **Inhibitor removal stage:** Half an inhibitor tablet was added to 800 µl of the supernatant and vortexed for 1 min immediately, then, centrifuged for 3 min at

14000 xg. All of supernatant was pipette into a new Eppendorf tube. The supernatant was centrifuged for other 3 minutes.

3- **Protein removal:** 400 µl of the supernatant was mixed with 20 µl of proteinase K and 400 µl of buffer AL was added and mixed for 15 seconds, then incubated at 70 ºC for one hour.

4- **Phenol Chloroform Clean-up:** The entire samples were poured into a 15 ml falcon tubes carefully, and added an equal volume of ice cold Tris-buffered phenol solution. The samples were mixed by hand and left on ice for 10 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed, then centrifuged for 5 minutes at 6000 xg. The aqueous layer was pipette off carefully and placed in new 1.5 ml Eppendorf tube.

5- **Precipitation:** 400 µl of ice-cold isopropanol was added. The samples were vortexed and placed in -20 °C freezer for overnight. Then, samples were centrifuged at 14000 xg for 30 minutes at 4 °C. The supernatant were pipette carefully and discarded. 500 µl of 70% molecular grade ethanol was added slowly, and discarded. The addition of 70% ethanol was repeated and discarded again. The pellets were dried for 5 minutes maximum. Finally, the DNA extracted was resuspended overnight at 4 °C by adding 30 µl of molecular grade water. The concentration of DNA and purity were determined using a Nanodrop-1000 Spectrophotometer.

The DNA concentration (ng /µl) in the sample was determined by using Nanodrop® ND-1000 a spectrophotometer at a wavelength 230 nm. DNA in grade water was used as a blank to re zero the device. The DNA was measured and the

average bacterial DNA >20 ng/ µl are good. Protein purity (A260/A280) and Humic acid purity (A260/ A230) >1.7 are good.

PCR amplification of the V3 region of 16S rRNA genes was undertaken with the reverse primer P2 (5'- ATT ACC GCG GCT GG-3') and the forward primer P3 with a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG GCC TAC GGG AGG CAG CAG-3'). These primers correspond to position 341 – 534 in the 16S rRNA of *E. coli* which produces a fragment of 193 base pair. Each PCR tube contained 1µl of primer P2 and P3 (50 pmol/µl, Eurofins MWG Operon, Germany), 3 µl DNA template, 25 µl of Ready Mix Taq DNA polymerase and were made up 50 µl with 20 µl of PCR grade water. The PCR thermal cycling was conducted under the following conditions: 94ºC for 10 min, then 30 cycles starting at 94ºC for 1 min, 65 ºC for 2 min, 72 ºC for 3 min. The annealing temperature decreased by 1 ºC every second cycle until 55 ºC and then remained at 55 ºC for the remaining cycles.

Eight microliter of the PCR products were then separated by electrophoresis on a 1.5% Agarose gel (Lonza, Rockland ME, USA). A mixture of 1.35 g of agarose powder and 90 ml of 1x TEA buffer (Tris/ EDTA/Acid) was dissolved in microwave for 1 min with mixing. Eight µl of PCR product was loaded in the wells of the gel with 4 µl of loading buffer. Eleventh µl of the 100bp DNA ladder (Fisher, UK) was used to assess the size of DNA products. The gel was run at 90 volts for 45 min and the bands were visualised with UV and photographed using Gray scale digital camera CFW-1312M (Tokyo, Japan) in the Universal Hood II, BIO-RAD Laboratories (Milan, Italy).

3.2.7.2 Denaturing gradient gel electrophoresis (DGGE)

The PCR products of the same length, but with different internal sequences, can be separated by DGGE, according to their melting properties. The DGGE was made using a DGGE-2001 system (CBS scientific, USA). Fifteen µl of PCR products were run on acrylamide gels (16cmX16cmX1mm) with a denaturing gradient of 40-60% (where 100% denaturing are 7M urea and 40% formamide). Loading buffer with 200 µl of green stain was added to the high gel solution (60%). One hundred µl of ammonium per sulphate (APS) was added to the high and low gel solutions. 50 µl of tetramethylethylindiamine (TEMED) was added to the gels and 16 ml of both gel solutions were added gradually using a Bio–Rad gradient delivery system (model 475) and a comb (30 wells) was inserted and gels were left for 20 min to completely set. All samples were run on the same gel to prevent issues of non-reproducibility. The outside lanes were not used. The gel was run at 60V for 16 hr at 60 ºC in 1x TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA). Visualizing of the DGGE band was achieved by high sensitivity and optimized gold staining method. The gel was soaked and incubated in fixation buffer 200ml 1x TAE containing 20 µl gold CYBER safe DNA stain (Invitrogen™, UK) for 25-30 minutes on an IKAO VIBRAX VXR basic shaking platform at 100 rpm/ min, at room temperature and scanned in a Bio-Rad Gel-Doc system and optimized for analysis of UV light. All the samples were triplicates per treatment. Identification of bacteria by sequencing PCR-DGGE fragments. DNA fragments of interest were excised aseptically from the polyacrylamide gel using sterile pipette tips, under the UV light, placed in 20 µl DNA grade water and incubated overnight at 4°C to allow elution of the DNA.

Five µl of eluted DNA was added to a master mix which included 12.5 µl of Ready Mix Taq polymerase, 1 µl of primer 2, 1 µl of primer 1 with no GC clamp and 10.5 µl of molecular grade water to make up 30 µl for re-PCR products. The mixture was run using the same program as PCR-DGGE. The PCR products were cleaned after checking the concentration of PCR product by using QIAquick PCR purification kit (QIAGEN, USA) to clean PCR product according to manufacturer's instructions. Briefly, 100 µl of Buffer PB was added to 20 µl of the PCR product and mixed. QIAquick spin column were placed in a provided 2 ml collection tubes. The samples were added to the QIAquick columns and centrifuged for 30–60 sec., to bind the DNA, then, flow-through was discarded and the QIAquick column was placed back into the same tube. 750 µl of buffer PE was added to the QIAquick column and centrifuge for 30-60 sec. to wash the samples. Flow-through was discarded and placed the QIAquick column back into the same tube, then, the column was centrifuged for an additional 1 min. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. 30 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane and centrifuged for 1 min to elute DNA, then, the DNA was stored in fridge at 4 °C overnight. The concentration of DNA was determined by using gel electrophoresis. Only 5 µl of diluted to 20-80 ng/µl of PCR product of C4 strain and 5 µl of one of the primers (5 pmol/ µl) in Eppendorf tube together was sent for sequencing centre of GATC biotechnology in Germany and the sequencing results send via their website: http://www.gatc-biotech.com/en/index.html. Sequence was compared to those in available databases by use of the BLAST (Basic Local Alignment Search Tool) in Gene Bank network services at http://blast.ncbi.nlm.nih.gov/Blast.cgi to determine its

approximate phylogenetic relationships. The major steps of DGGE are presented in Figure 3.3.

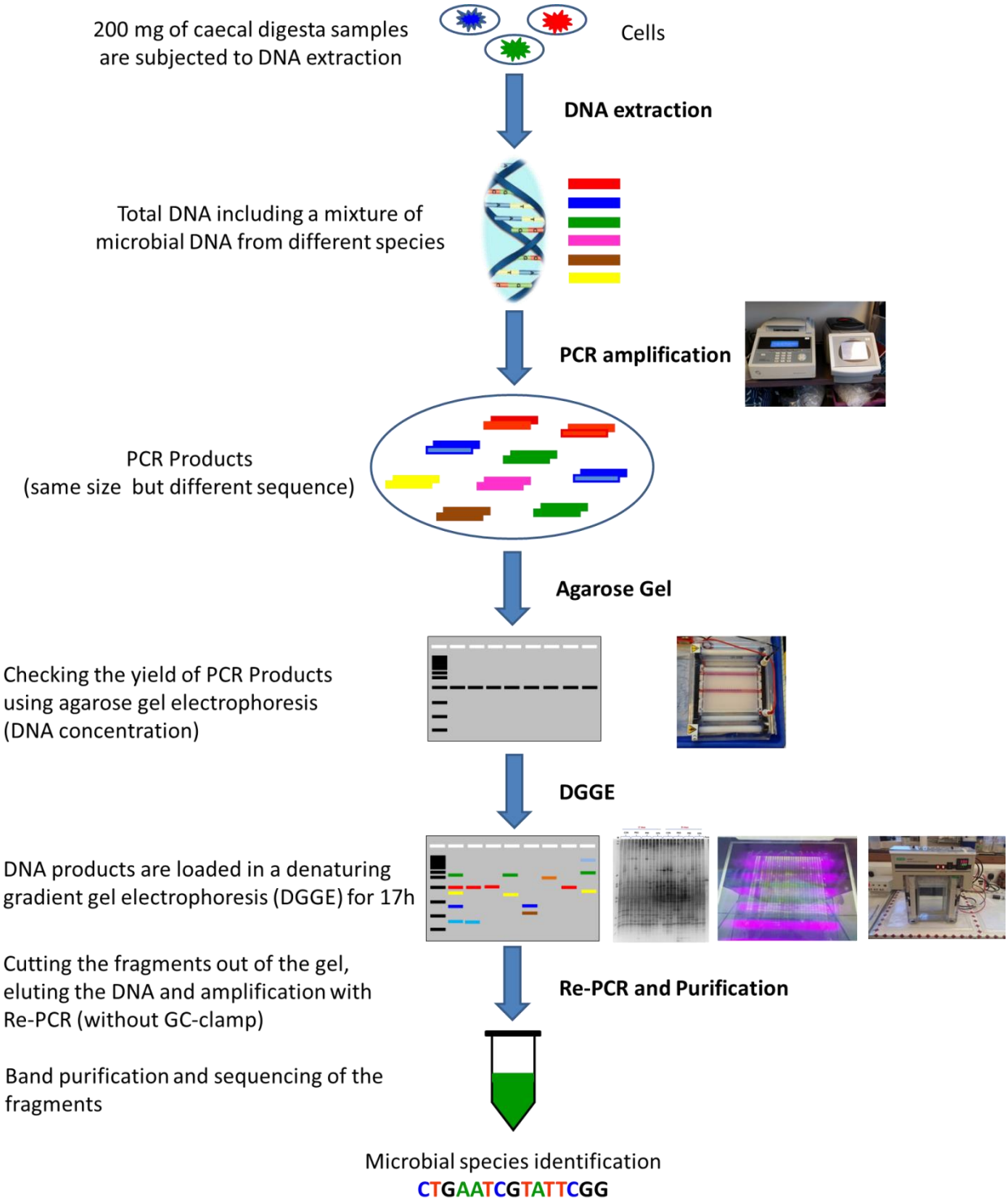


Figure 3.3: Schematic representation of the principal steps of the denaturation gradient gel electrophoresis (DGGE) process of PCR amplified DNA.

3.2.8 Gut Histology

On 14 and 21 days, three chicks from each treatment were randomly selected and killed by cervical dislocation. The samples for histology were taken from the jejunum at the junction of the Meckel's diverticulum and washed with PBS buffer (pH 7.0), and then fixed in 10% neutral buffer formalin. The samples were dehydrated by inundation with methanol 50%, 70%, 90%, 100% and 100% in an automatic tissue processor Leica TP1020 for 21 h (Leica, Germany), then embedded in fresh paraffin wax using a Leica EG1150H (Leica, Germany) to obtain a solid block containing the tissues for sectioning. The samples placed in wax small blocks (5 X 3 X 3 cm) and sectioned using a Leica RM2235 microtome type (Leica, Germany), serial sections were cut at a thickness of five µm. Sections were separated on water bath at 50 °C, for two minutes. The samples were placed on a microscope slide and left to dry. Slides containing the samples were placed in a slide holder and put it in an autostainer Leica XL (Leica, Germany) with Haematoxylin and Eosin (HE). Haematoxylin has a blue colour and stains the nucleic acids (nucleus). Eosin is pink colour and stains protein in cytoplasm and extracellular matrix. The samples were dried and covered with cover slide using DPX. The stained slides were examined under light microscope and photographed at 10X magnification by an Olympus Vanox-T microscope with digital camera mounted (E-620). The images were measured by Image J software. The aim of histology was to measure the villus height and crypt depth (µm) of jejunum at different age. Villus height was represented by the distance from the crypt opening to the tip of the villus, whereas crypt depth was determined from the base of the crypt to the level of the opening (Pelicano *et al*., 2005; Baurhoo *et al*., 2007).

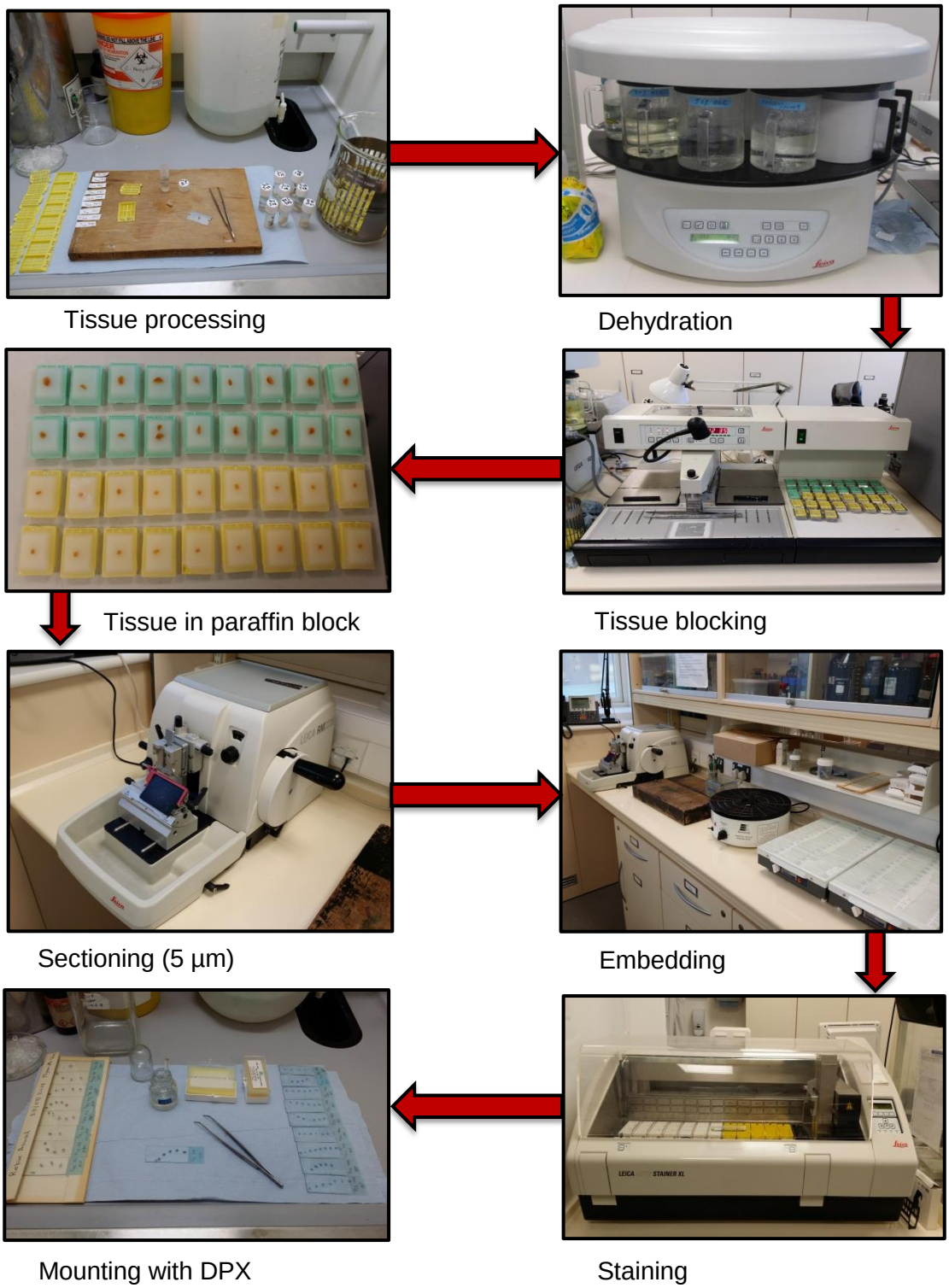


Figure 3.4: Major steps of the tissues sectioning for histological studies using different instruments.

3.2.9 Histology of Bursa of Fabricius

At days 14 and 21, three chicks from each treatment were randomly selected and killed by cervical dislocation. The bursa of Fabricius was taken and washed with PBS buffer (pH 7.0) then fixed in 10% neutral buffer formalin. The same procedure conducted when applied for gut histology. The diameter of sixty Bursa follicles were measured by Image J software per treatment, and the average of these values were used. Also, the body weight (g) and bursa weight (g) were recorded for each individual bird to determine the relative weight of the Bursa of Fabricius according to the following equation.

$$\textbf{Relative weight of the bursa} = \frac{\text{Bursa weight (g)}}{\text{Live body weight (g)}} \times 100$$

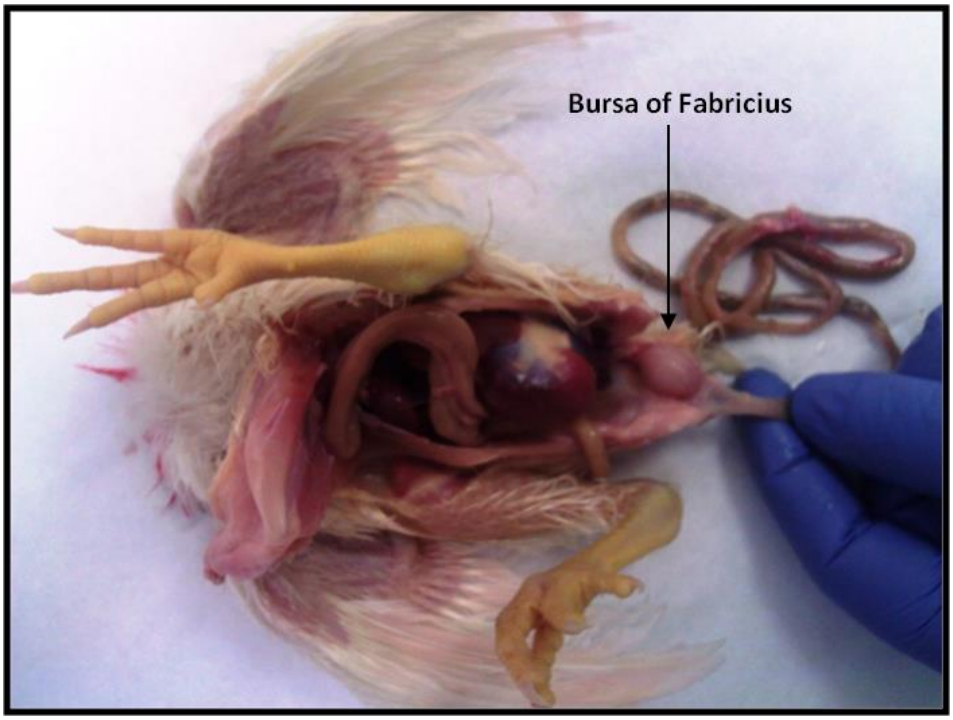


Figure 3.5: The bursa of Fabricius in chickens.

3.2.10 Haematology

The blood samples were collected from the wing vein or directly from the heart after killing the chicks of three birds per treatment at 14 and 21 days of age. The blood samples were collected by using one ml syringe and 23 gauge needles in test tubes with anticoagulant Di-Potassium ethylene diamine tetra acetic acid (K_2EDTA). Blood smears were made by dropping 5µl of fresh whole blood onto a glass slide; the end of the second slide was placed against the surface of the slide with the blood drop, at an angle of 45°. By drawing the “spreader slide” up against the drop of blood, it spread across the end of the slide by capillary attraction and filled the angle between the two slides. The “spreader slide” was then pushed back along the other slide (Dacie & Lewis, 1995). The prepared smears were left to dry at room temperature. Slides were stained using May Grunwald Giemsa stain. Slides were fixed in Methanol for 15 minutes after that slides were put in May Grunwald Sorensens Buffer solution 1:1 for 5 minutes then rinsed in Sorensens Buffer (pH 6.8) three times. Then slides were put in working solution 1 part of Giemsa stain and 9 part of Sorensens Buffer (pH 6.8) for 10 minutes then rinsed in Sorensens Buffer three times. Slides were allowed to dry at room temperature. Counting was accomplished by observing the slides under the light microscope (Olympus Vanox-T microscope) using oil immersion at a final magnification of x1000. To determine the counts of heterophil and lymphocyte, a minimum of 100 cells per sample were examined by light microscopy. All blood counts were examined by the same investigator. The results are presented as the percentage of each cell occurring in each sample. The H/L ratio was examined by dividing the number of heterophils by the number of lymphocytes (Gross and

Siegel, 1983). Photographs of slides were also taken using an Olympus Vanox-T microscope with digital camera mounted (E-620) at a total magnification of x1000.

3.3 Statistical analysis

Data obtained were statistically analysed using one way analysis of variance (ANOVA) Minitab statistics software version 16.0 Statistical analyses (Minitab, Plymouth, UK). The one-way ANOVA test (Tukey's Multiple Comparison test) was used to determine significant differences at 0.05 levels among the different parameters. Data are presented as mean ± standard deviation (SD). Comparisons of intestinal microbial communities DGGE between treatment diets were done using software package the Plymouth Routines In Multivariate Ecological Research (PRIMER 6, Primer-E Ltd, Plymouth Marine Laboratory, Plymouth, UK). Cluster analysis was used to check the observed groupings, and half matrix similarity analysis was also displayed as a measure of the similarity of replicates within and between treatments. The species richness and the microbial diversity were determined by using Margalef index and Shannon index, respectively. All data were tested by a normality test.

3.4 Results

3.4.1 Feed composition

The chemical analysis of the diets after adding inulin is presented in Table 3.3. No significant differences were found in T2 and T3 compared to T1 for moisture, lipid, ash, protein contents and gross energy level of the different experimental diet.

Table 3.3: Chemical composition analysed of each broiler diet samples.

Items	Treatments[1]		
	T1[2]	T2	T3
Dry matter %	88.65±0.04	88.54±0.09	88.69±0.09
Moisture (%)	11.34±0.04	11.44±0.09	11.30±0.09
Protein (%)	22.36±0.13	22.68±0.26	21.77±0.44
Lipid (%)	8.66±0.14	8.51±0.17	8.46±0.19
Ash (%)	4.70±0.05	4.60±0.01	4.66±0.04
NFE (%)[3]	52.91±0.33	52.71±0.07	53.81±0.63
Gross energy (MJ per kg)	17.84±0.04	17.78±0.09	17.60±0.05

[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[2] Results are mean values from three replications ± standard deviations.
[3] Nitrogen-free extracts (NFE) = 100 – (moisture + crude protein + crude lipid + ash).

3.4.2 Live body weight

Live body weight are presented in Table 3.4. Chicken weight increased by 6.47% and 8.19% for T2 and T3, respectively compared with control group at the end of experiment. There were no significant differences among all treatments. While, mathematically LBW was improved in both types of inulin treatments compared with control treatment.

Table 3.4: Effect of commercial and JA inulin on weekly live body weight of SPF Leghorn chicks during the experiment.

Time (Weeks)	Treatments[1]			P. values
	T1	T2	T3	
0	38.82±1.07 [a]	38.05±1.17 [a]	39.24±0.96 [a]	0.07
1	80.81±8.41 [a]	86.48±8.58 [a]	83.94±8.68 [a]	0.38
2	139.95±10.94 [a]	152.56±12.42 [a]	145.29±10.59 [a]	0.08
3	472.00±27.30 [a]	504.70±20.02 [a]	511.96±17.34 [a]	0.13

[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[a] Means with the same superscript in the same row are not significantly different ($P<0.05$).

3.4.3 The measurement of pH value

The pH values of the ileal and caecal digesta of chicks are shown in Table 3.5. There were significant differences among treatment groups. The dietary inulin supplementation 1% from T2 and T3 significantly improved ($P<0.05$) the level of pH (7.30 and 7.31 in ileum and 5.77 and 5.89 in caecum digesta, respectively) compared with the control treatment (7.47 and 6.21 in ileal and caecal digesta, respectively). However, there were no significant differences observed between the dietary inulin supplementation in both part of GIT.

Table 3.5: Effect of dietary inulin supplementation on the pH value of ileum and caecum contents of chicks at 21 days of age.

Treatment[1]	pH value	
	Ileum	Caecum
T1	7.47±0.08 [a]	6.21±0.10 [a]
T2	7.30±0.00 [b]	5.77±0.13 [b]
T3	7.31±0.01 [b]	5.89±0.06 [b]
P. values	<0.001	<0.001

1 T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[a,b] Means in the same column with different superscripts are significantly different ($P < 0.05$).

3.4.4 Changes in the Short-Chain Fatty Acids and Lactate Concentrations

The concentrations of short-chain fatty acids and lactate, at the end of experiments are presented in Tables 3.6. The concentration of lactic acid significantly (P< 0.05) increased in inulin from Jerusalem artichoke compared with control and Frutafit groups, but there is no difference observed between control and Frutafit group. The concentration of butyrate and propionic acids significantly (P< 0.01) increased in both types of inulin compared with the control group. While, there is no significant differences observed among all treatments for acetic acid at the end of the experiment.

Table 3.6: Concentration (mmol/L) of short-chain fatty acids in caecal digesta at the end of the experiment[1] (d 21).

SCFA	Treatment[2]			P. value
	T1	T2	T3	
Lactic acid	0.20±0.03 [b]	0.14±0.251 [b]	0.47±0.01 [a]	<0.001
Acetic acid	9.12±1.81 [a]	10.27±1.38 [a]	13.11±2.11 [a]	0.082
Propionic acid	0.98±0.16 [b]	1.77±0.28 [a]	2.27±0.43 [a]	0.007
Butyric acid	2.25±0.36 [b]	10.29±1.54 [a]	14.41±2.83 [a]	0.001

[1] Values are means ± SD of triplicate determination.
[2] T1: control, T2: Frutafit inulin (1%), T3 Inulin from JA (1%).
[a,b] Means within same row with different superscripts are significantly different (P<0.05).

3.4.5 Microbial enumeration of the caecum

Table 3.7 shows the effects of both types of dietary inulin supplementation on the microflora in the caecal digesta of SPF chicks at 14, 18 and 21d of age. The results showed that there were no statistically significant differences between all treatments for total aerobics, Lactobacilli, Bifidobacteria and coliform. While, the numbers of total anaerobic bacteria significantly (P<0.05) increased in the caecal digesta of chicks 14 days age compared with control group.

At 18 and 21 days, the contents of beneficial intestinal bacteria (Total anaerobic, Lactobacilli and Bifidobacteria) were changed and higher in both types of dietary inulin supplementation compared to the control group. While, there were no significant differences between both types of inulin among all contents of microorganisms in the caecum digesta at 14, 18 and 21 days of broiler's age. No *salmonella* were detected in all treatment at different days of age.

Table 3.7: Bacterial counts (Log_{10} CFU mL^{-1}) at 14, 18 and 21 days of age in caecal digesta of SPF chicks.

Time (Days)	Microbes	Treatments		
		T1	T2	T3
14	Total anaerobic	8.22 ± 0.07^{bB}	9.29 ± 0.07^{aA}	9.36 ± 0.06^{aA}
	Total aerobic	8.14 ± 0.05^{aA}	7.93 ± 0.24^{aA}	8.02 ± 0.04^{aA}
	Lactobacillus spp.	8.15 ± 0.12^{aA}	8.64 ± 0.42^{aB}	8.78 ± 0.37^{aA}
	Bifidobacterium spp.	8.37 ± 0.05^{aB}	8.43 ± 0.02^{aC}	8.44 ± 0.01^{aC}
	Total Coliform	7.75 ± 0.18^{aA}	7.62 ± 0.08^{aA}	7.69 ± 0.16^{aA}
	Salmonella	n.d.	n.d.	n.d.
18	Total anaerobic	8.83 ± 0.02^{bA}	9.27 ± 0.11^{aA}	9.19 ± 0.15^{aA}
	Total aerobic	7.89 ± 0.06^{aB}	7.78 ± 0.13^{aA}	7.77 ± 0.09^{aA}
	Lactobacillus spp.	8.13 ± 0.18^{bA}	9.27 ± 0.07^{aAB}	9.24 ± 0.07^{aA}
	Bifidobacterium spp.	8.69 ± 0.15^{bA}	9.10 ± 0.08^{aB}	9.04 ± 0.07^{aB}
	Total Coliform	7.66 ± 0.10^{aA}	7.58 ± 0.03^{aA}	7.60 ± 0.08^{aA}
	Salmonella	n.d.	n.d.	n.d.
21	Total anaerobic	8.90 ± 0.05^{bA}	9.30 ± 0.09^{aA}	9.26 ± 0.08^{aA}
	Total aerobic	7.94 ± 0.05^{aB}	7.74 ± 0.11^{aA}	7.81 ± 0.19^{aA}
	Lactobacillus spp.	8.09 ± 0.08^{bA}	9.29 ± 0.10^{aA}	9.22 ± 0.13^{aA}
	Bifidobacterium spp.	8.72 ± 0.08^{bA}	9.34 ± 0.09^{aA}	9.36 ± 0.09^{aA}
	Total Coliform	7.72 ± 0.17^{aA}	7.67 ± 0.17^{aA}	7.64 ± 0.15^{aA}
	Salmonella	n.d.	n.d.	n.d.

[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%). [a,b] Means in the same row and age with different superscripts are significantly different ($P < 0.05$). [A, B, C] Means in the same column and treatment with different age with different superscripts are significantly different ($P < 0.05$).

n.d.: Not detected.

3.4.6 PCR-DGGE of caecum digesta

The spectrophotometric assay showed that all the results of DNA concentrations in caeca samples were more than 80 ng /µl. The protein contamination of 260/280 was higher than 1.7 as well as the humic acid of 260/230.

Figure 3.6 shows the PCR–DGGE bacterial profiles of the digesta from the caecum of chickens at 14 (A) and 21 (B) days of age. Many different bands are shown in the DGGE image and the gel bands which are called operative taxonomy units (OTU) in each sample.

The similarity of bacterial population within and between the treatments were measured by nonmetric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 3.7.

Both analyses of caecal bacteria populations showed more similarity within samples from same treatments than those from other groups. The half matrix similarity of caeca DGGE fingerprints is shown in Table 3.8 indicates the average similarity within the control treatment is 60.73% at day 14 and 61.98% in day 21, commercial inulin 48.76% at day 14 and 57.32% at day 21, inulin from Jerusalem artichoke 69.24% at day 14 and 63.04% at day 21. The average bacterial population similarity between control groups at day 14 and 21 was 61.35%, while the commercial inulin was 53.04% and inulin from JA was 66.14%.

The richness of microbiota increased with bird age and added inulin from JA source to the diet. There were 32 DNA bands detectable in the samples of inulin from JA of 14 days old broiler chicks and the number of DNA bands was

increased to 36 when the chicks became 21 days old. While the average bands of commercial inulin and control groups were decreased (Table 3.9).

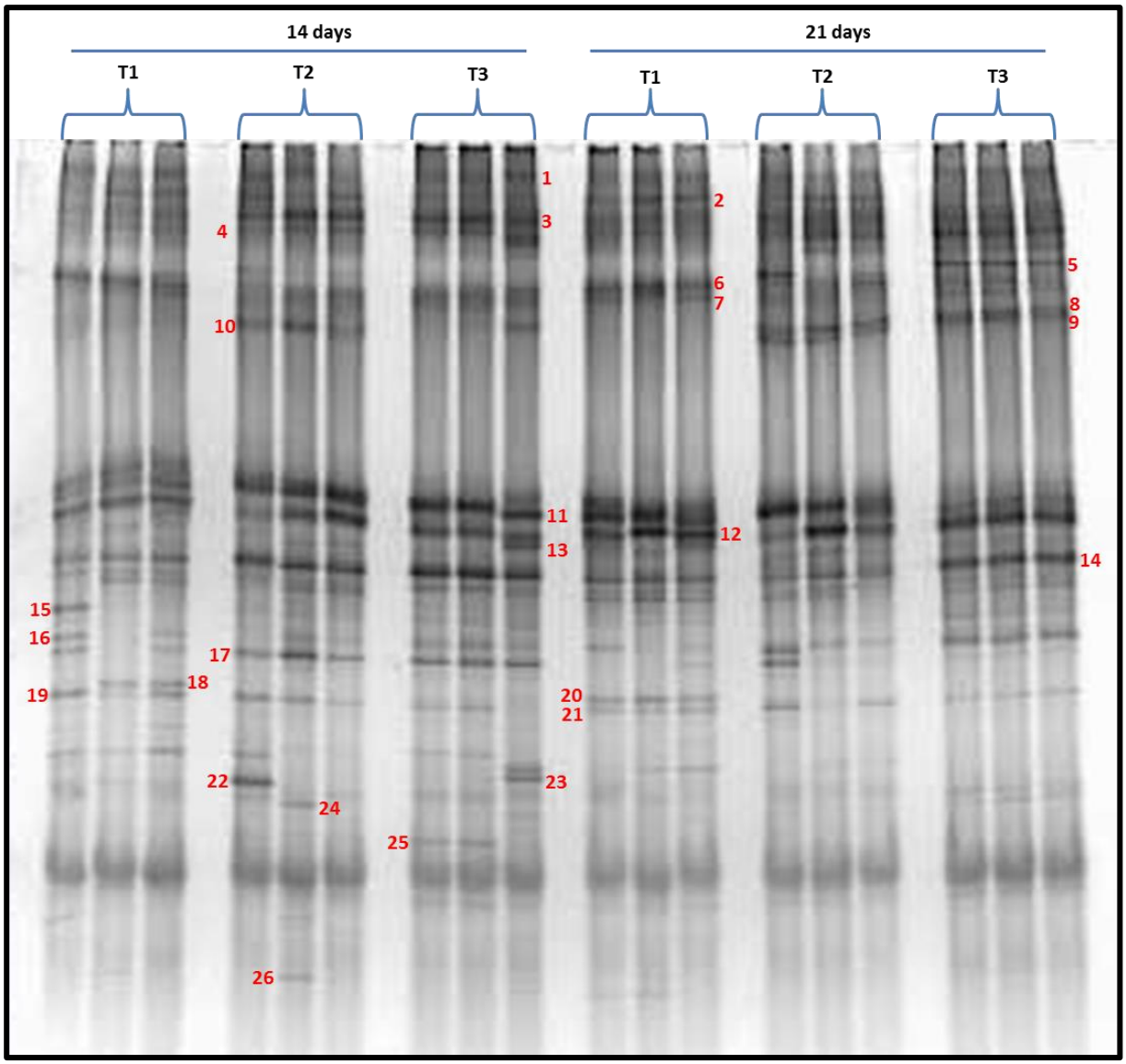
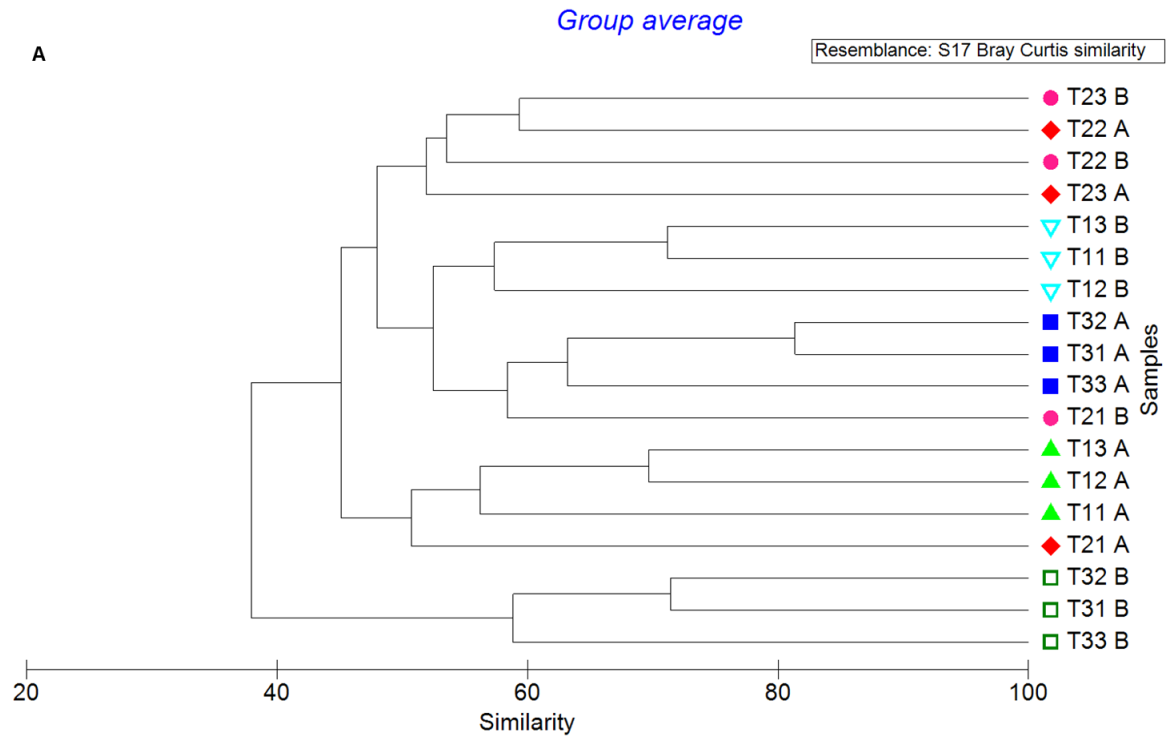


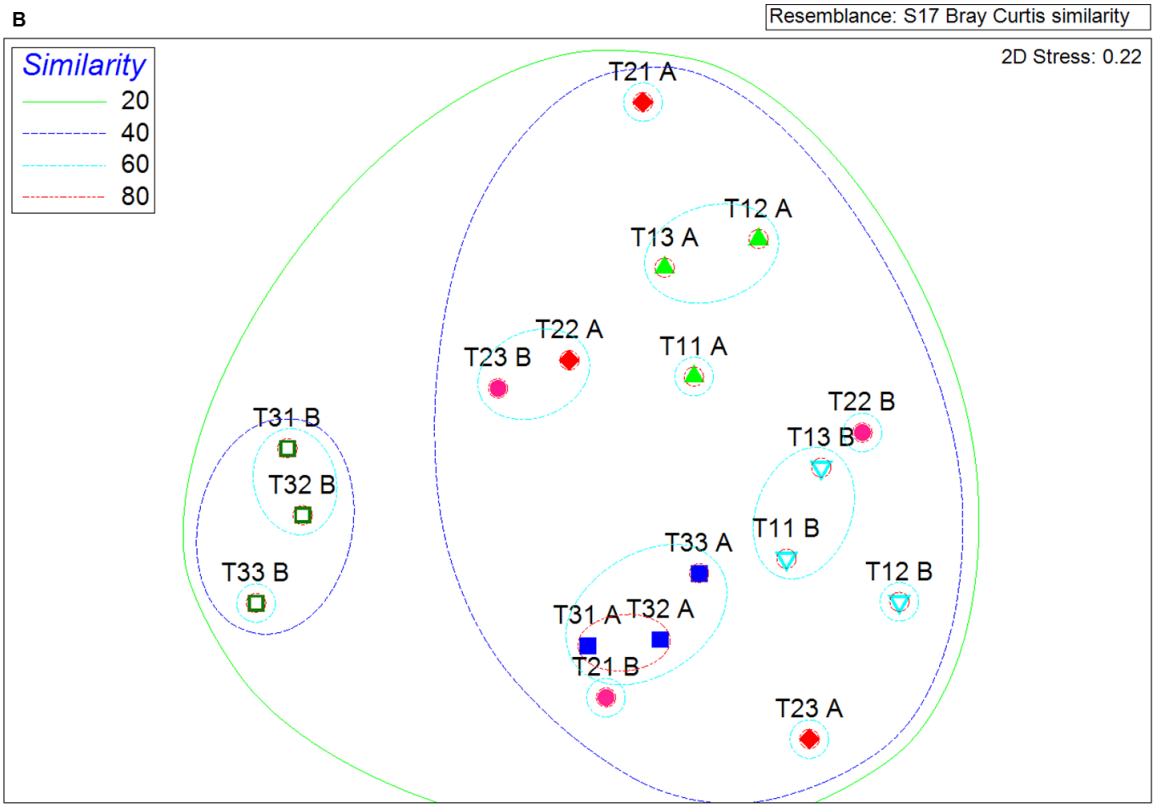
Figure 3.6: DGGE fingerprints of caecum digesta of treated and control group chicks at 14 and 21 days of age. Numbers represent the bands or operative taxonomy unites (OUT) excised and sequenced. T1: control, T2: commercial inulin (Frutafit) and T3: inulin from Jerusalem artichoke.

Table 3.8: The half matrix similarity of bacterial population of DGGE fingerprints of caeca showing the similarities between the replicates treatment.

Group	T11 A*	T12 A	T13 A	T21 A	T22 A	T23 A	T31 A	T32 A	T33 A	T11 B	T12 B	T13 B	T21 B	T22 B	T23 B	T31 B	T32 B	T33 B
T11 A	100																	
T12 A	50	100																
T13 A	62.5	69.70	100															
T21 A	44.44	49.23	58.46	100														
T22 A	50.75	52.17	55.07	50.00	100													
T23 A	42.62	38.10	34.92	38.71	57.58	100												
T31 A	45.90	38.10	44.44	38.71	51.52	46.67	100											
T32 A	50.00	51.61	45.16	36.07	52.31	47.46	81.36	100										
T33 A	52.94	48.57	51.43	43.48	54.79	53.73	59.70	66.67	100									
T11 B	60.00	41.94	51.61	32.79	43.08	54.24	54.24	55.17	60.61	100								
T12 B	45.45	50.00	41.18	32.84	42.25	52.31	46.15	46.88	50.00	59.38	100							
T13 B	49.18	47.62	50.79	41.94	39.39	43.33	50.00	57.63	47.76	71.19	55.38	100						
T21 B	51.61	37.50	37.50	38.10	41.79	45.90	62.30	60.00	52.94	56.67	45.45	59.02	100					
T22 B	39.34	47.62	47.62	45.16	48.48	53.33	46.67	47.46	41.79	50.85	46.15	60.00	55.74	100				
T23 B	54.24	32.79	52.46	46.67	59.38	44.83	51.72	42.11	43.08	45.61	38.10	48.28	57.63	58.62	100			
T31 B	43.08	38.81	41.79	36.36	42.86	34.38	40.63	34.92	45.07	44.44	31.88	37.50	40.00	31.25	41.94	100		
T32 B	29.85	40.58	40.58	35.29	41.67	33.33	48.48	40.00	30.14	36.92	33.80	45.45	44.78	39.39	46.88	71.43	100	
T33 B	28.99	47.89	39.44	28.57	32.43	29.41	44.12	38.81	32.00	32.84	38.36	35.29	40.58	29.41	39.39	55.56	62.16	100

Note: T1 = control, T2 = commercial inulin and T3= Inulin from JA. 1-3 replicate number in each treatment. (n=18). * 14 (A) and 21 (B) days of age.


A
Group average
Resemblance: S17 Bray Curtis similarity
T23 B
T22 A
T22 B
T23 A
T13 B
T11 B
T12 B
T32 A
T31 A
T33 A
T21 B
T13 A
T12 A
T11 A
T21 A
T32 B
T31 B
T33 B
Samples
20
40
60
80
100
Similarity


B
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.22
Similarity
20
40
60
80
T21 A
T12 A
T13 A
T22 A
T23 B
T11 A
T22 B
T13 B
T31 B
T32 B
T11 B
T33 A
T33 B
T12 B
T31 A
T32 A
T21 B
T23 A

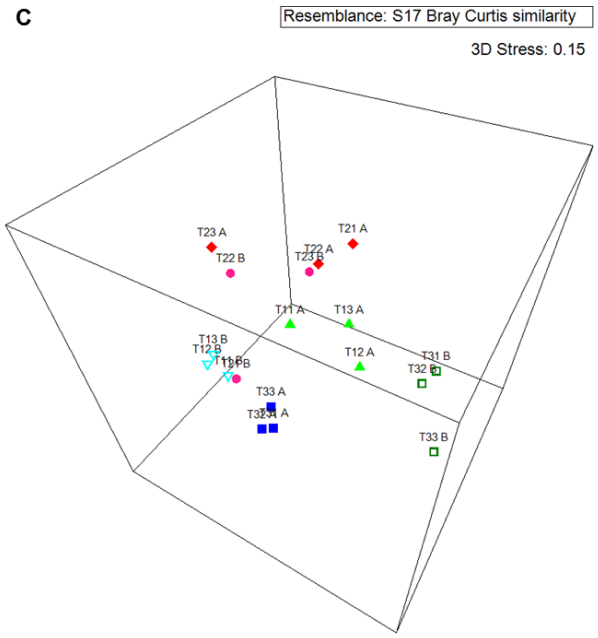


Figure 3.7: (A) Cluster analysis (B and C) non-metric multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in poultry caeca. T1= control, T2 = commercial inulin and T3= inulin from JA, 1-3 denotes replicate number in each sample. (n=18).

Table 3.9: Band numbers of bacterial community based on the PCR-DGGE DNA fingerprinting and similarity within treatments.

Time (Days)	Treatment[1]	Band number	Similarity
14	T1	32.33±1.15 [a]	60.73±9.96 [a]
	T2	32.66±3.05 [a]	48.76±9.50 [a]
	T3	32.00±4.35 [a]	69.24±11.05 [a]
	P. value	0.967	0.121
21	T1	31.33±3.21 [ab]	61.98±8.21 [a]
	T2	29.66±1.52 [b]	57.32±1.46 [a]
	T3	36.00±2.00 [a]	63.04±7.97 [a]
	P. value	0.039	0.567

[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[a] Means with the same superscript in the same column and age are not significantly different (P<0.05).

Diversity analysis of caecal microflora showed in table 3.10. The Shannon index and Margalef index indicate the diversity and richness of alimentary canal microflora of SPF chicks, respectively. These indexes were used to display the microbial population diversity and richness in the caeca. The diversity index of bacterial community based on the PCR-DGGE DNA fingerprinting indicated that; no significant differences in Shannon index and the Margalef index was observed in broilers fed with different diets at 14 d of age. At 21 d of age, birds fed the diets containing inulin from JA had greater Shannon index and Margalef index than birds fed commercial inulin and control groups. The Shannon index of inulin from JA group reached 3.58, which was obviously higher than control group and commercial inulin that 3.44 and 3.38 at 21 d of age. Therefore, the richness and diversity of inulin from JA group was distinctly higher than commercial inulin and control groups.

Table 3.10: Diversity index of bacterial community in caecal digesta based on the PCR-DGGE DNA fingerprinting at different day of age.

Time (Days)	Treatment[1]	Shannon index[2]	Margalef index[3]
14	T1	3.476±0.03 [a]	9.013±0.24 [a]
	T2	3.483±0.09 [a]	9.079±0.63 [a]
	T3	3.459±0.13 [a]	8.937±0.90 [a]
	P. value	0.952	0.965
21	T1	3.441±0.10 [ab]	8.801±0.66 [ab]
	T2	3.389±0.05 [b]	8.455±0.32 [b]
	T3	3.582±0.05 [a]	9.765±0.40 [a]
	P. value	0.041	0.040

[a,b] Means with the same superscript in the same column and age are not significantly different (P<0.05).
[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[2] Shannon diversity index: H‟ = -SUM(pi*Log(pi)).
[3] Margalef species richness: d = (S -1)／log (N).

The results of the trial sequence analysis shown in Table 3.11. The most family BLAST results in caecum were related to *Clostridium* spp., *Ruminococcus* spp. strains. For example, band numbers 8, 10, 16, 18, 19 and 26 related to *Clostridium* spp. and band numbers 2, 9, 12, 15 and 21 related to *Ruminococcus* spp. strains. Otherwise, band number 14 which was related to *Lb. crispatus* in all treatments, but had more density in both types of inulin compared with control groups at 14 and 21 days of age.

Table 3.11: Summary results of sequencing analysis bands of PCR-DGGE fingerprints of chicken caecum samples.

Band Number	NCBI Accession number	Max. Identity (%)	NCBI BLAST matches
1	GU412282.1	94	*Lachnospiraceae bacterium* oral taxon 419 clone DO097
2	NR_036777.1	98	*Ruminococcus torques* strain VPI B2-51
3	GQ493042.1	90	Uncultured bacterium clone PM1t2
4	NR_029097.1	97	*Oribacterium sinus* strain AIP 354.02
5	JN803476.1	82	Uncultured organism clone SRM
6	GU412296.1	98	*Lachnospiraceae bacterium* oral taxon 419 clone RA002
7	EU452782.1	97	Uncultured bacterium clone H80N1
8	NR_118730.1	97	*Clostridium symbiosum* strain ATCC 14940
9	NR_116747.1	99	*Ruminococcus faecis* strain Eg2
10	AB622849.1	97	*Clostridium* sp. Clone-49 gene
11	NR_029146.1	100	*Sedimentibacter hydroxybenzoicus* strain JW/Z-1
12	NR_036800.1	96	*Ruminococcus gnavus* strain ATCC 29149
13	GU102314.1	96	Uncultured bacterium clone BFV08
14	KC757156.1	100	*Lactobacillus crispatus* strain CLS01
15	NR_044265.1	100	*Ruminococcus gauvreauii* strain CCRI-16110
16	NR_119085.1	98	*Clostridium polysaccharolyticum* strain DSM 1801
17	NR_043551.1	100	*Lactonifactor longoviformis* strain ED-Mt61/PYG-s6
18	NR_025796.1	100	*Clostridium jejuense* strain HY-35-12
19	NR_075043.1	100	*Amphibacillus xylanus* NBRC 15112 strain NBRC 15112
20	EU311586.1	86	Uncultured alpha proteobacterium isolate DGGE gel band CHBn23
21	AJ318889.1	97	*Ruminococcus* sp. 16442
22	NR_026103.1	97	*Clostridium populeti* strain 743A
23	NR_044048.1	98	*Coprococcus comes* ATCC 27758 strain
24	JF667250.1	97	Uncultured bacterium clone GDIC2IK01AH6W8
25	NR_113319.1	96	*Anaerostipes butyraticus* strain JCM 17466
26	AB702931.1	99	*Clostridiales bacterium* CIEAF 017 gene

3.4.7 Histology of Jejunum

Table 3.12 showed that treatments had highly significant (P<0.01) effects on villus length of jejunum at 14 and 21 days old SPF chicks. The both types of inulin significantly (P<0.01) increased the villus length compared to the control treatment. While there were no significant differences between T2 and T3 in the length of villi. Figures 3.8 and 3.9 illustrated that clearly the differences between the diet inulin supplementation with control treatment at days 14 and 21, respectively. In comparison with the control basal diet, the inclusion of both inulin had no effect (P = 0.35) on crypt depth, at 14 days of age. While significantly increased (P = 0.005) crypt depth in T3 which received the diet containing 10 g of inulin/kg from JA on T1 and T2.

Table 3.12: Effects of dietary inulin supplementation on villus height (µm), crypt depth (µm) in the Jejunum of SPF chicks at 14 and 21 d of age.

Time (Days)	Parameters	Treatments			P. value
		T1	T2	T3	
14	Villus height	471.22±31.91^{b}	597.93±66.92^{a}	615.11±89.24^{a}	<0.001
	Crypt depth	71.91±11.38^{a}	74.75±11.64^{a}	78.63±14.71^{a}	0.35
21	Villus height	533.20±20.84^{b}	690.09±70.62^{a}	696.13±96.66^{a}	<0.001
	Crypt depth	67.42±17.41^{b}	74.61±8.53^{ab}	84.61±13.47^{a}	0.005

[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[a,b] Means in the same column and age with different superscripts are significantly different ($P < 0.05$).

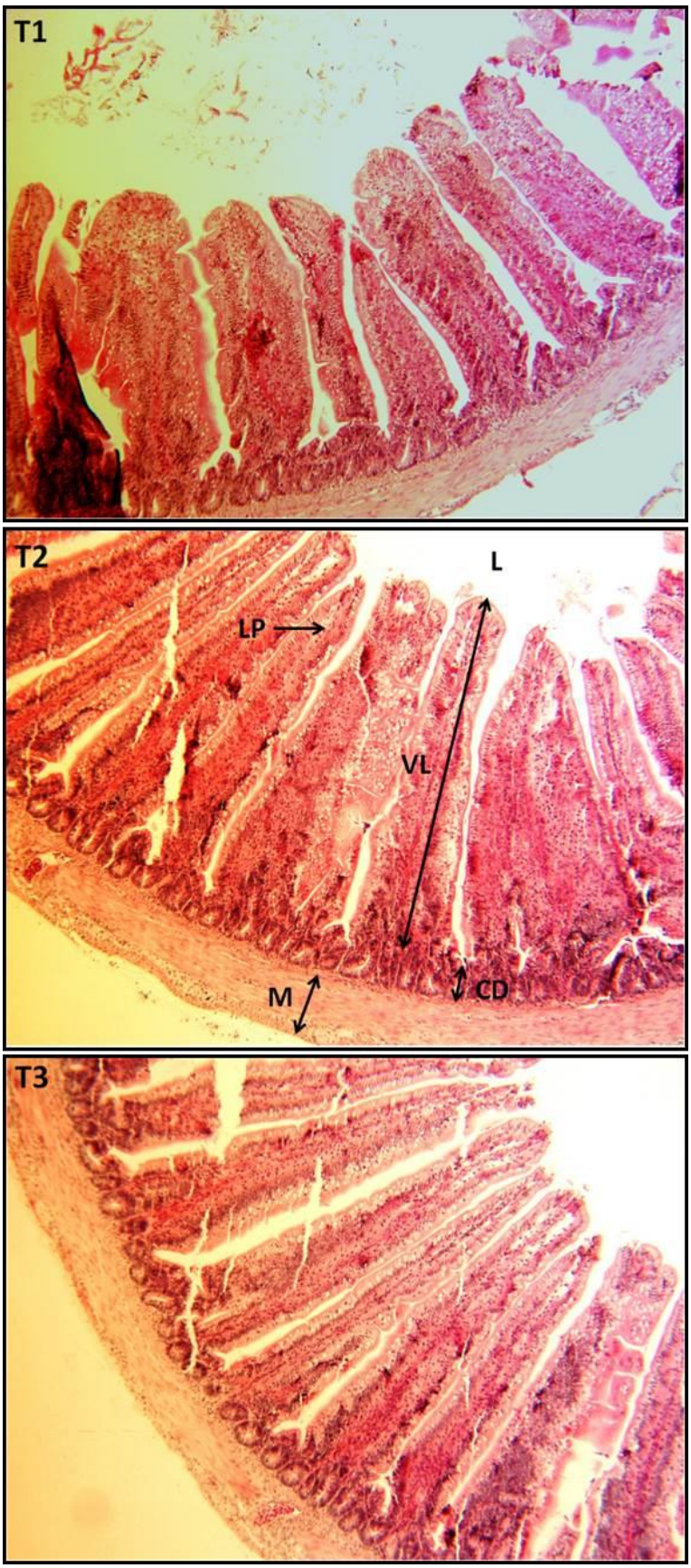


Figure 3.8: Haematoxylin and eosin stained section of jejunum of chicks fed diets containing inulin at 14 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA. L: Lumina, LP: Lamina propria, VL: Villus length, CD: Crypt depth, M: Muscularis. (10X Magnification).

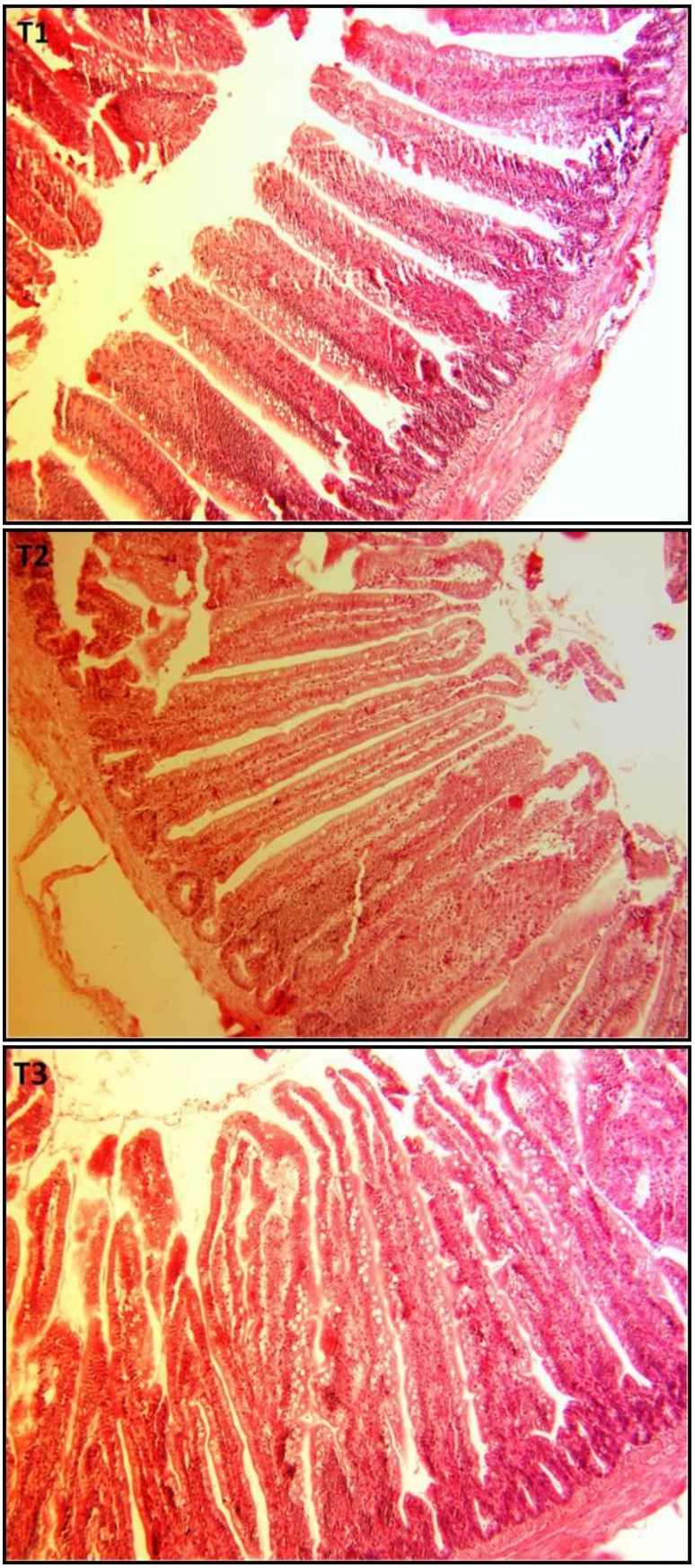


Figure 3.9: Haematoxylin and eosin stained section of jejunum of chicks fed diets containing inulin at 21 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

3.4.8 Relative weight of Bursa of Fabricius

Figure 3.10 illustrated that relative weight of Bursa of Fabricius from the chicks treated with dietary inulin supplementation compare to the control treatment. No statistical ($P<0.05$) differences in the relative weight of BF observed among inulin supplementation and control treatment, at different days of age. While, the higher weight of BF was observed in both commercial inulin and inulin from JA at 14 and 21 days of age.

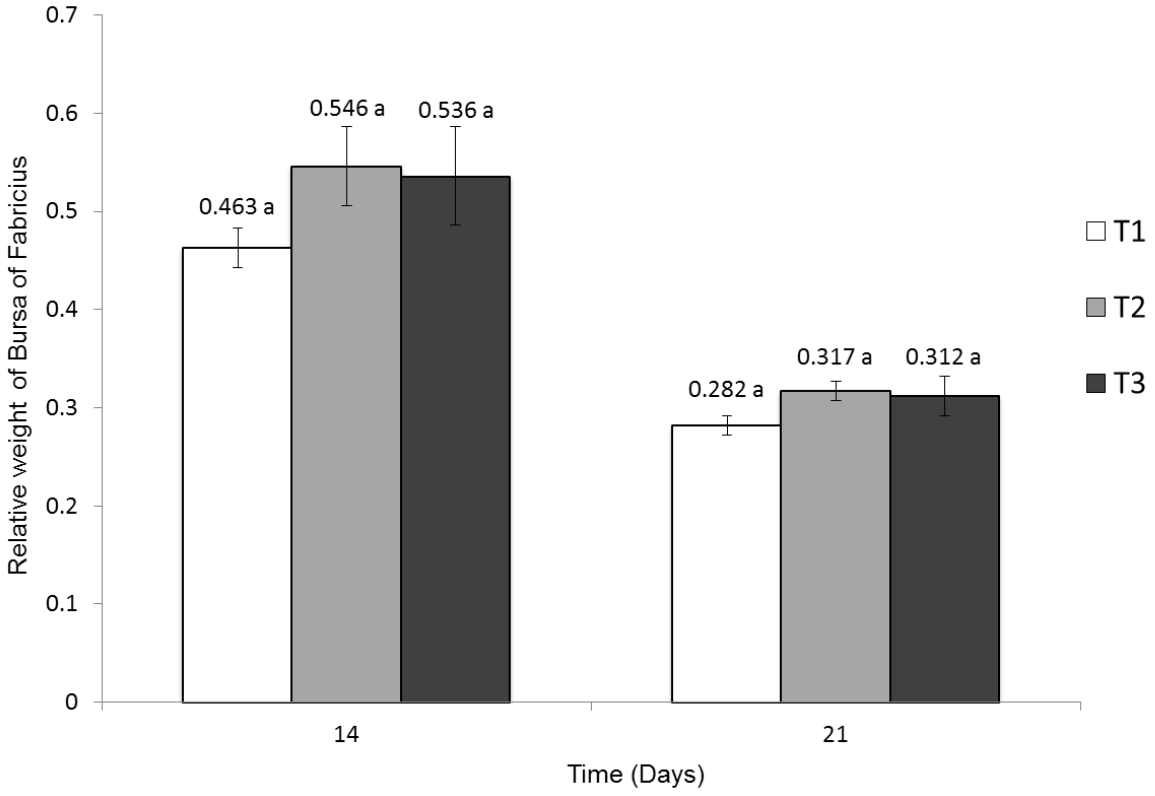


Figure 3.10: Effect of inulin on relative weight of BF.

3.4.9 Histology of Bursa of Fabricius

Figure 3.11 showed the results of the Bursa Histology measured in chicks at 14 and 21 days of age. Also, the results of tissue sections of the Bursa of Fabricius of three treatments were described in microscopic photos (Figures 3.12 and 3.13). In comparison with the control basal diet with the inclusion of both type of inulin had a great effect on the size of follicle of Fabricius at 14 and 21 days except the JA inulin compared with control at 14 day. The diameters of follicles of Fabricius were increased significantly ($P<0.05$) in dietary inulin supplementation (10 g of inulin/kg) compared to the control treatment, at 21 days of age. While, the results showed that no significant differences observed between the both types of inulin treatment for growth of follicles of Fabricius at different day of age.

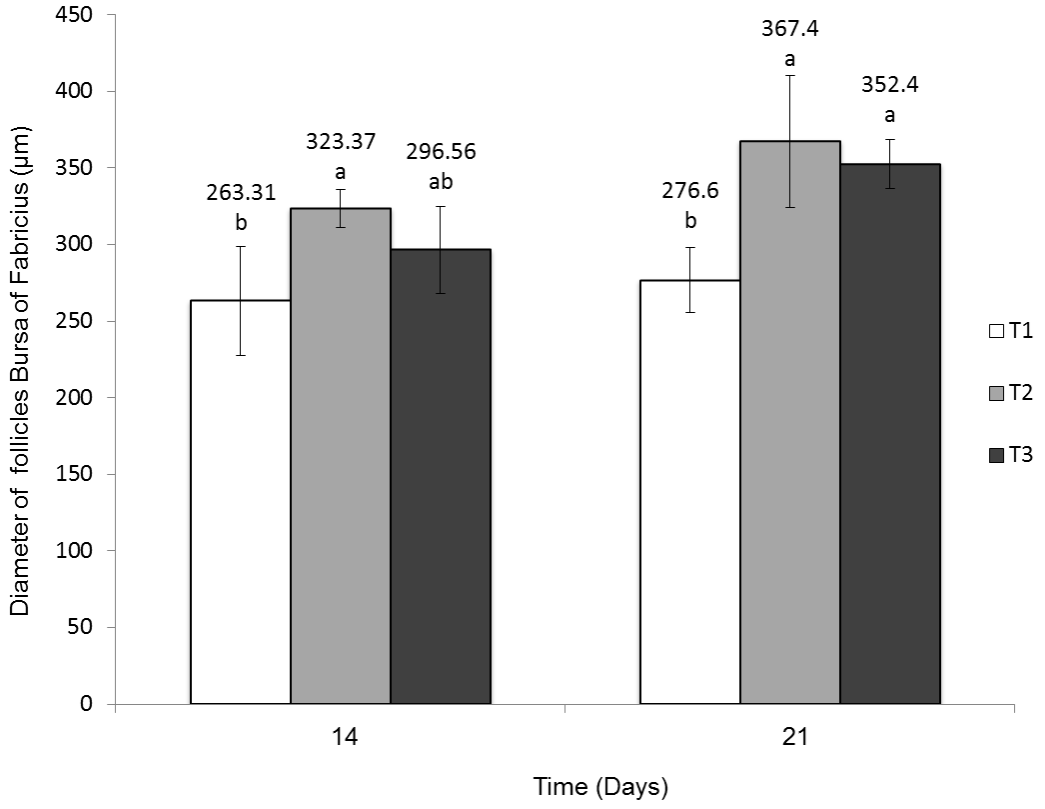


Figure 3.11: The effects of inulin on diameter of follicles of Fabricius in SPF chicks fed diets containing inulin at 14 and 21 days of age.

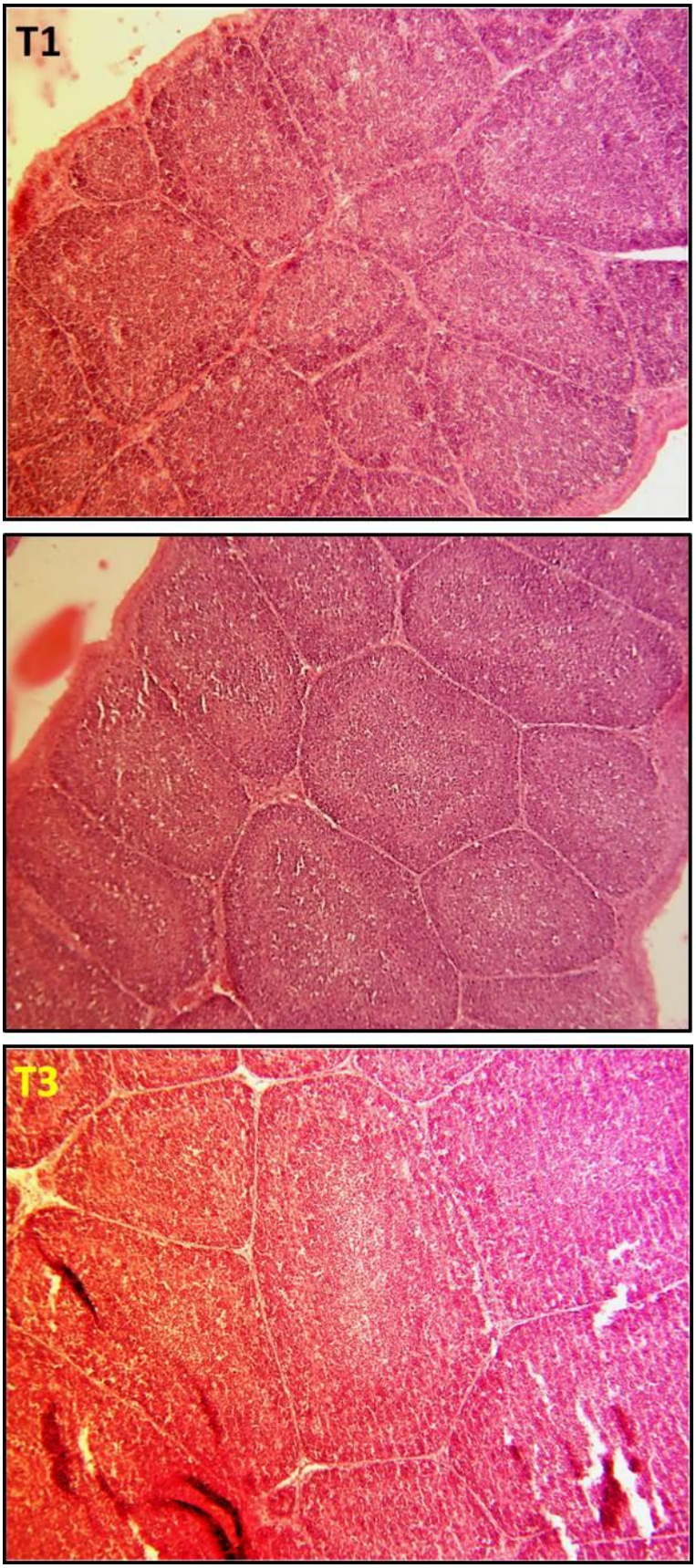


Figure 3.12: Bursa of Fabricius in chicks fed diets containing inulin at 14 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

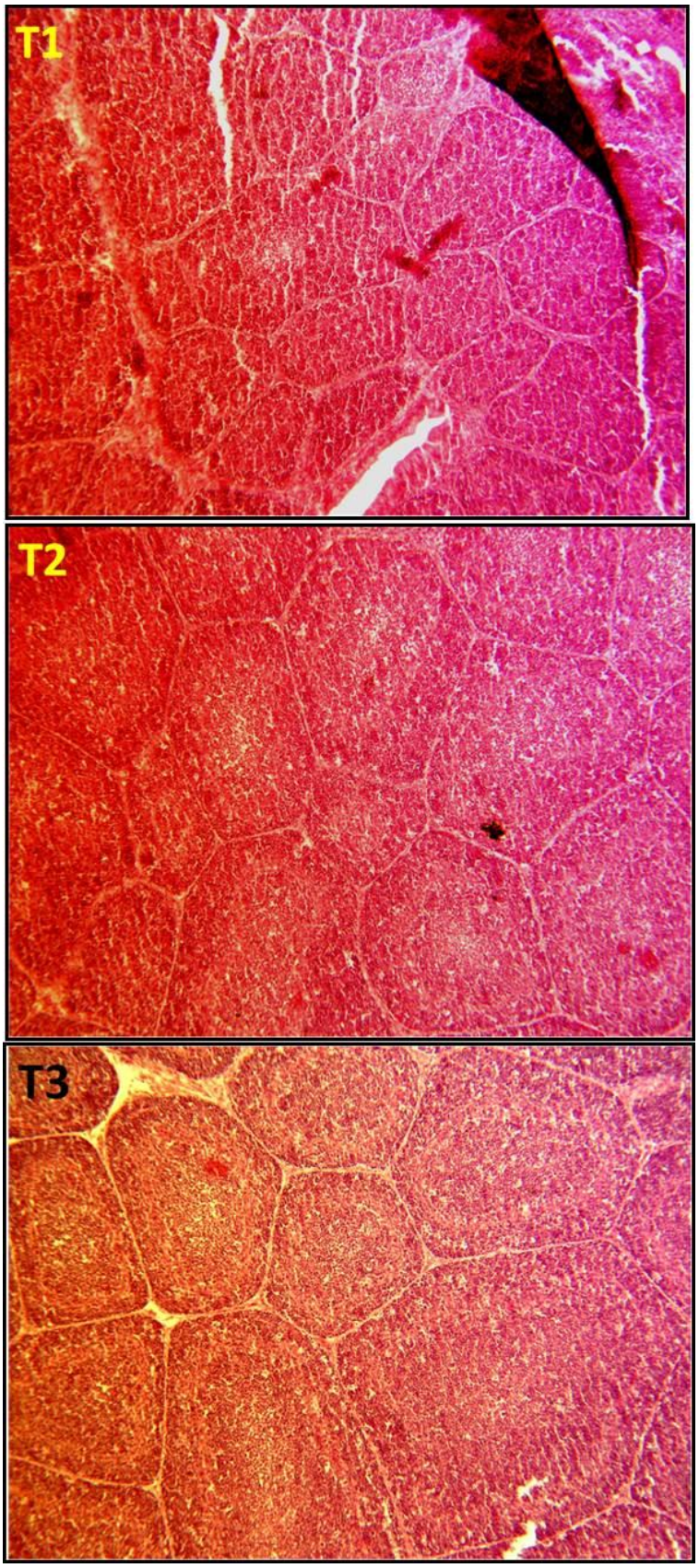


Figure 3.13: Bursa of Fabricius in chicks fed diets containing inulin at 21 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

3.4.10 Haematology

The treatments had highly significant effects on Lymphocyte count, Heterophil count and H/L ratio at 14 and 21 day of age. Both type of inulin significantly decreased the H/L ratio compared with the control treatment. There were no significant differences between T2 and T3 diet supplemented treatments. While, the better H/L ratio was observed for chicks fed inulin from Jerusalem artichoke being 0.30 and 0.28 at 14 and 21 day, respectively.

Table 3.13: Results of WBC's counts and heterophil/lymphocyte ratio in all treatment groups on day 14 and 21 of trial.

Time (Days)	Treatment	Lymphocyte[2]	Heterophils	H/L ratio
14	T1	60.33±1.52 ^{b}	26.66±1.52 ^{a}	0.43±0.02 ^{a}
	T2	68.00±2.64 ^{a}	24.00±2.00 ^{ab}	0.34±0.02 ^{b}
	T3	69.66±3.51 ^{a}	21.66±2.08 ^{b}	0.30±0.03 ^{b}
	P. value	0.011	0.048	0.002
21	T1	59.66±0.57 ^{b}	27.66±1.52 ^{a}	0.46±0.03 ^{a}
	T2	73.33±1.52 ^{a}	23.33±1.52 ^{b}	0.31±0.01 ^{b}
	T3	73.66±1.52 ^{a}	20.66±1.52 ^{b}	0.28±0.01 ^{b}
	P. value	<0.001	0.004	<0.001

[1] T1: control, T2: commercial inulin (1%), T3 Inulin from JA (1%).
[2] Results are mean values from three replications ± standard deviations.
[a,b] Means with the same superscript in the same column and age are significantly different (P<0.05).

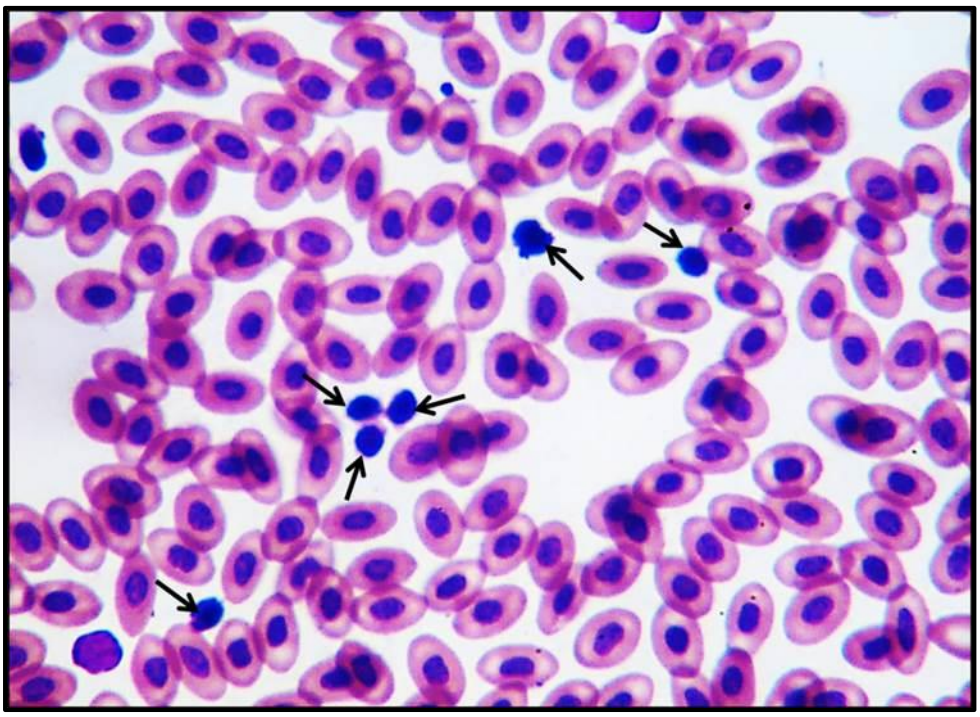


Figure 3.14 Blood film of chicken showed red blood cells (RBCs) have a nucleus and the arrow is lymphocytes, May Grunwald Giemsa stains used with magnification X100.

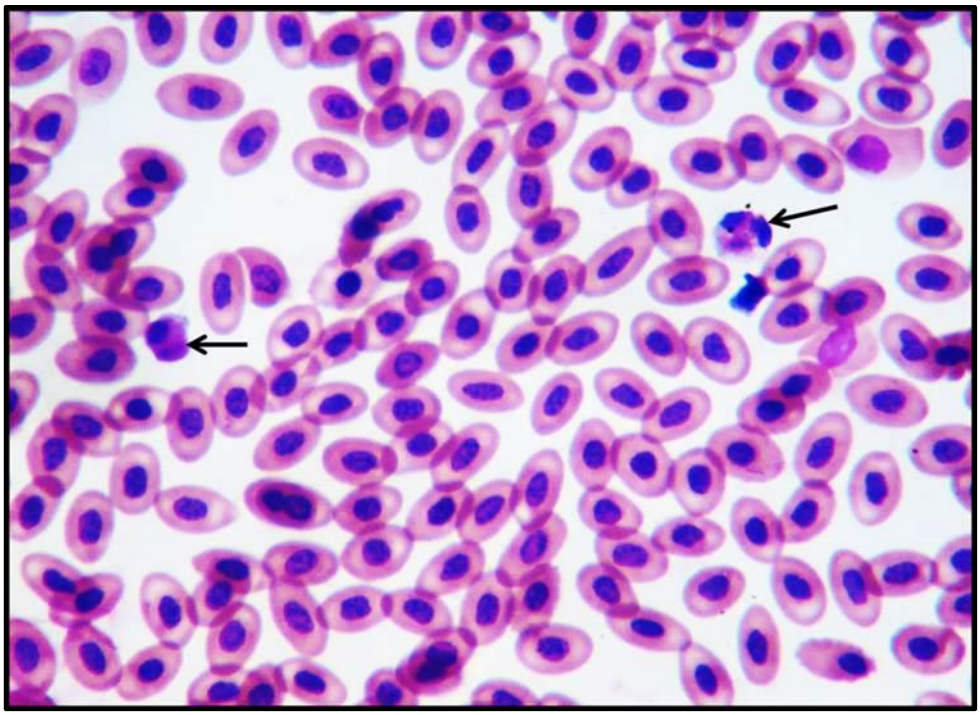


Figure 3.15: Blood film of chicken showed the red blood cells have a nucleus and the arrow is Heterophil, May Grunwald Giemsa stains used with magnification X100.

3.5 Discussion

The aim of this study was to investigate the effect of inulin from Jerusalem artichoke tubers on microbial population and histology of the chicken gut. There were no effects of the treatments on BW, but the total weight was mathematically more than control feed. The live body weight at 21 day was increased about 6.47% and 7.80% for T2 and T3, respectively compared with the control group. This result agreed with the results of some researchers (Yusrizal and Chen, 2003; Biggs *et al*., 2007 and Rehman *et al*., 2007a; Rehman *et al*., 2008 and Velasco *et al*., 2010; Elrayeh and Yildiz, 2012). While, others found that BW significantly increased by adding inulin as prebiotic to the diet (Waldroup *et al*., 1993; Williams *et al*., 2008 and Rebole *et al*., 2010).

The population of the intestinal bacterial community can be changed by a variety of factors, diet being one of them as it acts as a substrate for the indigenous intestinal microflora (Rehman *et al*., 2007b). Inulin can stimulate the growth of the intestinal bacteria as well as alter the ratio of various SCFA (Rehman *et al*., 2008). The present study showed the influence of dietary inulin supplementation on the microbial population of the caecum as detected by traditional culture technique and molecular technique of intestine in SPF chickens. Beneficial bacteria were increased by adding either commercial inulin or inulin from JA tubers. The higher Lactobacilli and Bifidobacteria observed in broiler fed both types of inulin may be due to the lowering the pH value in the intestine and increasing the production of SCFA which have bacteriostatic and bactericidal properties as observed in this trial (Fuller, 2001).

Amit-Romach *et al*. (2004) indicated that in young chicks the major species present in the small intestines and caeca was Lactobacilli and Bifidobacteria, with

other beneficial bacteria population becoming more dominant in the caeca at older age. Rebole *et al.* (2010) showed that Bifidobacteria in broilers caecum digesta in laying hens significantly increased after adding inulin to their diets.

Park and Park (2012) demonstrated that the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. in caecum was stimulated by adding inulin to the diet compared with the control group, while the growth of *E. coli* and *Salmonella* was clearly inhibited ($P<0.05$).

The normal gut microflora in farm animals is important because of its effect on the production of livestock and the quality and safety of livestock products. In poultry, the caecal microflora can protect chickens against bacterial infection; a healthy microflora present in the small intestine contributes significantly to small intestinal function, including digestion and nutrient absorption (Kabir *et al*., 2004; Gil De Los Santos *et al*., 2005; Mountzouris *et al*., 2007)

Inulin related carbohydrates are not dissolved in the small intestine of birds and reached the lower part of digestive system where it reduces the numbers of harmful microorganisms *E. coli* and *Salmonella* and selectively promotes the growth of beneficial microorganisms lactobacilli and bifidobacteria (Yusrizal and Chen, 2003; Park and Park, 2011). One other reasons to reduce the number of pathogens, they could attached with the prebiotics instead of attaching to intestinal epithelial cells and, therefore, move through the intestine without colonization (Newman, 1994).

The DGGE gel band numbers in the inulin from Jerusalem artichoke was higher than the control and commercial inulin group. The high species richness in gut microflora is associated with decreased ability of pathogens to colonize the gut (Dillon *et al.*, 2005). Rehman *et al*. (2008) recently demonstrated that inulin did

not affect the overall bacterial communities, but did alter the metabolic activity of the microbiota. When assessing antimicrobial alternatives such as prebiotics, the impact on the microbial profiles, the metabolic activity of the bacteria, and the subsequent effects on bird performance and nutrient utilization must be considered. DGGE as a technique was a very helpful tool to study the bacterial population diversity and for following up sequencing.

The difficulties associated with the cultivation of entire intestinal microflora have become challenge in assessing the intestinal microbial population in animals. Hence the knowledge of intestinal microbiota based on traditional culture techniques seems to be incomplete (Gong *et al*., 2002). The application of PCR-DGGE technique was described to monitor the changes in the caecal microbiota of chicks fed an inulin supplemented diet. This is a genetic fingerprinting technique that examines the microbial diversity based upon electrophoresis of PCR-amplified 16S rDNA fragments with gels containing a linear gradient of DNA denaturants (Muyzer *et al*., 1993). The PCR product banding pattern is indicative of the number of bacterial species or assemblages of groupings consisting of species that are present and thus allow visualization of the genetic diversity of microbial populations. These amplified fragments may be referred to as PCR products, fragments, bands. This technique acts as an appreciate method for the evaluation of microbial ecosystems. Additionally, it also allows the analysis of large number of samples and detection of shifts predominant microbial populations. This molecular fingerprinting technique has been used successfully to describe the variation in bacterial population or intestinal microbial community of broilers (Gong *et al*., 2002; Knarreborg *et al*., 2002; Zhu *et al*., 2002; Amit-Romach *et al*., 2004; Hume *et al*., 2006). In the present study, sequencing that

returned with an ideal result for caecum digesta showed that six of 26 bands detected from DGGE profile were belonged to *Clostridium* spp., five were related to *Ruminococcus* spp. and six of them related to uncultured bacteria. In poultry caeca the highest viable bacterial count and most complex microbiota exist (Huyghebaert, 2003). Amit-Romach *et al*. (2004) reported that at the first three weeks of chicken intestine 30% of intestinal bacteria belonged to *E. coli* and *Clostridium* spp. strains. Almost one-third of the bacteria in young chicken caecum consisted of *E. coli* and *Clostridium* species. The results in their study also indicated that in young chicks the major species present in the small intestines and ceca was lactobacilli, with a bifidobacteria population becoming more dominant in the ceca at older age.

Dietary addition of both types of inulin caused a major increase in the villus height in the jejunum when compared with control treatment. In the present study the villus height at 14 day was increased about 21.2% and 23.4% for T2 and T3 respectively compared with the control group. At 21 day of age also villus height was increased about 22.73% and 23.4% compared with the control group. An increase in villi height in the jejunum has been previously reported in broilers fed a prebiotic-based diet compared with control treatment (Iji *et al*., 2001; Rehman *et al*., 2007a). The current study findings suggest that lactobacilli, bifidobacteria and total anaerobic bacteria are improved by the dietary supplementation of commercial inulin or inulin from JA. So, it has important implications for villus height, because long villi are correlated with improved gut health. At d 14 and 21 birds fed the T1 diet had shorter villi than those fed the T2 and T3 diets. Both diets T2 and T3 had higher cecal populations of beneficial bacteria as well, and this could explain the higher villi observed in T2 and T3. Xu *et al*. (2003) also

reported that broilers fed Fructooligosaccharide 4g/kg diet had higher villi in the jejunum and ileum than control diet, as well as with high population of *Bifidobacteria* and *Lactobacillus* strains. Similarly, inulin has been found to increase the villus length and crypt depth of the jejunum in broiler chicks (Rehman *et al*., 2007a).

The Bursa of Fabricius is an organ of the immune system and is responsible for maturation of B lymphocytes (Alloui *et al*., 2005). Glick *et al*. (1956) showed that removal of the bursa in newly hatched chicks severely weakened the ability of the adult birds to produce antibodies. The size of the bursa is an indication of the immune functions and the relative weight of bursa to live body weight was recorded to compare the results between different treatments. In the present study, the results showed that no significant differences observed between the treatments for growth of Bursa of Fabricius. But the total weight of BF was mathematically more than control group. Elrayeh and Yildiz (2012) reported that in their study supplementation of 0.7% inulin in the diet of broilers did not affect the weight of Bursa of Fabricius compared to the control treatment. Dezaji *et al*. (2013) reported that addition of prebiotic to broilers diet did not show any significant effect on BF weight compared with control group. However, in the present study the follicle diameter of BF was significantly ($P<0.05$) increased in both types of inulin compared with the control group. Withers *et al*. (2005) have observed that there are two distinct types of follicle in the recovering bursa, large follicles with a cortex and medulla, and small follicles without these structural compartments. Birds with only small follicles did not produce detectable antibodies against IBDV or subsequently administered antigen. The presence of the larger follicles was correlated to ability to produce Ig responses. In contrast,

the small follicles were not able to support the complete programme of bursal B-cell development.

Stress could cause an increase in the stimulation of the adrenal gland to produce hormones which has a direct effect to analyse a lymphatic cell which causes an increase in H/L ratio (Gross and Siegel, 1983). Thus H/L ratio could be used as an indicator for the health of animals and any increase of H/L ratio refers to an increase in stress case (James and Stanley, 1989). In the present study the H/L ratio at 14 day was decreased about 20.93% and 30.23% for T2 and T3 respectively compared with the control group. At 21 day of age also H/L ratio was decreased about 32.60% and 39.13% compared with the control group. The lower H/L ratio in the experimental treatments may be because the inulin addition to the diet could inhibit the nutritional stress or any stress which causes an increase in H/L ratio (Karoglu and Drudage, 2005). AL-Kassie *et al*. (2008) who found a significant decrease in H/L ratio of broiler fed on the diet supplemented with 10g/kg of prebiotic (*Taraxacum officiale*) at 42 days compared with the control. Heterophil granules contain antimicrobial substances that can be released through degranulation to kill phagocytized bacteria (He *et al*. 2005). Lymphocytes are a type of White Blood Cells (WBCs) which form part of the body's immune system and help the body fight of infection. Lymphocytes attack foreign bodies by either producing antibodies or swallowing pathogen.

3.6 Conclusions

Inulin can stimulate the growth of the intestinal bacteria as well as may effect on the intestinal histology. The aim of this study was to investigate the influence of inulin from commercial (Frutafit® HD, Netherlands) and Jerusalem artichoke tubers (*Helianthus tuberosus*) as prebiotic supplementation on diversity of the caecal microflora, jejunum histology and immune organ of specific pathogen free (SPF) chicks. At 21 days of age the contents of beneficial bacteria in caecal digesta (Total anaerobic, lactobacilli and bifidobacteria) were increased in both types of dietary inulin supplementation compared with control group. Diversity analysis of PCR-DGGE DNA fingerprinting revealed that the richness and variety of caecal microflora in chicks fed inulin from JA were better than that in commercial inulin and control treatment. Both types of inulin significantly ($P<0.05$) increased villus height and crypt depth compared to the control treatment at different days of age. In addition, the diameter of follicles of Fabricius were increased significantly ($P<0.05$) in dietary inulin supplementation compared to the control treatment at 14 and 21 days of age. No significant differences were observed between both types of inulin treatment for villus height and growth of follicles of Fabricius at different days of age. This investigation has found that inulin which was extracted from Jerusalem artichoke had a similar result when compared with commercial inulin and could be a suitable candidate for an inulin source in broiler diets.

CHAPTER FOUR

The effects of dietary probiotic supplementation on gut microflora, histology and immune functions of broiler chickens.

4.1 Introduction

This study was designed to investigate the impact of *Lactobacillus animalis* isolated from healthy chicken gut in dry feed on the microbial population, structure and diversity of intestinal microflora and histology of broiler chickens.

The development and use of probiotics for poultry is based on the knowledge that the microflora in the gut participates in resistance to enteric infections and suppresses the growth of pathogenic bacteria. It has been shown to be participate in protection against a variety of pathogenic bacteria including *Escherichia coli*, *Salmonella* Enterica strain, *Campylobacter* spp. and *Clostridium* spp. (Jin *et al*., 1997; Murry *et al*., 2006; Ragione *et al*., 2004).

The development of molecular approaches has allowed the study of microbial groups that had previously remained undetected due to the limitations of standard classical microbiological method. Such limitations may be due to species-species interdependence in certain situations, and is due to a lack of knowledge with respect to actual nutritional requirements of these non-culturable microbes (Muyzer, 1999). Therefore, adopting molecular microbial ecology techniques will improve the chances of a successful analysis of the microbial community in its entirety. The purpose of the molecular microbial ecology investigations was to identify changes in the bacterial community as influenced by a probiotic additive to dry feed.

Most of the reported research on probiotics focuses on the use of various strains of *Lactobacillus*. The *Lb. animalis* strain isolated from chicken GI tract showed probiotic properties. The present study was conducted to investigate the effect of *Lactobacillus animalis* on performance, gut microflora and histology of broiler chickens and compare it with a commercially available probiotic Bactocell®.

4.2 Material and Methods

4.2.1 Experimental design and treatments

One hundred and two one-day-old male Hubbard broiler chicks were obtained from a commercial hatchery (P D Hooks Hatcheries Kentisbere, Devon, UK). The chicks were divided into three treatments (34 birds / treatment) and housed in nine pens of identical size (100 x 80 cm) in a deep litter system with a wood shaving floor and equipped with feeders and drinkers. Each treatment had three replicates (two replicates of 11 birds/ pen and one replicate of 12 birds) in a completely randomized design. Basal diets were formulated according to NRC standard (1994). The birds had free access to water and feed. The climatic conditions and lighting program followed the commercial recommendation. One hundred milligram of freeze dry *Lb. animalis* and Bactocell were added to one kg of feed in a sterile bag and mixed well to give a final concentration of 10^{10} CFU/kg of product. The duration of the trial was 28 d.

Chicks were assigned to the following treatments:

Control = Control group feed standard broiler diets. **(CON)**

Probiotic = Standard broiler diets + 100 mg/kg 2.62×10^{10} CFU/kg commercial probiotic Bactocell® (*Pediococcus acidilactici*). **(PRO1)**

Probiotic = Standard broiler diets + 100 mg *Lactobacillus animalis* / kg of diet, containing 1.72×10^{10} CFU/kg. **(PRO2)**

Table 4.1: The composition of standard broiler diets which was used for the trial.

Ingredients	Composition of diet (g/ 100 g)	
	Starter (1-21 days)	Grower (22-28 days)
Maize meal	54.75	60.19
Soybean meal	27.38	26
Fish meal	11.41	6.92
Soya oil	4.57	5
Limestone	0.20	0.20
Di calcium phosphate	0.11	0.11
Salt	0.17	0.17
Lysine	0.11	0.11
DL-Methionine	0.05	0.05
Chick premix [1]	1.25	1.25
Calculated values[2]		
ME (kcal/kg)	3178.00	3277.23
CP %	22.61	20.36
Lysine %	1.40	1.20
Methionine %	0.43	0.46
Calcium %	1.24	1.13
Available phosphate %	0.56	0.53

[1]The chick premix (MINSAL P330 Chick, Derbyshire, England) provided the following per kilogram of diet: 800000 IU of vitamin A, 240000 IU of vitamin D3, 2581 mg of Iron, 126 mg of Iodine, 40 mg of Cobalt, 1600 mg of Copper, 10322 mg of Manganese, 6667 mg of Zinc and 44.5 mg of Selenium.

[2]Food requirements were estimated according to (NRC, 1994).

4.2.2 Freeze drying *Lactobacillus animalis*

A *Lactobacillus* strain identified as *Lactobacillus animalis* that had been isolated from chicken caecum and had been selected for its probiotic properties in our laboratory referred to a Strain No. C4 in chapter three was stored at -80ºC. The easiest way to introduce probiotic into feed is in a lyophilized freeze dried form. One litre of an overnight culture of *Lb. animalis* in MRS broth incubated at 37ºC was centrifuged at 6000 rpm for 10 min. The sediment was mixed with 10 ml PBS and kept in freezer at -20 ºC for 24 hrs. The samples were transferred to a freeze dryer (Edward, Modulyo, England) at -60 ºC. The viability of the resulting freeze-dried culture was determined by mixing 100 mg with 0.9 ml PBS, followed by serial dilution and plating onto MRS agar incubated overnight at 37ºC. The viability of the freeze dried culture was very good; with a yield of 1.72×10^{13} CFU/g of freeze dried material. Bacteria were kept in freezer -20 ºC until further use.

4.2.3 Characteristics studied

4.2.3.1 Production performance

At one day old and at the end of each week, birds were weighted by a digital balance and feed consumption were monitored weekly and feed conversion ratio was calculated as feed consumed per unit of weight gain. The performance was calculated using the equations:

Weight gain (g) = BW at the end of the week - BW at the beginning of the week

Feed conversion ratio = Feed intake / weight gain

At the end of the experimental period (28 days) the European Production Efficiency Factor (EPEF) was calculated, based on the age of broilers at sacrifices (days), the average live body weight (kg / head), viability (%) and feed conversion ratio:

$$\textbf{EPEF} = \frac{\text{Liveability (\%) x live body weight at end trail (Kg) x 100}}{\text{Age of end trial (days) x Feed conversion ratio}}$$

4.2.3.2 Gut microflora analysis

At day 14, nine chicks per treatment were killed and the rest are killed at the end of the trial (day 28). Post-mortem 1 gm of gut contents from the ileum and caecum of nine chicks per treatment were aseptically removed. These were used for the assessment of gut microflora population changes using standard microbiology (culture techniques) as described in Section 3.2.7 and molecular microbiology as described in Section 3.2.8, except for DNA extraction. In this study DNA was extracted by a new extraction kit, as follows: A QIAamp fast stool mini kit (QUIAGEN, West Sussex, UK) method was used for DNA extraction with some modification to the manufacturer's instruction. Two hundred mg of sample was prepared in a sterilized Eppendorf tube, and DNA extracted by the following four stages:

1. Lyse and Inhibitor removal stage: 200 mg of samples were weighted in a 2 ml Eppendorf tube, and the samples were placed on ice. 500 µl of fresh lysozyme solution (50mg/ml TE buffer) was added, and then the samples were incubated at 37 ºC for 30 minutes and vortexed for 15 seconds with centrifugation for 5 min at 14000 xg. One ml Inhibit EX Buffer was added and

mixed for 1 minute. The mixture was placed on a hot plate at 90 ºC for 5 minutes and vortexed for 15 seconds with centrifugation for 1 min at 14000 xg.

2. Protein removal: 15 µl of proteinase K was pipetted into a new 2 ml Eppendorf tube. Then, 200 µl of supernatant was pipetted from step 1 into the 2 ml Eppendorf tube containing proteinase K. Then, 200 µl of Buffer AL was added and mixed for 15 seconds, then incubated at 70°C for 10 min
3. Precipitation: 200 µl of ethanol (96–100%) was added to the lysate, and mixed by vortexing. 600 µl lysate from the last step was Carefully applied to the QIAamp spin column. The cap of column was closed and centrifuged at 14000 xg for 1 min. Then, the QIAamp spin column was placed in a new 2 ml collection tube, and the filtrate with tube was discarded. 600 µl lysate was added again until all of the lysate has been loaded on the column.
4. Clean-up: The QIAamp spin column was carefully opened and 500 µl of Buffer AW1 was added. Then, the mixture was centrifuged at 14000 xg 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 14000 xg for 3 min. The collection tube containing the filtrate was discarded. The QIAamp spin column was transferred into a new, labelled 1.5 ml Eppendorf tube and pipetted 200 µl Buffer ATE directly onto the QIAamp membrane. Incubate for 1 min at room temperature, then centrifuge at 14000 xg for 1 min to elute DNA. Finally, the DNA extracted was stored at 4 °C for short term storage. The concentration of DNA and purity were determined using a Nanodrop-1000 Spectrophotometer.

All other procedures as described in Section 3.2.8. Including polymerase chain reaction (PCR) followed by agarose gel electrophoresis (AGE), and denaturant grade gel electrophoresis (DGGE) analysis and lastly gene sequences. Selected bands (OTU) of DGGE gel were aseptically separated and sequenced according to whether the band represented many groups or was a unique band for particular groups. BLAST at NCBI was used to confirm the species of the bacteria. The pH and SCFA were measured as described in Section 3.2.5 and 3.2.6, respectively.

4.2.3.3 Histological examination

Chick's intestine (Jejunum sections) and Bursa of Fabricius were taken and used for assessment of histological examination as described in Section 3.2.9 and 3.2.10, respectively.

4.3 Statistical analysis

All data were subjected to one way analysis of variance (ANOVA) using Minitab statistics software and Primer-6 software as described in Section 3.3.

4.4 Results

4.4.1 Freeze dried *Lactobacillus animalis*

In this study the viability of the freeze dried culture was very good; with a yield of 1.72×10^{13} CFU/g of freeze dried material. One gram of freeze dried bacteria was produced from one litre of overnight broth of *Lb. animalis* culture. The viability results of freeze-dried bacteria kept in the freezer were very high surviving percentage.

4.4.2 Effects of probiotic on performance parameters

The effects of probiotic supplementations on growth performance parameters are summarized in Table 4.2 – 4.5. There was no significant ($P>0.05$) difference in body weight of broilers among experimental groups at first week. The body weight of broilers supplemented with PRO2 was significantly ($P<0.05$) higher than broilers in PRO1 and control group on day 14. At 21 and 28 days, broilers supplemented with both types of probiotics had significantly ($P<0.01$) higher body weight compared with the control group. However, the difference in body weight of broilers between both probiotic groups was not significant ($P>0.05$).

Table 4.3 shows the weekly and average weight gain of broiler chicks during the experiment. There were no significant differences observed among all treatments in weekly weight gain of broiler chicks. However, final weight gain was significantly ($P<0.01$) higher in PRO1 (7.62%) and PRO2 (7.84%) groups compared with the control group.

Table 4.4 shows the weekly and average feed intake of broiler chicks during the experiment. The effect of probiotic supplementations were not significant (P>0.05) on broiler chickens weekly and average feed intake compared with the control group.

Table 4.5 shows the Weekly and average feed conversion ratio (FCR) of broiler chicks during the experiment. There was no significant (P>0.05) difference in FCR of broilers among experimental groups at first and fourth weeks. The FCR of broilers supplemented with both types of probiotics were significantly (P<0.05) improved in comparison with broilers in control group at second week of age. Also, at 21 day of age, only probiotic1 recorded significantly improved feed conversion ratio compared with control and probiotic2 groups. The chicks in both types of probiotic groups showed a significant (P<0.05) improvement in final feed conversion ratio compared to the control group.

Figure 4.1 shows the European production efficiency factor (EPEF) of broiler chickens during the experiment. In all treatments, there were no mortalities. The treatment had highly significant effect (p<0.01) on the EPEF at the end of the experiment. The chicks in probiotic1 (311.03) and probiotic2 (309.87) showed significant increases in EPEF compared with control groups (260.06). While, there were no significant differences observed between both probiotics at the end of experiment.

Table 4.2: Effect of probiotic supplementation on weekly and final live body weight (g) of broiler chicks (Mean ± standard division).

Time (Weeks)	Treatment			P. value
	CON	PRO1	PRO2	
0	42.2±2.73 [a]	42.3±3.44 [a]	42.2±2.58 [a]	0.992
1	160.3±16.96 [a]	163.7±16.47 [a]	164.8±15.14 [a]	0.492
2	344.2±35.43 [b]	362.5±34.42 [ab]	365.9±33.70 [a]	0.024
3	754.4±62.56 [b]	819.8±68.74 [a]	817.7±67.86 [a]	0.001
4	1204.2±71.5 [b]	1301.8±86.1 [a]	1305.0±79.6 [a]	<0.001

[a, b] : means within each row had the different subscript were differ significantly (P<0.05).

Table 4.3: Effect of probiotic supplementation on weekly and final body weight gain (g) of broiler chicks (Mean ± standard division).

Time	Treatment			P. value
	CON	PRO1	PRO2	
1st week	118.27±5.69 [a]	121.28±2.82 [a]	122.70±0.84 [a]	0.385
2nd week	184.17±8.73 [a]	198.93±4.27 [a]	201.25±14.74 [a]	0.164
3rd week	411.21±25.04 [a]	457.61±8.48 [a]	450.53±31.54 [a]	0.107
4th week	449.95±11.77 [a]	481.23±23.12 [a]	487.57±21.17 [a]	0.110
Final WG	1163.6±36.9 [b]	1259.1±13.8 [a]	1262.1±21.4 [a]	0.005

[a, b] : means within each row had the different subscript were differ significantly (P<0.05).

Table 4.4: Effect of probiotic supplementation on weekly and accumulative feed intake (g) of broiler chicks (Mean ± standard division).

Time	Treatment			P. value
	CON	PRO1	PRO2	
1st week	157.26±10.25 [a]	151.13±5.94 [a]	145.18±4.19 [a]	0.206
2nd week	276.88±8.64 [a]	260.86±9.58 [a]	259.86±15.58 [a]	0.215
3rd week	540.33±25.57 [a]	518.64±24.34 [a]	524.37±17.55 [a]	0.520
4th week	954.1±16.8 [a]	953.8±28.4 [a]	970.4±23.5 [a]	0.631
Final FI	1928.5±26.8 [a]	1884.4±36.5 [a]	1899.8±44.0 [a]	0.382

[a] : means within each row had the different subscript were differ significantly (P<0.05).

Table 4.5: Effect of probiotic supplementation on weekly and feed conversion ratio of broiler chicks (Mean ± standard division).

Time	Treatment			P. value
	CON	PRO1	PRO2	
1st week	1.33±0.07 a	1.24±0.07 a	1.18±0.02 a	0.081
2nd week	1.50±0.03 a	1.31±0.05 b	1.29±0.04 b	0.003
3rd week	1.31±0.07 a	1.13±0.03 b	1.16±0.08 ab	0.036
4th week	2.12±0.08 a	1.98±0.07 a	1.99±0.06 a	0.126
Final FCR	1.65±0.05 a	1.49±0.04 b	1.50±0.05 c	0.011

a, b, c : means within each row had the different subscript were differ significantly (P<0.05)

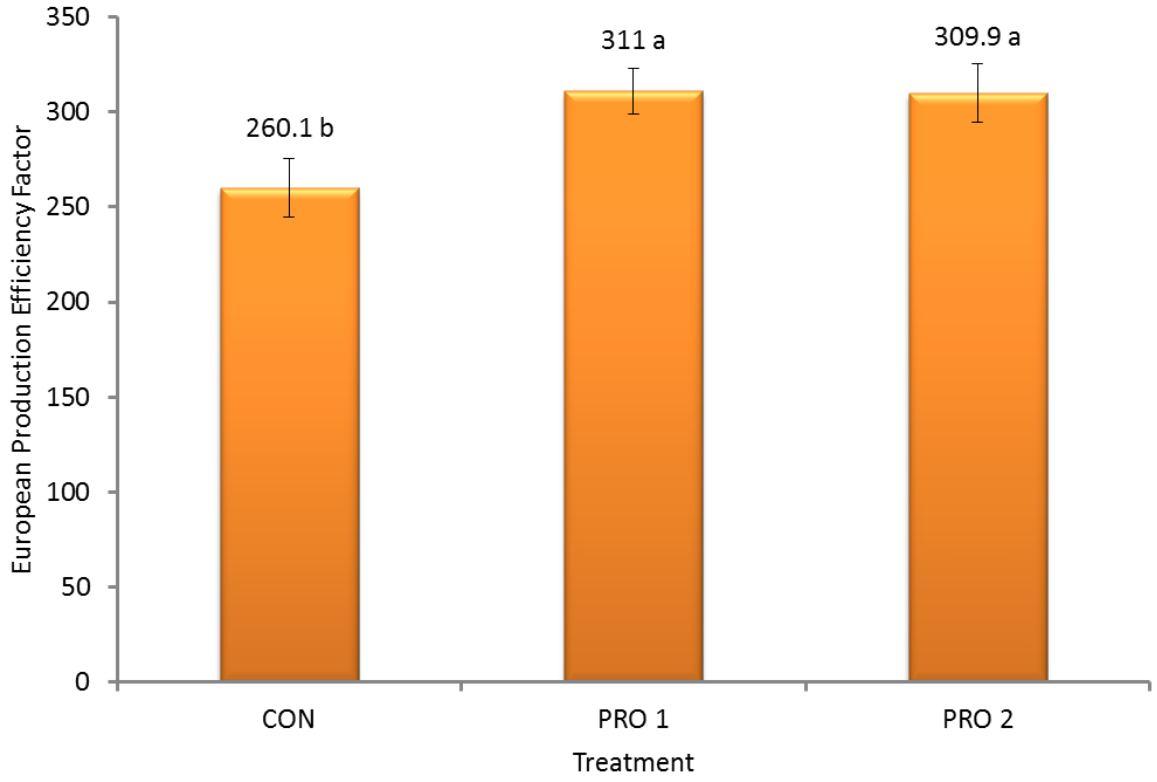


Figure 4.1: Effect of probiotic supplementation on European Production Efficiency Factor of broiler chickens (Mean ± standard division).

4.4.3 Effects of probiotics on pH value

Table 4.6 showed the pH values of the digesta in the ileum and caecum 14 and 28 days of age. There were significant differences among treatment groups. At 14 days of age, the dietary PRO1 supplementation significantly decreased (P<0.05) the level of pH 6.18 compared with the control and PRO2 groups 7.35 and 6.54, respectively in ileal digesta. Also, at the end of experiment, the pH value in ileum was reduced in both probiotic groups compared with the control group. However, there was no significant difference (P>0.05) in both type of probiotics. In caeca, PRO2 had highly significant difference (P<0.05) effect on the pH value 5.53 compared to the control group 6.55 at 14 days. However, there were no significant differences between both types of probiotic supplementations.

Table 4.6: Effect of probiotic supplementation on pH value in ileum and caecum of broiler chicks (Mean ± standard division).

Time (Days)	Treatment[1]	pH value	
		Ileum	Caecum
14	CON	7.35±0.03 [a]	6.55±0.38 [a]
	PRO1	6.18±0.48 [b]	5.87±0.55 [ab]
	PRO2	6.54±0.39 [ab]	5.53±0.08 [b]
	P. values	0.020	0.049
28	CON	6.69±0.21 [a]	6.26±0.39 [a]
	PRO1	5.81±0.20 [b]	6.14±0.17 [a]
	PRO2	5.60±0.48 [b]	5.61±0.27 [a]
	P. values	0.015	0.074

[a, b] means within each column had the different subscript were differ significantly (P<0.05).

4.4.4 Changes in the Short-Chain Fatty Acids and Lactate Concentrations

Table 4.7 showed the effects of both types of probiotic supplementation on the short chain fatty acid in the ileal and caecal digesta of broiler chicks at the end of experiment. The results showed that there were no significant (P>0.05) differences in lactic acid and propionic acid in ileal and caecal digesta among all treatments, respectively. However, in the ileal digesta, the concentration of acetic acid and propionic acid were significantly (P<0.01) increased in both type of probiotics compared with control group.

In the caecal digesta, lactic acid increased significantly (P<0.01) in PRO2 compared with PRO1 and control groups. While, acetic acid were significantly (P<0.05) increased in both types of probiotic compared with control group. On the other hand, the propionic acid in ileum and the acetic acid in caecal digesta were significantly (P<0.05) increased in PRO1 compared with PRO2.

Table 4.7: Influence of supplementation of probiotic on the short-chain fatty acid (mmol/L) profile in the caecal and ileum digesta of broilers at the end of the experiment.

Position	Treatment	SCFA		
		Lactic acid	Acetic acid	Propionic acid
Ileum	CON	7.665±0.65 [a]	5.027±0.57 [b]	2.008±0.35 [c]
	PRO1	9.544±0.70 [a]	9.214±1.39 [a]	5.937±0.47 [a]
	PRO2	9.296±1.34 [a]	7.887±0.32 [a]	3.743±0.24 [b]
	P. value	0.101	0.003	<0.001
Caecum	CON	1.419±0.23 [b]	10.240±0.87 [c]	1.591±0.35 [a]
	PRO1	1.860±0.29 [b]	17.732±0.32 [a]	2.071±0.20 [a]
	PRO2	2.586±0.10 [a]	15.144±1.36 [b]	2.048±0.25 [a]
	P. value	0.002	<0.001	0.140

[a,b] Means within same row with different superscripts are significantly different (P<0.05).
[1] Values are (mmol/L) means ± SD of triplicate determination.

4.4.5 Effects of probiotics on microflora

4.4.5.1 Microbial enumeration by conventional based method

Table 4.8 and 4.9 showed the effects of both types of probiotic supplementation on the microflora in the ileal and caecal digesta of broiler chicks at 14 and 28d of age. The results showed that both type of probiotics significantly ($P<0.01$) increased total anaerobic bacteria, *Lactobacillus* spp. and *Bifidobacterium* spp. at 14 and 28 days of age in ileal and caecal digesta. While, the numbers of total aerobic bacteria were significantly ($P<0.05$) decreased in the ileal and caecal digesta at 28 days of age compared to the control group. Also PRO1 and PRO2 significantly ($P<0.05$) decreased numbers of total coliforms in the ileal and caecal digesta of at 14 and 28 days of age compared to the control group, except probiotic1 in the ileum at 28 day.

There were significantly ($P<0.01$) increased numbers of total anaerobic bacteria, *Lactobacillus* spp. and *Bifidobacteria* spp. in ileum and anaerobic bacteria and *Lactobacillus* spp. in caecum at 28 days compared to 14 days of age in all treatments. However, the numbers of total aerobic bacteria and coliforms were decreased significantly ($P>0.05$) at 28 days compared to 14 days of age for all treatments

Table 4.8: Bacterial counts (Log_{10} CFU mL^{-1}) at 14 and 28 days of age in ileum digesta of broiler chickens.

Microbes		days	Treatments			P. value between treatment
			CON	PRO1	PRO2	
Total anaerobic		14	7.21±0.19 ^{bB}	7.65±0.09 ^{aB}	7.450±0.13 ^{abB}	0.029
Total aerobic			7.84±0.10 ^{aA}	7.64±0.08 ^{abA}	7.51±0.10 ^{bA}	0.017
Lactobacillus spp.			9.20±0.05 ^{bB}	9.57±0.03 ^{aB}	9.60±0.03 ^{aB}	<0.001
Bifidobacterium spp.			9.10±0.04 ^{bB}	9.24±0.02 ^{aB}	9.28±0.03 ^{aB}	0.002
Total Coliform			7.68±0.05 ^{aA}	6.97±0.08 ^{bA}	7.08±0.05 ^{bA}	<0.001
Salmonella			n.d.	n.d.	n.d.	--
Total anaerobic		28	8.16±0.14 ^{bA}	8.56±0.07 ^{aA}	8.74±0.06 ^{aA}	0.001
Total aerobic			7.18±0.04 ^{aB}	6.87±0.11 ^{bB}	6.92±0.03 ^{bB}	0.004
Lactobacillus spp.			9.60±0.11 ^{bA}	9.97±0.03 ^{aA}	9.94±0.03 ^{aA}	0.001
Bifidobacterium spp.			9.82±0.09 ^{bA}	10.01±0.03 ^{aA}	10.03±0.04 ^{aA}	0.012
Total Coliform			6.98±0.06 ^{aB}	6.65±0.16 ^{abB}	6.63±0.16 ^{bB}	0.034
Salmonella spp.			n.d.	n.d.	n.d.	--
p. value within treatment	Total anaerobic		0.002	<0.001	<0.001	
	Total aerobic		0.001	0.001	0.001	
	Lactobacillus spp.		0.005	<0.001	<0.001	
	Bifidobacterium spp.		<0.001	<0.001	<0.001	
	Total Coliform		<0.001	0.037	0.010	
	Salmonella spp.		--	--	--	

^{a,b,c} Means in the same raw and age with different superscripts are significantly different ($P < 0.05$). $^{A, B, C}$ Means in the same raw and treatment with different age with different superscripts are significantly different ($P < 0.05$).
n.d. : Not detected.

Table 4.9: Bacterial counts (Log_{10} CFU mL^{-1}) at 14 and 28 days of age in caecal digesta of broiler chickens.

Microbes		days	treatments			P. value between treatment
			CON	PRO1	PRO2	
Total anaerobic		14	7.64±0.15 ^{bB}	8.27±0.24 ^{aB}	8.39±0.13 ^{aB}	0.005
Total aerobic			7.90±0.15 ^{aA}	7.73±0.05 ^{abA}	7.51±0.18 ^{bA}	0.045
Bifidobacterium spp.			8.34±0.08 ^{bB}	8.77±0.03 ^{aB}	8.79±0.03 ^{aB}	<0.001
Bifidobacterium spp.			9.80±0.04 ^{bA}	10.04±0.07 ^{aA}	10.16±0.08 ^{aA}	0.002
Total Coliform			8.72±0.16 ^{aA}	7.94±0.05 ^{bA}	7.58±0.11 ^{cA}	<0.001
Salmonella			n.d.	n.d.	n.d.	--
Total anaerobic		28	8.89±0.04 ^{bA}	9.04±0.04 ^{aA}	8.95±0.04 ^{abA}	0.017
Total aerobic			7.42±0.04 ^{aB}	6.96±0.08 ^{bB}	6.99±0.05 ^{bB}	<0.001
Bifidobacterium spp.			9.89±0.05 ^{bA}	10.07±0.03 ^{aA}	10.06±0.04 ^{aA}	0.004
Bifidobacterium spp.			9.83±0.04 ^{bA}	10.09±0.03 ^{aA}	10.08±0.03 ^{aA}	<0.001
Total Coliform			7.19±0.03 ^{aB}	6.91±0.08 ^{bB}	7.00±0.07 ^{bB}	0.007
Salmonella spp.			n.d.	n.d.	n.d.	--
p. value within treatment	Total anaerobic		<0.001	0.005	0.002	
	Total aerobic		0.006	<0.001	0.010	
	Bifidobacterium spp.		<0.001	<0.001	<0.001	
	Bifidobacterium spp.		0.55	0.325	0.241	
	Total Coliform		<0.001	<0.001	0.002	
	Salmonella spp.		--	--	--	

^{a,b} Means in the same raw and age with different superscripts are significantly different ($P < 0.05$). $^{A, B, C}$ Means in the same raw and treatment with different age with different superscripts are significantly different ($P < 0.05$).
n.d. : Not detected.

4.4.5.2 Microbial population of the ileum and caecum by molecular method

4.4.5.2.1 Spectrophotometric assay

After DNA extraction, all the results of DNA concentrations in ileum and caecum samples were more than 20ng/µl, from 14 and 28 days. The protein and humic acid contamination was higher than 1.7.

4.4.5.2.2 PCR-DGGE analysis

The amplified DNA template from the caecal and ileal samples appeared as single bands by the agarose gel electrophoresis as shown in the example in Figure 4.2. A single band is desirable for successful PCR.

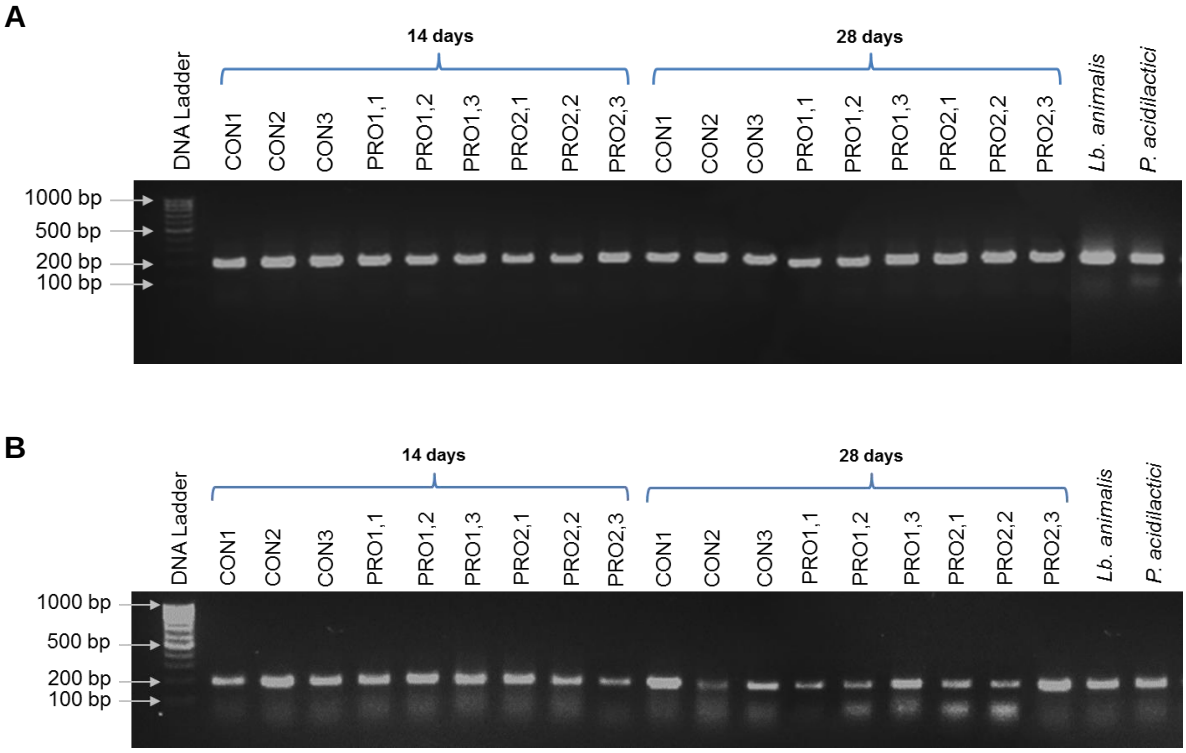


Figure 4.2: PCR amplified product of DNA templates of the Caecum (A) and ileum (B) samples at different days and pure *Lb. animalis* and *P. acidilactici* strains.

4.4.5.2.3 DGGE analysis of ileum bacterial community

Figure 4.3 shows the PCR–DGGE bacterial profiles of the digesta from the ileum of chickens at 14 (A) and 28 (B) days of age. Many different bands are shown in the DGGE image and the gel bands which are called operative taxonomy units (OTU) in each sample.

The similarity of bacterial population within and between the treatments were measured by nonmetric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 4.4.

The both analyses of ileal bacteria populations showed more similarity within samples from the same treatments than those from other groups. The half matrix similarity of ileal DGGE fingerprints is shown in Table 4.10 indicates the average similarity within the control treatment is 81.78% at day 14 and 71.38% in day 28, probiotic1 81.67% at day 14 and 74.22% at day 28, probiotic2 74.53% at day 14 and 84.62% at day 28. The average bacterial population similarity between control groups at day 14 and 28 was 76.58%, while the probiotic1 was 77.94% and probiotic2 was 79.57%.

The average numbers of bands of both type of probiotics significantly ($P<0.05$) increased compared with control group at 14 days old. 17.33 DNA bands were detected in the pro2 samples at 14 days old chicks and the number of DNA bands increased to 21.66 when the chicks became 28 days old (Table 4.11).

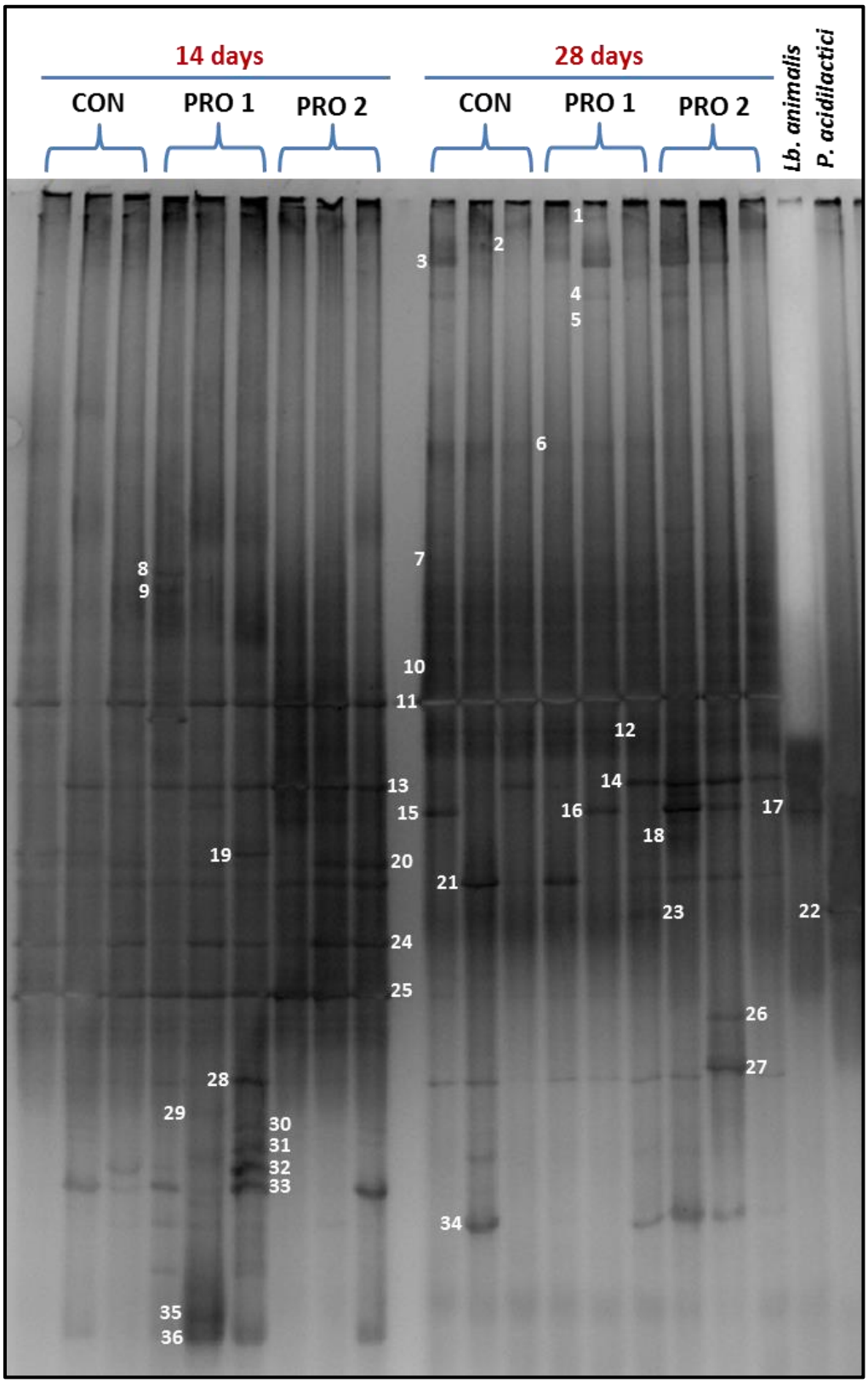


Figure 4.3: DGGE fingerprints of ileum digesta of treated and control group chicks at 14 and 28 days of age. Numbers represent the bands or operative taxonomy units (OUT) excised and sequenced.

Table 4.10: The half matrix similarity of bacterial population of DGGE fingerprints of ileum showing the similarities between the replicates treatment.

Group	CON A1	CON A2	CON A3	PRO1 A1	PRO1 A2	PRO1 A3	PRO2 A1	PRO2 A2	PRO2 A3	CON B1	CON B2	CON B3	PRO1 B1	PRO1 B2	PRO1 B3	PRO2 B1	PRO2 B2	PRO2 B3
CON A1	100																	
CON A2	76.19	100																
CON A3	90.91	78.26	100															
PRO1 A1	76.92	66.67	85.71	100														
PRO1 A2	64.29	68.97	66.67	82.35	100													
PRO1 A3	64.00	76.92	74.07	83.87	78.79	100												
PRO2 A1	69.23	51.85	64.29	68.75	70.59	58.06	100											
PRO2 A2	66.67	51.61	62.50	72.22	73.68	57.14	88.89	100										
PRO2 A3	69.23	81.48	71.43	68.75	70.59	77.42	62.50	72.22	100									
CON B1	38.10	18.18	34.78	44.44	34.48	30.77	59.26	58.06	37.04	100								
CON B2	32.00	23.08	29.63	45.16	42.42	40.00	51.61	62.86	51.61	69.23	100							
CON B3	63.64	43.48	58.33	57.14	46.67	44.44	71.43	68.75	57.14	78.26	66.67	100						
PRO1 B1	52.17	33.33	48.00	55.17	45.16	42.86	68.97	66.67	48.28	83.33	71.43	88.00	100					
PRO1 B2	40.00	23.08	37.04	45.16	36.36	33.33	58.06	62.86	45.16	84.62	73.33	74.07	78.57	100				
PRO1 B3	57.14	41.38	53.33	64.71	61.11	54.55	76.47	78.95	58.82	68.97	72.73	80.00	77.42	66.67	100			
PRO2 B1	45.71	38.89	43.24	53.66	60.47	50.00	68.29	75.56	58.54	61.11	70.00	64.86	68.42	75.00	79.07	100		
PRO2 B2	46.67	38.71	43.75	55.56	57.89	51.43	72.22	80.00	61.11	64.52	80.00	75.00	78.79	80.00	73.68	84.44	100	
PRO2 B3	46.67	38.71	43.75	55.56	57.89	51.43	66.67	75.00	61.11	70.97	80.00	75.00	72.73	80.00	78.95	84.44	85.00	100

Note: CON = control, PRO1 = *Pediococcus acidilactici*, PRO2 = *Lactobacillus animalis*. A= at day 14, B= at day 28, 1-3 refers to replicate number in each case.

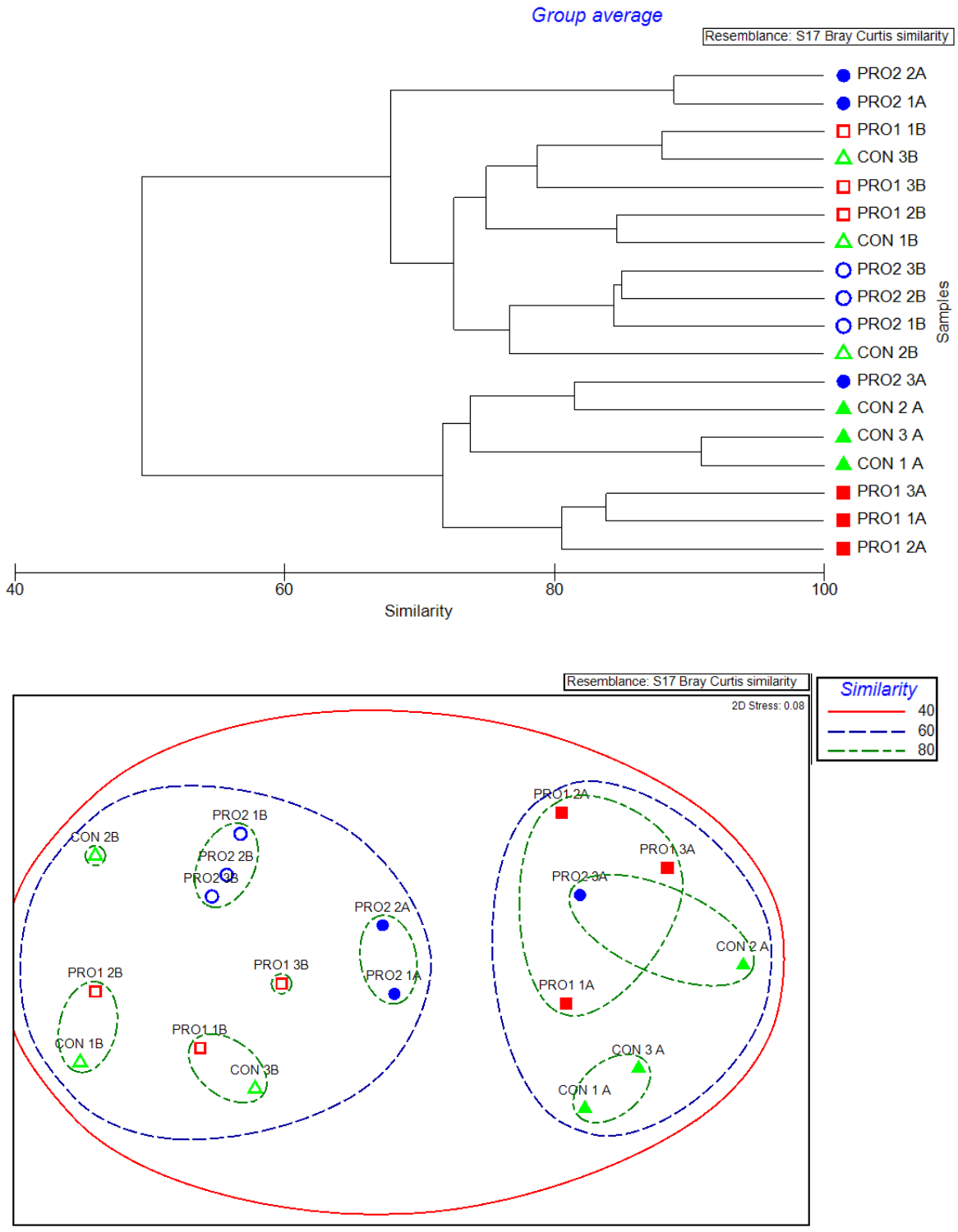


Figure 4.4: (Top) Cluster analysis (Bottom) non-metric multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in poultry ileum. A: 14 days, B: 28 days old of broilers. 1-3 denotes replicate number in each sample.

Table 4.11: Band numbers of ileum bacterial community based on the PCR-DGGE DNA fingerprinting and similarity within treatments (Mean ± SD).

Time (Days)	Treatment	Band number	Similarity
14	CON	11.00±1.00 [b]	81.78±7.96 [a]
	PRO1	16.33±1.52 [a]	81.67±2.60 [a]
	PRO2	17.33±2.30 [a]	74.53±13.3 [a]
	P. value	0.008	0.567
28	CON	12.66±2.08 [b]	71.38±6.08 [b]
	PRO1	15.33±2.51 [b]	74.22±6.56 [ab]
	PRO2	21.66±2.88 [a]	84.62±0.32 [a]
	P. value	0.012	0.045

[a,b] Means with the different superscript in the same column and age are significantly different (P<0.05).

Diversity and richness of ileum microflora were analysed by using Shannon index and Margalef index, respectively. These indexes were used to display the microbial population diversity and richness in the ileum, data showed in Table 4.12. The diversity and richness index of bacterial community based on the PCR-DGGE DNA fingerprinting indicated that; at 14 day of age, both types of probiotic significantly (P<0.01) increased Shannon and Margalef index compared with birds fed control group. However, only PRO2 increased significantly (P<0.01) diversity and richness of ileal microflora compared with control group, at 28 days of age. There were no significant differences between both type of probiotics at 14 and 28 days.

Table 4.12: Diversity index of bacterial community in ileum digesta based on the PCR-DGGE DNA fingerprinting at different day of age (Mean ± SD).

Time (Days)	Treatment	Shannon index[1]	Margalef index[2]
14	CON	4.16±0.25 [b]	2.39±0.09 [b]
	PRO1	5.48±0.36 [a]	2.79±0.09 [a]
	PRO2	5.72±0.53 [a]	2.84±0.12 [a]
	P. value	0.007	0.004
28	CON	4.58±0.51 [b]	2.53±0.15 [b]
	PRO1	5.24±0.60 [ab]	2.72±0.16 [ab]
	PRO2	6.71±0.64 [a]	3.07±0.12 [a]
	P. value	0.012	0.013

[a,b] Means with the same superscript in the same column and age are not significantly different (P<0.05).
[1] Shannon diversity index: H" = -SUM(pi*Log(pi)).
[2] Margalef species richness: d = (S -1)/log (N). (S: Total species, N: Total individuals)

The results of the sequence analysis are shown in Table 4.13. A positive sequencing was returned for 30 bands out of the 36 PCR fragments. The other samples sequencing quality were below the required standard and sequencing data was zero.

Inclusion of PRO2 in the diet was found to alter microbiota composition. The BLAST results of the ileum most genera were belonged *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Ruminococcus* spp., *Escherichia* spp., *Clostridium* spp., *Acidaminobacter* spp. and *Enterobacter* spp. strains. For example, band number 3 was related to *Lb. gasseri* and it was detected in two lines of PRO2 compared with PRO1 and control groups. Band number 6 which was related to *Escherichia coli* and detectable in all the samples of broiler chickens at 28 day, and had an increased density in control group compared with both types of probiotics. Band numbers 8 and 9 were related to *Pediococcus stilesii* and *Pediococcus pentosaceus*, respectively, were detected more density

in PRO1. The band number 12 was related to *Lb. acidophilus*, it was appeared in all samples only at 28 days of broilers age. The band number 14 was related to *Lb. gigeriorum*; the density of this band in the PRO2 of days 14 and 28 was higher when compared with the birds fed PRO1 and control groups. The band number 23 was related to *Pediococcus acidilactici* only appeared in PRO1 based *Pediococcus acidilactici* which was on one line with the pure band (Band number 22). This result confirms the survival of *Pediococcus acidilactici* in chicken GI tract. The band number 25 was *Escherichia coli* and it was found in all treatments at 14 day, while it was completely gone at 28 day in PRO2 group.

Table 4.13: Summary results of sequencing analysis bands of PCR-DGGE fingerprints of chicken ileum samples.

Band Number	NCBI Accession number	Max. Identity (%)	NCBI BLAST matches
1	NR_075051.1	98	*Lactobacillus gasseri* strain ATCC 33323
2	NR_113904.1	100	*Enterococcus faecium* strain NBRC 100486
3	NR_041920.1	98	*Lactobacillus gasseri* strain ATCC 33323
4	NR_113338.1	99	*Lactobacillus plantarum* strain NBRC 15891
5	NR_075045.1	98	*Lactobacillus acidophilus* NCFM strain NCFM
6	NR_074891.1	100	*Escherichia coli* O157:H7
7	NR_117574.1	99	*Lactobacillus johnsonii* strain CIP 103620
8	NR_04240.1	98	*Pediococcus stilesii* strain FAIR-E 180
9	NR_041640.1	99	*Pediococcus pentosaceus* ATCC 25745
10	NR_025273.1	100	*Lactobacillus johnsonii* strain ATCC 33200
11	NR_028683.1	94	*Acidaminobacter hydrogenoformans* strain glu 65
12	NR_113638.1	99	*Lactobacillus acidophilus* strain NBRC 13951
14	NR_117057.1	99	*Lactobacillus gigeriorum* strain CRBIP 24.85
16	NR_042111.1	98	*Lactobacillus* gallinarum strain ATCC 33199
17	AB911530.1	100	*Lactobacillus animalis* gene strain: JCM 8692
18	NR_113924.1	100	*Enterococcus gallinarum* strain NBRC 100675
21	NR_075064.1	95	*Lactobacillus johnsonii* NCC 533 strain *or*
22	NR_042057.1	100	*Pediococcus acidilactici* strain DSM 20284
23	NR_042057.1	99	*Pediococcus acidilactici* strain DSM 20284
24	NR_118568.1	98	*Enterobacter cloacae* strain ATCC 13047
25	NR_114042.1	100	*Escherichia coli* strain NBRC 102203
26	NR_113244.1	100	*Clostridium butyricum* strain JCM 1391
27	NR_113261.1	98	*Lactobacillus gallinarum* strain JCM 2011
28	KF504995.1	99	Uncultured *Lactobacillus* sp. clone 4394
29	NR_125539.1	100	*Pectobacterium carotovorum* subsp. actinidiae strain KKH3
32	NR_102794.1	99	*Enterobacter cloacae* strain DSM 30054
33	NR_119274.1	98	*Lactobacillus crispatus* strain DSM 20584
34	NR_119036.1	99	*Romboutsia lituseburensis* strain ATCC 25759
35	EF587947.1	93	Uncultured *Enterococcus* sp. isolate DGGE gel band 7v3
36	NR_104559.2	99	*Enterococcus gallinarum* strain LMG 13129

4.4.5.2.4 DGGE analysis of caecum bacterial community

Figure 4.5 shows the PCR–DGGE bacterial profiles of the digesta from the caecum of chickens at 14 (A) and 28 (B) days of age. Many different bands are shown in the DGGE image. The similarity of bacterial population within and between the treatments were measured by nonmetric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 4.6.

The both analyses of caecal bacteria populations showed more similarity within samples from same treatments than those from other groups. The half matrix similarity of caecal DGGE fingerprints is shown in Table 4.14 indicates the average similarity within the control treatment is 82.1% at day 14 and 84.11% in day 28, PRO1 88.64% at day 14 and 82.48% at day 28, PRO2 89.7% at day 14 and 94.39% at day 28. The average bacterial population similarity between control groups at day 14 and 28 was 83.1%, while the PRO1 was 85.56% and PRO2 was 92.04%.

The average bands numbers of PRO2 significantly ($P<0.05$) increased compared with control and PRO1 groups at 14 days old. While, both type of probiotic supplementations caused no statistically significant differences in the number of PCR-DGGE bands within the caecal digesta, at 28 days old (Table 4.15).

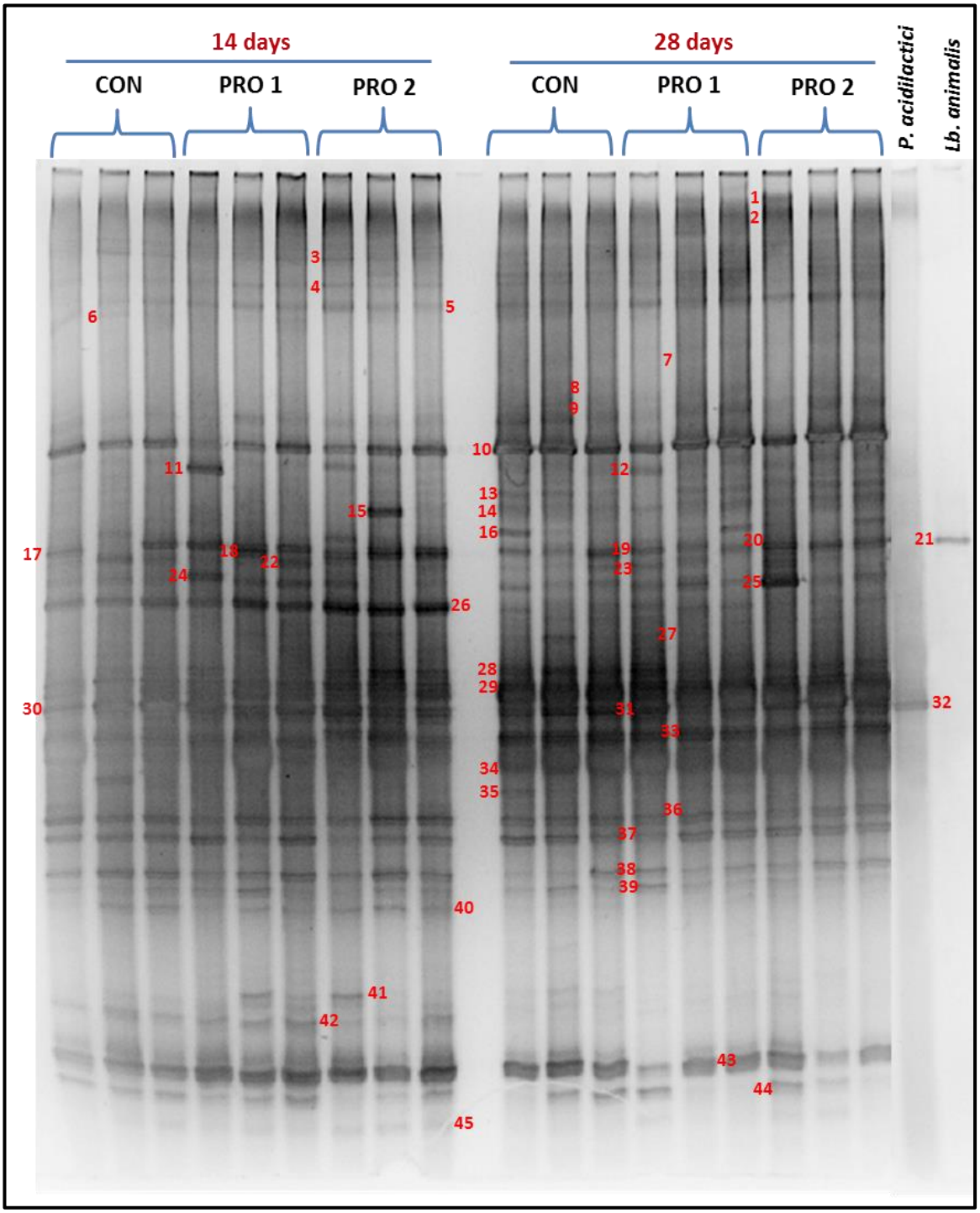


Figure 4.5: DGGE fingerprints of caecum digesta of treated and control group chicks at 14 and 28 days of age. Numbers represent the bands or operative taxonomy units (OUT) excised and sequenced.

Table 4.14: The half matrix similarity of bacterial population of DGGE fingerprints of caeca showing the similarities between the replicates treatment.

Group	CON A1	CON A2	CON A3	PRO1 A1	PRO1 A2	PRO1 A3	PRO2 A1	PRO2 A2	PRO2 A3	CON B1	CON B2	CON B3	PRO1 B1	PRO1 B2	PRO1 B3	PRO2 B1	PRO2 B2	PRO2 B3
CON A1	100																	
CON A2	85.71	100																
CON A3	78.26	82.35	100															
PRO1 A1	72.34	76.92	93.88	100														
PRO1 A2	80.00	76.00	85.11	87.50	100													
PRO1 A3	81.82	73.47	82.61	85.11	93.33	100												
PRO2 A1	70.59	78.57	75.47	74.07	76.92	74.51	100											
PRO2 A2	67.92	75.86	80.00	78.57	77.78	75.47	90.00	100										
PRO2 A3	72.00	72.73	80.77	79.25	78.43	80.00	84.21	94.92	100									
CON B1	76.00	72.73	69.23	67.92	74.51	72.00	73.68	74.58	78.57	100								
CON B2	71.43	75.41	72.41	71.19	70.18	67.86	69.84	76.92	80.65	87.10	100							
CON B3	65.31	70.37	82.35	84.62	80.00	81.63	75.00	79.31	83.64	80.00	85.25	100						
PRO1 B1	67.92	75.86	80.00	82.14	74.07	71.70	73.33	77.42	71.19	67.80	70.77	75.86	100					
PRO1 B2	62.30	69.70	69.84	71.88	61.29	62.30	76.47	80.00	77.61	77.61	79.45	75.76	82.86	100				
PRO1 B3	64.29	75.41	75.86	74.58	70.18	67.86	82.54	86.15	83.87	74.19	79.41	78.69	76.92	87.67	100			
PRO2 B1	64.29	72.13	72.41	74.58	73.68	71.43	88.89	92.31	87.10	74.19	73.53	75.41	83.08	84.93	88.24	100		
PRO2 B2	60.00	70.77	70.97	73.02	68.85	66.67	86.57	89.86	84.85	72.73	77.78	76.92	81.16	90.91	94.44	94.44	100	
PRO2 B3	62.07	73.02	73.33	75.41	71.19	68.97	86.15	89.55	87.50	75.00	80.00	79.37	77.61	88.00	94.29	91.43	97.30	100

Note: CON = control, PRO1 = *Pediococcus acidilactici*, PRO2 = *Lactobacillus animalis*. A= at day 14, B= at day 28, 1-3 refers to replicate number in each case.

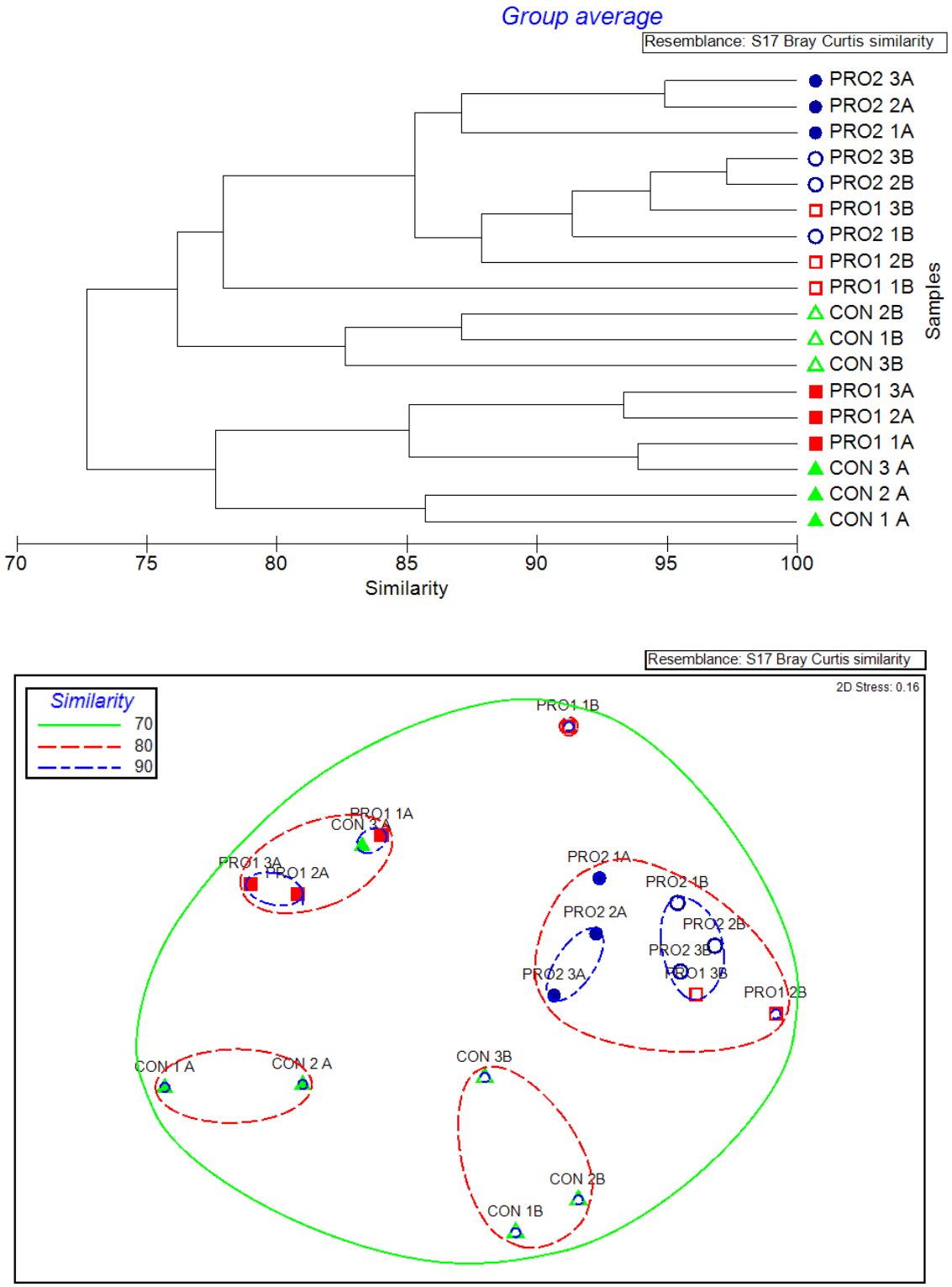


Figure 4.6: (Top) Cluster analysis (Bottom) non-metric multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in poultry caecum. A: 14 days, B: 28 days old of broilers. 1-3 denotes replicate number in each sample.

Table 4.15: Band numbers of caecum bacterial community based on the PCR-DGGE DNA fingerprinting and similarity within treatments (Mean ± SD).

Time (Days)	Treatment	Band number	Similarity
14	CON	24.33±2.51 [b]	82.10±3.73 [a]
	PRO1	23.33±1.52 [b]	88.64±4.23 [a]
	PRO2	29.33±1.52 [a]	89.70±5.35 [a]
	P. value	0.018	0.161
28	CON	29.66±3.78 [a]	84.11±3.68 [ab]
	PRO1	34.66±4.04 [a]	82.48±5.38 [b]
	PRO2	36.00±2.00 [a]	94.39±2.93 [a]
	P. value	0.132	0.024

[a,b] Means with the different superscript in the same column and age are significantly different (P<0.05).

Diversity and richness of caecum microflora were analysed by using Shannon index and Margalef index. Data showed in Table 4.16. The diversity and richness index of bacterial community based on the PCR-DGGE DNA fingerprinting indicated that; at 14 day of age, PRO2 significantly (P<0.01) increased the Shannon index compared with birds fed PRO1 and control group. However, only PRO2 significantly (P<0.01) increased the richness of caecal microflora compared with PRO1. At 28 days of age, both type of probiotic supplementations caused no statistically significant differences in the diversity and richness of PCR-DGGE within caecal digesta.

Table 4.16: Diversity index of bacterial community in caecum digesta based on the PCR-DGGE DNA fingerprinting at different day of age (Mean ± SD).

Time (Days)	Treatment	Shannon index[1]	Margalef index[2]
14	CON	7.30±0.55 [b]	3.18±0.1 [ab]
	PRO1	7.08±0.33 [b]	3.14±0.06 [b]
	PRO2	8.38±0.32 [a]	3.37±0.05 [a]
	P. value	0.018	0.019
28	CON	8.45±0.79 [a]	3.38±0.12 [a]
	PRO1	9.48±0.82 [a]	3.54±0.11 [a]
	PRO2	9.76±0.40 [a]	3.58±0.05 [a]
	P. value	0.132	0.129

[a,b] Means with the same superscript in the same column and age are not significantly different (P<0.05).
[1] Shannon diversity index: H" = -SUM(pi*Log(pi)).
[2] Margalef species richness: d = (S -1)/log (N). (S: Total species, N: Total individuals)

A number of bands (45) were excised from the PCR-DGGE gel and 41 samples were subjected to sequence and BLAST analysis (Figure 4.5). The purification of four bands was not good enough to send for sequencing as recommended by GATC company around (20-80 ng/µl).

The BLAST results of the caecum showed most species were related to *Clostridium* spp., *Lactobacillus* spp., *Ruminococcus* spp., *Eubacterium* spp., *Coprococcus* spp., *Anaerostipes* spp., *Stomatobaculum* spp., *Enterococcus* spp. and *Roseburia* spp. strains (Table 5.17). There were some notable changes in the composition of the caecal microbiota samples compared with the ileal digesta. Most species of the caecum digesta were related to *Clostridium* spp. For example, the sequences of the band numbers 2, 5, 14, 15, 23, 25, 30, 36, 40 and 45 were related to *Clostridium* spp. On the other hand, some bands represented uncultured bacteria as shown in table 4.17.

Table 4.17: Summary results of sequencing analysis bands of PCR-DGGE fingerprints of chicken caecum samples.

Band Number	NCBI Accession number	Max. Identity (%)	NCBI BLAST matches
1	AB279894.1	83	Uncultured *Lactobacillus* sp. isolate: DGGE band: 6b
2	NR_119085.1	99	*Clostridium polysaccharolyticum* strain DSM 1801
3	NR_044265.1	98	*Ruminococcus gauvreauii* strain CCRI-16110
4	NR_104559.2	97	*Enterococcus gallinarum* strain LMG 13129
5	NR_118669.1	99	*Clostridium herbivorans* strain 54408
6	NR_113319.1	99	*Anaerostipes butyraticus* strain JCM 17466
7	NR_074986.1	97	*Lactobacillus crispatus* ST1 strain ST1
8	NR_114779.2	97	*Enterococcus cecorum* strain LMG 12902
9	GQ116215.1	100	Uncultured bacterium clone nbw689c09c1
10	JX527944.1	91	Uncultured *Ruminococcaceae* bacterium clone
11	KC354212.1	100	Uncultured *Lactobacillus* sp. clone
12	AB863735.1	100	*Lactobacillus plantarum* gene
14	NR_025796.1	100	*Clostridium jejuense* strain HY-35-12
15	NR_026103.1	98	*Clostridium populeti* strain 743A
16	FJ508667.1	92	Uncultured bacterium
17	NR_036777.1	100	*Ruminococcus torques* strain VPI B2-51
18	NR_116863.1	98	*Anaerostipes butyraticus* strain 35-7
19	FJ504484.1	100	Uncultured bacterium
20	HE975050.1	98	Uncultured chicken cecal bacterium
21	JQ961836.2	97	Uncultured *Lactobacillus* sp.
23	KF503105.1	99	Uncultured Clostridiales bacterium clone 2288
24	AB470799.1	100	Uncultured bacterium
25	NR_118669.1	100	*Clostridium herbivorans* strain 54408
26	JN021871.1	97	Uncultured bacterium
27	NR_118676.1	95	*Eubacterium xylanophilum* strain ATCC 35991
28	NR_104799.1	100	*Anaerostipes hadrus* strain DSM 3319
29	NR_117792.1	100	*Stomatobaculum longum* strain ACC2
30	NR_118669.1	100	*Clostridium herbivorans* strain 54408
31	NR_113924.1	100	*Enterococcus gallinarum* strain NBRC 100675
32	NR_042057.1	100	*Pediococcus acidilactici* strain DSM 20284
34	NR_125571.1	100	*Oribacterium asaccharolyticum* strain ACB7
35	NR_042832.1	98	*Roseburia faecis* strain M72/1
36	NR_113199.1	98	*Clostridium aminovalericum* strain JCM 11016
37	NR_044049.1	97	*Coprococcus eutactus* strain ATCC 27759
38	NR_117758.1	98	*Roseburia intestinalis* strain DSM 14610
39	NR_028740.1	97	*Clostridium xylanovorans* strain HESP1
40	NR_117711.1	96	*Clostridium formicaceticum* strain DSM
41	KJ616351.1	96	Uncultured bacterium
42	HG326857.1	96	Uncultured bacterium
43	HG326857.1	99	Uncultured bacterium
45	NR_113323.1	96	*Clostridium bifermentans* strain JCM

4.4.6 Effects of probiotic on jejunum histology

The effect of probiotic supplementations on jejunum histology are presented in Table 4.18. Results showed that treatments had highly significant (P<0.001) effects on villus length of jejunum at 14 and 28 days old broiler chicks. The both types of probiotic significantly (P<0.001) increased the villus length and crypt depth compared to the control treatment. While there were no significant differences between PRO1 and PRO2 in the length of villi and crypt depth.

Table 4.18: Effect of probiotic supplementation on villus height (µm), crypt depth (µm) in the Jejunum of SPF broiler chicks at 14 and 21 d of age.

Time (Days)	Parameters	Treatments			P. values
		CON	PRO1	PRO2	
14	Villus height	577.73±35.08 [b]	629.10±30.67 [a]	636.77±29.54 [a]	<0.001
	Crypt depth	84.36±7.68 [b]	97.38±5.99 [a]	96.25±6.63 [a]	<0.001
28	Villus height	668.01±29.14 [b]	719.68±24.45 [a]	737.84±19.02 [a]	<0.001
	Crypt depth	91.73±5.27 [b]	98.97±5.11 [a]	100.44±4.07 [a]	<0.001

[a,b] Means in the same row and age with different superscripts are significantly different ($P < 0.05$).

4.4.7 Relative weight of Bursa of Fabricius

Figure 4.7 illustrated that relative weight of Bursa of Fabricius (BF) from the chicks treated with dietary probiotic supplementation compared to the control treatment. No statistical (P>0.05) differences in the relative weight of BF observed between probiotic supplementations and control treatment, at 14 day of age. The higher weight of BF was observed in both type of PRO1 and PRO2 at 14 and 28 days of age. While, the relative weight of BF significantly (P<0.01) increased in PRO2 compared to PRO1 and control group, at 28 days of experiment.

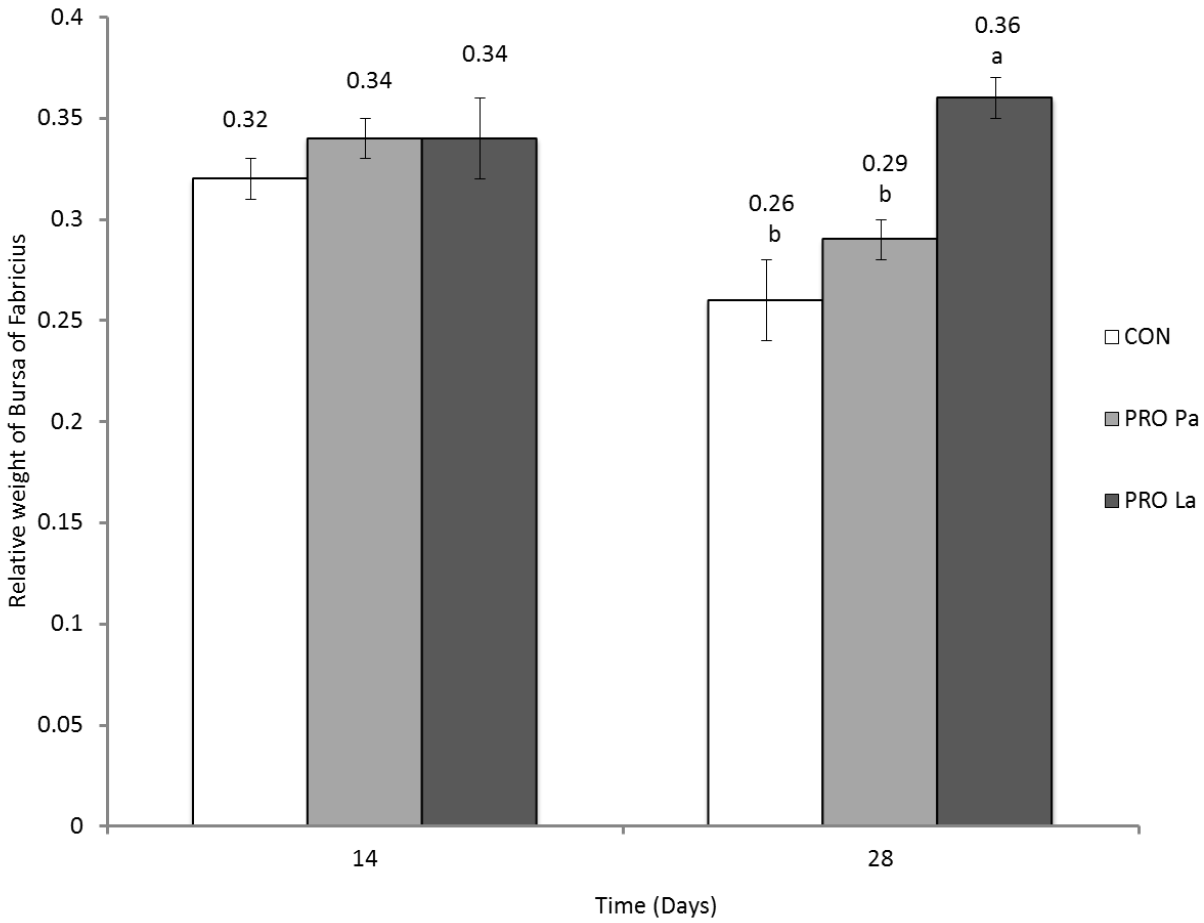


Figure 4.7: Effect of probiotic supplementation on Bursa of Fabricius weight of broiler chicks (Mean ± standard division).

4.4.8 Histology of Bursa of Fabricius

Figure 4.8 showed the results of the Bursa Histology measured in broilers at 14 and 28 days of age. In comparison with the control basal diet with the inclusion of both type of probiotics had a great effect on the size of follicle of Fabricius at 14 and 28 days. The diameters of follicles of Fabricius were increased significantly ($P<0.05$) in dietary probiotic supplementations (100 mg of probiotic/kg) compared to the control treatment, at 14 and 28 days of age. While, the results showed that no significant differences observed between the both types of probiotic treatment for growth of follicles of Fabricius at different day of age.

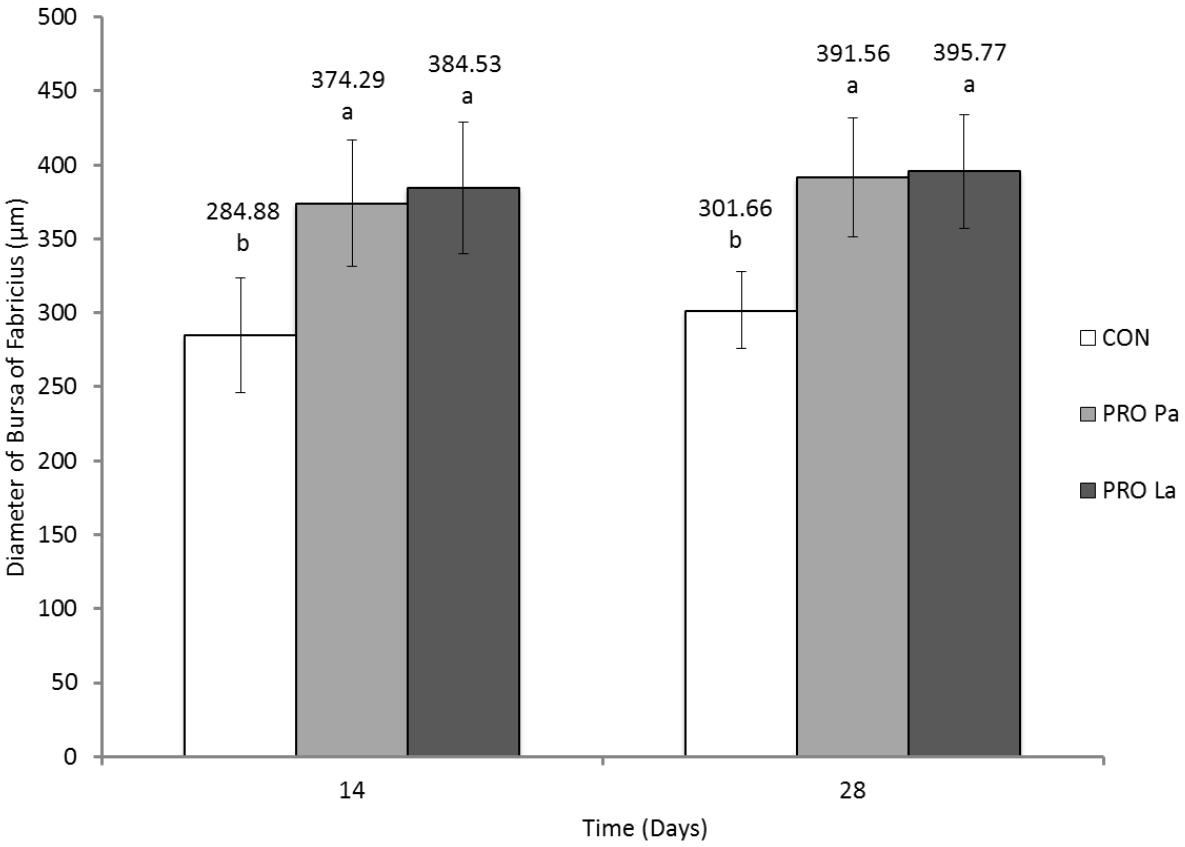


Figure 4.8: Effect of probiotic supplementation on diameter of Bursa of Fabricius of broiler chickens (Mean ± standard division).

4.5 Discussion

The use of probiotics to improve poultry performance and health is increasing due to the recent ban on antimicrobial growth promoters in different production systems. The present study confirmed that beneficial effects of dietary inclusion of probiotic *Lb. animalis* and *P. acidolactici* on gut health and intestinal microflora (i.e. increase beneficial bacteria and improve gut histology). These beneficial effects were directly associated with improvements in production performance of broiler chickens. So far, a variety of microbial species have been used as probiotics in poultry (Ewing and Cole, 1994; Patterson and Burkholder, 2003). In broiler nutrition, probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have a beneficial effect on broiler performance (Jin *et al*., 1998; Zulkifli *et al*., 2000; Kalavathy *et al*., 2003; Kabir *et al*., 2004; Gil De Los Santos *et al*., 2005), modulation of intestinal microflora and pathogen inhibition (Rada and Rychly, 1995; Pascual *et al*., 1999).

The results of the present study showed that average weight gain was significantly ($P<0.05$) increased in PRO1 and PRO2 (7.58% and 7.8%) respectively compared with control. Feed conversion ratio was significantly ($P<0.05$) improved by the dietary supplementation of both types of probiotic compared with the control. European production efficiency factor was significantly ($P<0.05$) increased in PRO1 and PRO2 groups (16.19% and 15.88%) respectively compared with the control group. This result are in agreement with findings of Mohan *et al*. (1996) showed that the use of probiotic containing a similar proportion of six strains of variable organisms namely *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Aspergillus oryzae*,

Streptococcus faecium and *Torulopsis* spp. in feed had a beneficial effect on body weight gain of broiler chicks from 4^{th} to 6^{th} week of age. Jin *et al*. (1996) found that inclusion of probiotic (*Lactobacillus* and *Bacillus subtilis*) in diet stimulated favorable microbial balance in gut and consequently improved feed efficiency and growth performance in broilers. Chiang and Hsieh (1995) reported that broilers fed probiotic-supplemented diet had better weight gain and feed efficiency when compared with the broilers fed the un-supplemented diet. Mountzouris *et al* (2010) observed that diets containing 10^8 cfu probiotic/kg increased body weight of broilers significantly compared with control. Jin *et al*. (1998) reported that the addition of *L. acidophilus* I26 strain or a mixture of 12 lactobacilli to the basal diet of broilers significantly increased their body weight for 0-6 weeks. Similar results on the beneficial effects of *Lactobacillus* cultures on the growth of chickens were also reported by several researchers (Jin *et al*., 1998; Zulkifli *et al*., 2000; Kalavathy *et al*., 2008; Mookiah *et al*., 2014).

In contrast, Awad *et al*. (2009) reported that addition of probiotic to broilers diet did not show any significant effect on body weight compared with control group. Lee *et al*. (2010) noted that *Bacillus* spp. as Direct-fed microbial did not significantly modify body weight gain compared with non-DFM-fed control. Also, several authors (Jung *et al*., 2008; Awad *et al*., 2009; Salianeh *et al*., 2011; Dizaji *et al*., 2013) reported that there were no significant differences in weight gains and body weight of chickens given diets with or without probiotics.

To maintain the intestinal microflora balance in animals it is important to prevent diseases by controlling the overgrowth of potentially pathogenic bacteria. The control of infections through a non-antibiotic approach is urgently requested. The natural bacterial flora (e.g. probiotic bacteria) represents a promising alternative

therapy. The present study showed the influence of dietary probiotic supplementation on the microbial population of the ileum and caecum digesta as detected by culture-based technique and molecular technique in broiler chicks. Beneficial bacteria were increased by adding either commercial probiotic Bactocell or *Lb. animalis* which was isolated from chicken caecum. The higher lactobacilli and bifidobacteria observed in broiler fed in both types of probiotic may be due to the lowering the pH value in the intestine and increasing the production of SCFA as observed in this experiment which has bacteriostatic and bactericidal properties (Fuller, 2001). These results are in agreement with Mountzouris *et al*. (2010) who found that probiotic supplementation (PoultryStar ME, Biomin GmbH, Herzogenburg Austria) in the diets of broilers had the highest *Bifidobacterium* spp. and *Lactobacillus* spp. concentrations compared with control group. Smirnov *et al*. (2005) showed that the use of probiotic (Pro, PrimaLac® StarLabs) 2 g/ kg of diet, containing the viable microorganisms *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* and *Enterococcus faecium* (minimum 1.0×10^8 cfu/g) significantly ($P<0.05$) increased the relative amounts of *Lactobacillus* species in the ileum by 147% compared with control. However, the probiotic did not significant affect the relative amounts of *Lactobacillus* species in the jejunum and duodenum in chickens at 14 days of age.

Probiotic supplementation of the intestinal microflora in poultry, especially with *Lactobacillus* species, showed beneficial effects on resistance to infectious agents such as *Escherichia coli* (Jin *et al*., 1996), *Salmonella* spp. (Pascual *et al*., 1999, Wali, 2012), *Campylobacter* spp. (Stern *et al*., 2001) and, more recently, *Eimeria acervulina* (Dalloul *et al*., 2003). Proposed mechanisms of pathogen inhibition by the probiotic microorganisms include competition for nutrients,

production of antimicrobial conditions and compounds (short chain fatty acids, low pH, and bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system (Rolfe, 2000).

The DGGE analysis separate DNA on the basis of sequence dissimilarities (Netherwood *et al*., 1999). The results of DGGE profiles revealed that the numbers of bacterial species (DGGE gel band numbers) in the ileum and caeca at 14 and 28 days of the PRO2 group was more than PRO1 and control groups. Indeed, the differences of bands in ileum and caeca indicated the changes in predominant microflora by type of feeds. In general, the band numbers in the both types of probiotic groups were higher but not significantly compared the control group, except PRO1 at 14 days in caeca, and this may be because adding *Lb. animalis* and *P. acidolactici* had a role in these changing. In the present study, the richness of bacteria species in both types of probiotics was higher than control group. The high species richness in gut microflora is associated with decreased ability of pathogens to colonize the gut (Dillon *et al.*, 2005).

In the present study, dietary addition of both types of probiotic caused a major increase in the villus height and crypt death in the jejunum when compared with control treatment. The villus height at 14 day was increased about 8.16% and 9.27% for PRO1 and PRO2 respectively compared with the control group. At 28 day of age also villus height was increased about 7.17% and 9.46% for PRO1 and PRO2 respectively compared with the control group. An increase in villus height in the jejunum has been previously reported in broilers fed a probiotic-based diet compared with control treatment (Jin *et al*., 1998; Zhang *et al*., 2005). Pelicano *et al*. (2005) showed that beneficial effects were observed in histological parameters of the intestinal mucosa with the use of probiotics at 21 days of age.

Higher villi in the jejunum ($p<0.01$) were observed when *Bacillus subtilis*-based probiotic was used compared to control diet. A previous study of Pelicano *et al*. (2003) also showed that villus height at 42 days of age was numerically higher in the jejunum of birds fed probiotics based on *Bacillus* spp. and *Lactobacillus* spp. in the diet and water when compared to control birds.

4.6 Conclusion

In conclusion, this study showed beneficial effects of dietary inclusion of *Lb. animalis* based probiotic. This strain which was isolated from a chicken caecum had potential probiotic properties as ability to improve growth performance of broilers compared with the control. Also, the increase of lactobacilli and bifidobacteria in the ileum and caecum digesta, increase villus height and crypt death of jejunum, reduction of pH in ileum, increase the size of follicle of Fabricius were observed by supplementation of PRO1 and PRO2 in the diet of broilers at the end of the experiment. The results from this study showed that both types of probiotic supported the growth of healthy of chicks and could be a suitable candidate as a source of probiotic in broiler diet. There is not difference between both types of probiotic when compared to each other.

CHAPTER FIVE:

The influences of probiotic, prebiotic and synbiotic on gut microflora, immune function, blood characteristics and meat quality of broiler chickens

5.1 Introduction

The aim of this study was to investigate the influence of dietary supplementation of a probiotic (*Lactobacillus animalis*), a prebiotic Jerusalem artichoke tuber (*Helianthus tuberosus*) and a combination of both (Synbiotic) on the production performance, organ weights, length of the small intestine, gut microflora, jejunum histology, immune organ and meat quality of Hubbard broiler chickens.

In the modern intensive poultry production, newly hatched chicks have little chance of contact with their mothers and consequently normal microflora is slow in colonizing the intestine (Fuller, 1989). The development and use of probiotics, prebiotics and synbiotics for poultry is based on the knowledge that the microflora in the gut participates in the resistance to enteric infections and suppresses the growth of pathogenic bacteria, where it has been shown to participate in protection against a variety of pathogenic bacteria including *Escherichia coli*, *Salmonella*, *Campylobacter*, *Clostridium* (Jin *et al*., 1997; Kalavathy *et al*., 2003, 2005, 2009; Murry *et al*., 2006; Dibaji *et al*., 2014).

In the present experiment, a strain identified as *Lactobacillus animalis* in chapter three that was isolated from chicken caecum, and was selected for its probiotic properties and combined with inulin prebiotic as a synbiotic. No previous research has been published on the use of *Lb. animalis* and inulin from Jerusalem artichoke tubers as called a synbiotic.

Few studies report the effects of probiotic, prebiotic and synbiotics on the gut microflora using molecular methods and development of the digestive system and meat quality of broiler chickens. Therefore, the present study evaluated the use of these three products on production performance, gut microflora, characteristics of intestinal tract and meat quality of broiler chickens.

5.2 Material and Methods

5.2.1 Experimental design and treatments

Seventy two one-day-old male broiler chicks (Hubbard strain) were used for this experiment in a 2 x 2 factorial design, considering two variables (Probiotic and Prebiotic) with two levels (0 and 1) in the diet. There were four treatments with three replications (six chicks per replicate). Some parameters were distributed in a 3 x 2 factorial design, considering three variables (Probiotic, Prebiotic and Time) with two levels (0 and 1) in the diet and different time (17 and 35 days). The chicks were obtained from a commercial hatchery (P D Hooks Hatcheries Kentisbere, Devon, UK) and housed in 100x80 cm pens. Wood shavings were used as floor bedding for the first two weeks and straw for last three weeks. The chicks were allocated assigned to receive one of four dietary treatments. Broiler diets in the form of ground were prepared as in chapter 4.12 (NRC, 1994). The feed and water were supplied *ad libitum*. The duration of the trial was 35 days. Chicks were assigned to the following treatments:

CON = Feed standard broiler diet (No Probiotic / No Prebiotic), **(Control)**

PRO = Standard broiler diet + 100 mg *Lactobacillus animalis* / kg of diet, containing 1.72×10^{10} cfu/kg (Probiotic / No Prebiotic), **(Probiotic)**

PRE = Standard broiler diet + 1% Jerusalem artichoke inulin (No Probiotic / Prebiotic) **(Prebiotic)**

SYN = Standard broiler diet + combination between 100 mg Probiotic (1.72×10^{10} cfu/kg) + 1% Prebiotic / kg diet (Probiotic / Prebiotic), **(Synbiotic)**

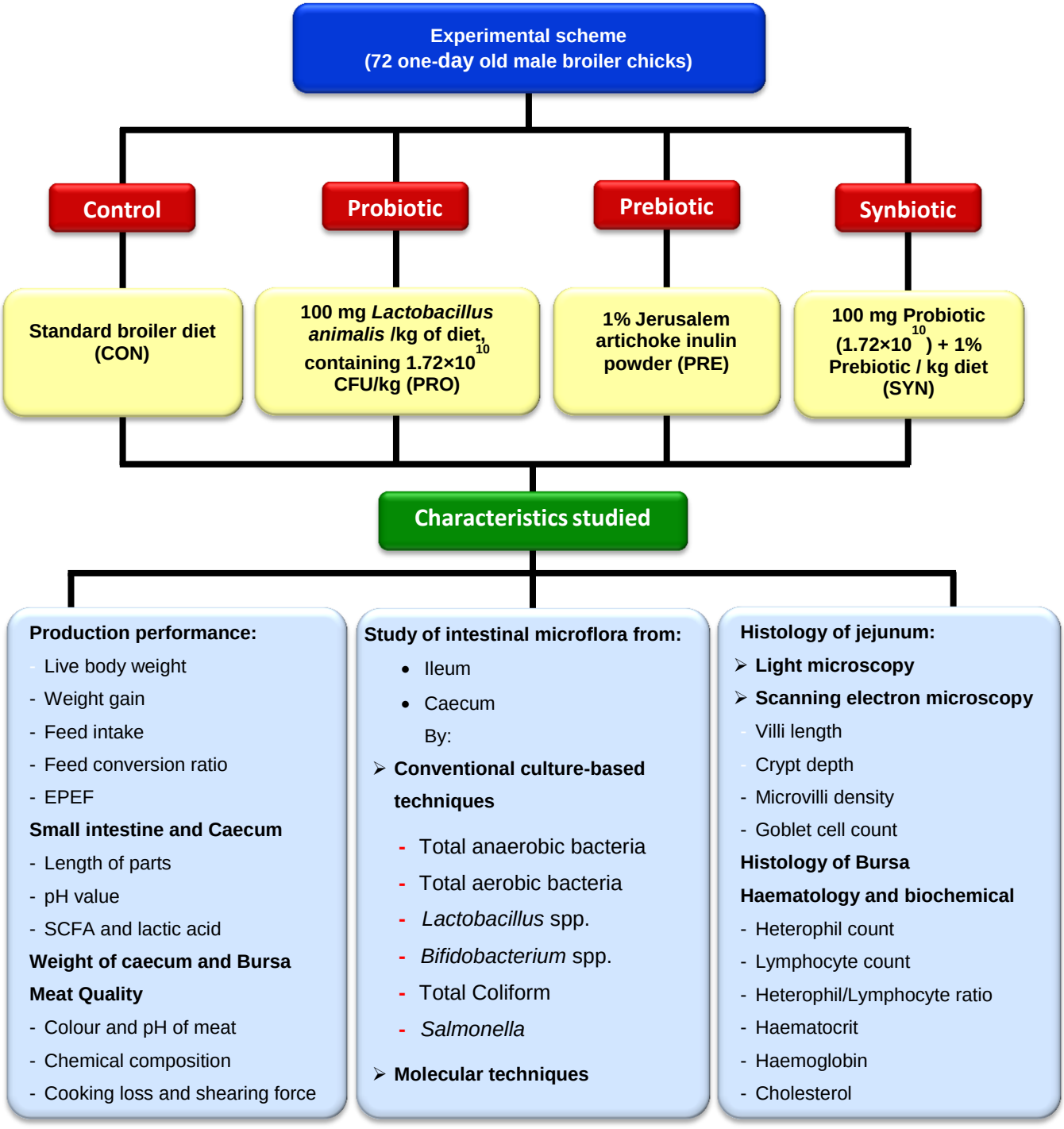


Figure 5.1: Layout of the feed trial.

5.2.2 Characteristics studied

4.2.2.1 Production performance

Body weight gain and feed consumption were monitored weekly and feed conversion ratio was calculated as feed consumed per unit of weight gain as described in Section 4.2.3.1.

5.2.2.2 Gut microflora analysis

At day 17, six chicks per treatment were killed and the rest are killed at the end of the trial (day 35). Post-mortem 1 gm of gut content (Ileum and caeca) from two chicks per replicate were aseptically removed and used for the assessment of gut microflora population changes using standard microbiology (culture techniques) and molecular microbiology as described in Section 3.2.7 and 3.2.8, except the DNA extraction as described in Section 4.2.3.2 including DNA extractions, polymerase chain reaction (PCR) followed by agarose gel electrophoresis (AGE), and denaturant grade gel electrophoresis (DGGE) analysis and lastly gene sequences. Selected bands (OTU) of DGGE gel were aseptically separated and sequenced according to whether the band represented many groups or a unique band for particular groups and match with BLAST at NCBI to confirm the name of the bacteria. The pH and SCFA measurements described in section 3.2.5 and 3.2.6 respectively.

4.2.2.3 Length of GI tract

Length of whole small intestine, duodenum, jejunum, ileum and caeca were measured using a tape measure and caecum and Bursa of Fabricius weights were recorded by using an electronic digital balance.

5.2.2.4 Histomorphology

5.2.2.4.1 Light microscopy (LM)

Chick's intestine (Jejunum sections) and Bursa of Fabricius were taken and used for assessment of histological examination by light microscopy as described in section 3.2.9 and 3.2.10, respectively. The samples stained with haematoxylin and eosin for measurement of villus length and crypt depth. While, for goblet cell measurement Alcian blue and Periodic acid stains (PAS) was used.

5.2.2.4.2 Scanning electron microscopy (SEM)

SEM samples were taken from the jejunum of three birds per treatment. Typically, intestinal samples from jejunum (0.5 mm) were excised and washed thoroughly in 1% Scarboxymethyl- L-cysteine for 30 Sec in order to remove epithelial mucus. Samples were then fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (1: 1 vol., pH 7.2, 3% NaCl). Fixative removal of samples was carried out by rinsing two times with distilled water for 15 min. Dehydration was achieved by placing samples in graded ethanol solutions (30%, 50%, 70%, 90%) for at least 15 min each and then twice in 100%. After the dehydration process samples were critically point dried with ethanol as the intermediate fluid and CO_2 as the transition fluid (Emitech K850; Kent, UK) for one hour. Dried samples are then mounted on aluminium stubs and gold coated using an Emitech K550 sputter coater (Kent, UK). Samples were then examined with a Jeol JSM 6610 LV scanning electron microscope at 15 kV (Jeol, Tokyo, Japan) (see figure 5.2). SEM images were taken with high magnification (x20000) and analysed using image J software in order to calculate the density of the microvilli (MD). A thresholding

technique for Images was used to differentiate the ratio between the microvilli covered area (M, foreground) to the background (B, background), MD=M/B, and was measured in arbitrary units (AU). Images were analysed blind to prevent bias and typically three images per sample were analysed.

Figure 5.2: Scanning electron microbiology unit at the University of Plymouth.

5.2.2.5 Haematological parameters

At 35 days of age, three birds from each treatment were selected and 1-1.5 ml blood samples were collected to determine the Haematocrit, haemoglobin, leukocyte counts and Heterophil/Lymphocyte ratio. The blood samples were collected in test tubes with anticoagulant (K_2EDTA). Leukocyte counts as described in chapter four, Section 3.2.11.

5.2.2.5.1 Haematocrit (Hct)

Haematocrit is used as an indicator of animal health and is the percentage of packed blood cells to plasma volume (Rao & Deshpande, 2005). In order to measure haematocrit fresh blood was drawn into heparinised haematocrit tubes by capillary rise and sealed with Cristaseal. Heparinized capillary tubes were filled to ¾ with blood. Capillaries were centrifuged at 12500 rpm for five min (Thermo, Heraeus Pico 17, Haematokritrotor, Germany). Haematocrit values were measured as the total percentage packed cell volume (PCV) using a Hawksley Micro-Haematocrit reader.

5.2.2.5.2 Haemoglobin (Hb)

Haemoglobin (Hb) concentration was calculated based on Drabkin's cyanide-ferricyanide solution as described by Rao & Deshpande (2005). Briefly, the Drabkin‘s reagent consists of dissolved 50mg of potassium cyanide, 20mg of potassium ferricyanide and 1g of sodium bicarbonate made the volume to 1l in a conical flask using distilled water and stored in a borosilicate glass bottle for later use.

The assay was performed by adding 20 µl of whole blood to 5 ml of Drabkin‟s reagent, and vortexes immediately. The haemoglobin was measured at 540 nm using a spectrophotometer (Thermo spectronic, Helious Epsilon, USA) against a blank containing 5 ml Drabkin‟s reagent and 20 µl distilled water. Haemoglobin absorbance was measured from a curve prepared from reference standards (cyanmethaemoglobin; Sigma diagnostic kit Nº 525 A). The values obtained are expressed in g/dl.

5.2.2.5.3 Cholesterol Determination

At the end of experiment, cholesterol samples were determined from whole blood using Accutrent® GC (Boehringer Mannheim GmbH, Germany) figure 5.3. The machine was calibrated with the code strip and then cholesterol content was measured in whole blood using strips inserted into the meter according to the manufacturer's instruction. The Accutrend® GC meter measures the intensity of the reaction colour within the reaction layer of the test strip by reflectance photometry and calculates the parameter concentration of the sample through a lot-specific algorithm. The result is displayed in mg/dl or mmol/l and stored automatically with time and date.

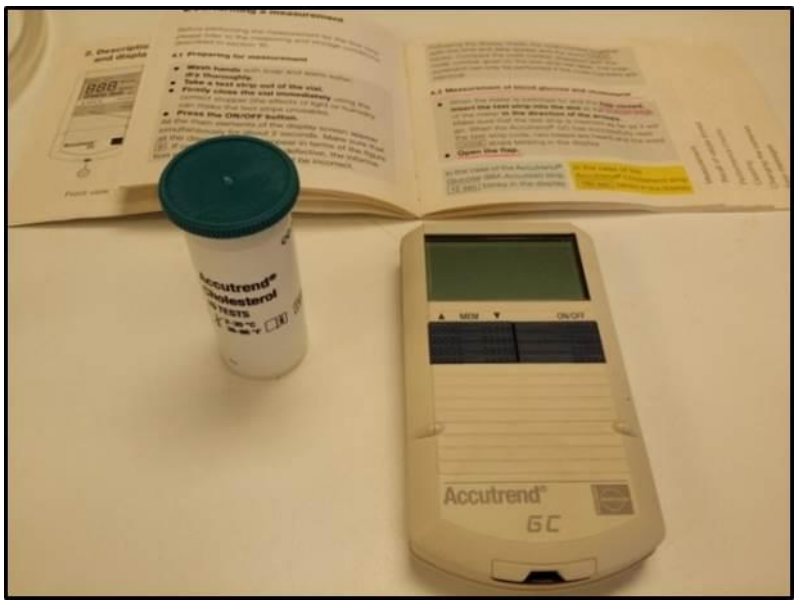


Figure 5.3: Accutrent® GC meter for cholesterol determination.

5.2.2.6 Meat quality

5.2.2.6.1 Chemical composition of meat

At the end of the experiment (35 days), three chickens from each treatment were randomly selected and slaughtered. Proximate chemical composition (moisture, protein, lipid and ash content) in triplicate of breast and leg were determined according to the standard methods with slight modification for automatic equipment and analytical instrumentation (AOAC, 2002).

5.2.2.6.1.1 Moisture

All samples were weighed and dried (in triplicate) at 105 ºC with a fan assisted oven (Gallenkamp Oven BS, Model; OV-160, England) until a constant weight was achieved. Percentage moisture was calculated by:

$$\textbf{Moisture (\%)} = \frac{\text{Wet weight (g)} - \text{Dry weight (g)}}{\text{Wet weight (g)}} \times 100$$

Dry matter or total solid was measured as: (100 - % moisture)

5.2.2.6.1.2 Ash

Ash (total mineral or organic matter) content was determined in duplicate by combusting known dry weight of sample (~500 mg) into a pre-weighed crucible. The crucibles were then incinerated in a muffle furnace (Carbolite, Sheffield, England) at 550 ºC for 12h until light grey ash results or to constant weight. Percentage ash was determined from the sample residue by:

$$\textbf{Ash (\%)} = \frac{\text{Sample residue (g)} - \text{crucible weight (g)}}{\text{initial sample weight (g)}} \times 100$$

5.2.2.6.1.3 Lipid

Lipid content was determined in duplicate using the Soxhlet extraction method. Samples were weighed (~3 g) and placed into a cellulose thimble lightly plugged with cotton wool and inserted into the condensers of a SoxTecTM extraction system (Tecator Systems, Högnäs, Sweden; model Soxtec 1043 and service unit 1046). Pre-weighed cups containing 40 mL of ether extract are clamped into the condenser and the extraction settings are moved to the boiling position for 30 min, after which extraction was set to the rinsing position for a further 45 min. The cups containing extracted lipid were then transferred to a fume cupboard for 30 min before final weighing (Figure 5.4). Lipid content was determined as:

$$\textbf{Total lipid (\%)} = \frac{\text{Final weight of cup (g)} - \text{Initial weight of cup (g)}}{\text{Initial weight of cup (g)}} \times 100$$

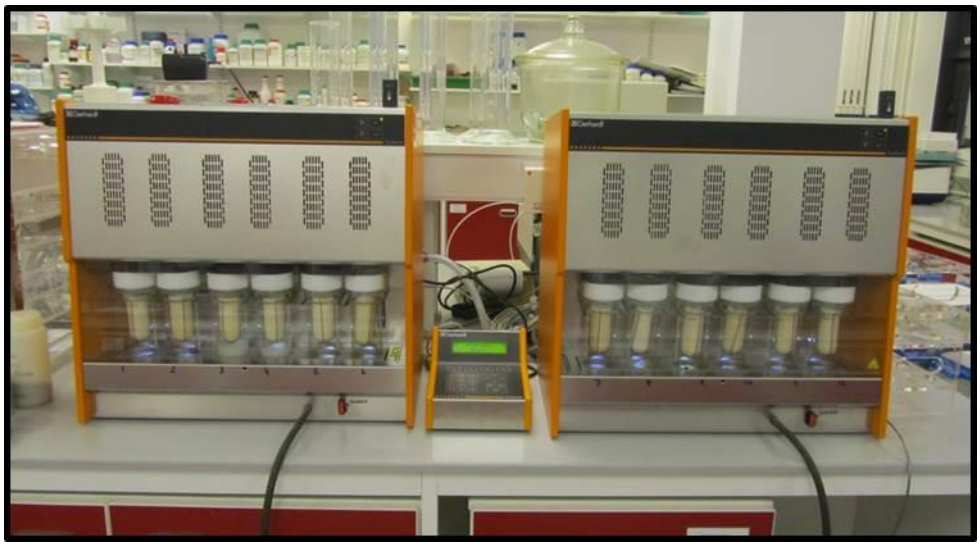


Figure 5.4: Soxhlet system operated in the nutrition laboratory of the University of Plymouth.

5.2.2.6.1.4 Protein

Determination of crude protein (CP) in breast and leg meat was done by the Kjeldahl method to gain the total nitrogen (N) content. This value is then multiplied by a factor 6.25 to calculate the crude protein content. Briefly, 100 mg of sample was weighed directly into a Kjeldahl digestion tube along with a catalyst tablet (3g K_2SO_4 , 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2; BDH Ltd. Poole, UK) and 10 mL of concentrated sulphuric acid (H_2SO_4) (Sp. Gr. BDH Ltd. Poole, UK). Digestion was performed with a Gerhardt Kejldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 100 ºC for 30 min, 225 ºC for 45 min and at 380° C for 60 min. The tube rack was removed from the heating block and allowed to cool down during the additional 30 min. After this digestion stage the samples are distilled using Vodapest 40 automatic distillation unit (Gerhardt Laboratory Instruments, 81 Bonn, Germany) (Figure 5.5). The distillate was neutralised with concentrated H_2SO_4 and from the titration value crude protein determined as;

$$\textbf{Crude protein (\%)} = \frac{[(ST - BT) \times 0.10 \times 14 \times 6.25]}{SW\ (mg)} \times 100$$

Where 0.10 is the molarity of the acid, 14 the relative atomic mass of nitrogen and 6.25 a constant relationship between N and the animal protein of the sample. ST is sample titre (mL), BT is blank titre (mL) and SW is the initial sample weight (mg).

Figure 5.5: Left is computerized digestion block and right is distillation unit of the Kjeldahl system utilized (Gerhardt Laboratory instruments) at the University of Plymouth.

5.2.2.6.2 The pH value of meat

The pH was measured using direct insertion of a Hanna Instruments electrode (Hanna Instruments, UK) into breast and leg muscles immediately after slaughter. An incision 0.5 to 1 cm deep was made to allow insertion of the electrode (Schneider *et al*., 2012).

5.2.2.6.3 Colour of meat

A Minolta CR-400 (Konica Minolta Sensing Americas Inc., Ramsey, NJ) colorimeter (aperture size: 8 mm; light source: illuminant D65) was used to assess the colour [CIE; lightness L*, redness a*, and yellowness b*] of breast and leg muscles, where L* is the chrome associated to meat lightness, a* is the chrome that ranges between green to red and b* is the chrome that ranges between blue and yellow, according to the methodology proposed by (Pelicano *et*

al., 2005). Standard calibration with black and white tiles was used before measurements. Colour was measured at the surface of individual breast and leg fillets in an area free from obvious colour defects (bruises, blood spots, or surface discolorations). Breast and leg meat colour were measured on three birds per treatment and were taken in different position on each samples and the average reading was recorded.

5.2.2.6.4 Cooking loss

Cooking loss (CL) was determined five hours after slaughter in an oven pre-warmed to 170°C, according to the methodology proposed by (Pelicano *et al*., 2003; Jeong *et al*., 2011). Raw breast meat samples were weighed and put in trays with aluminium grills previously dried in an incubator. The trays were placed inside the oven until sample core temperature reached 75°C. Temperature was monitored with thermocouple inserted to the thickest parts of one of the middle breast samples on the tray. The thermocouple was attached to a digital thermometer/logger (Comark, model 2502, Sper Scientific Ltd., Scottsdale, AZ) during cooking. After cooking, the fillets were removed from the trays, individually covered with foil, and stored overnight at 3°C in plastic bags. The following day, the cooked breasts were brought to room temperature and weighed again to determine cooking loss. CL was calculated as the difference between the initial and the final sample weights.

5.2.2.6.5 Shearing Force

The samples used to determine cooking loss were the same as those used to evaluate shearing force. Shear force was determined using a Texture Analyser (Texture Analyser, Model TA-HDi®, England) connected to a Warner-Bratzler blade according to the method of (Jeong *et al*., 2011). A texture analyser was calibrated with a 100-kg load cell; the Warner-Bratzler blade was set at 10 mm/s, and the test was triggered by a 10-g contact force. After all samples were at room temperature, they were cut into slices of approximately 2.0 × 2.0 × 1.3 cm^3 and placed in a way that the fibres were oriented perpendicularly to the Warner-Bratzler blade. Three shear force measurements per breast fillet were made, and the shear force value (N) was calculated as (kgf).

Figure 5.6: Texture analyser unit at the University of Plymouth.

5.3 Statistics analysis

All data were analysed by 2×2 and 3×2 factorial design using Minitab statistics software. Primer-6 software was used for composition of bacterial profile as described in Section 3.3.

5.4 Results

5.4.1 Performance parameters

Table 5.1 showed the effect of probiotics, prebiotics and synbiotics on live body weight of broiler chickens during the experiment. The results showed the chickens weight increased by 4.47%, 2.21% and 5.26% for probiotic, prebiotic and synbiotic, respectively compared with control group at the end of the experiment.

Table 5.2 refer to the estimation of coefficients of probiotic, prebiotic and instruction between probiotic and prebiotic on live body weight. Probiotic, prebiotic and interaction between both showed no significant differences in live body weight for the initial weight, first and third week of age of broiler chickens. However, only the treatment of probiotic had highly significant effect ($p<0.01$) on live body weight at second, fourth and fifth week of age. The chicks treated with synbiotics showed highest live body weight than the other groups at the end of experiment. While, there was no significant interaction between probiotic and prebiotic on live body weight, which indicates that probiotic utilization in the diet had independent effect on the live body weight at the end of the experiment.

Table 5.3 showed that weekly and average weight gain of broiler chicks during the experiment. There was an increase in final weight gain of about 79 g, 39 g and 94 g per chicks of probiotic, prebiotic and synbiotic, respectively compared with the control group.

The estimated coefficients in table 5.4 showed the probiotic, prebiotic and interaction between both showed no significant differences in weekly weight gain of broiler chicks. While, the coefficients of probiotic and prebiotic were

significantly increased in final weight gain. However, there was no significant interaction between probiotic and prebiotic on the final weight gain.

The weekly and average feed intakes of broiler chicks during the experiment are shown in table 5.5. The results showed the final feed intake was decreased for prebiotics and synbiotics compared with the probiotics and control groups.

The estimated coefficients in table 5.6 showed the weekly and final feed intake were decreased for prebiotics and synbiotics compared to the probiotics and control groups. Probiotic was significantly ($P<0.01$) reduced the feed intake in the first and third weeks. Prebiotic had a highly significant ($P<0.01$) effect on all weeks and final feed intake. However, there was no significant interaction between probiotic and prebiotic on weekly and final feed intake.

Table 5.7 showed the additives supplementations had an effect in feed conversion ratio at 35 days of age. The estimated coefficients table 5.8 showed the chicks with probiotic and prebiotic groups had a significant ($P<0.01$) improvement in final feed conversion ratio. While, there was no significant interaction between probiotic and prebiotic on the final feed conversion ratio.

Table 5.9 showed the European production efficiency factor (EPEF) and mortality of broiler chickens during the experiment. In all treatments, there were no mortalities. The estimated coefficients table 5.10 showed the probiotic and prebiotic had significant improvement on the EPEF at the end of the experiment. However, there was no significant interaction between the probiotic and prebiotic on EPEF.

Table 5.1: Effect of probiotic, prebiotic and synbiotic on weekly and final live body weight (g) of broiler chicks (Mean ± standard division).

Time (Weeks)	Treatment			
	CON	PRO	PRE	SYN
0	40.3±2.60	41.2±2.81	40.3±2.80	40.1±2.51
1	161.7±13.16	168.8±11.73	162.9±16.78	169.9±13.82
2	380.3±41.36	396.4±48.33	377.8±43.78	411.8±46.26
3	725.3±51.99	755.6±45.23	729.2±56.35	762.6±31.84
4	1160.5±41.5	1223.6±41.2	1174.3±44.7	1221.6±47.7
5	1686.2±52.0	1765.2±68.8	1724.4±44.3	1779.9±50.9

Table 5.2: Estimated coefficients for weekly live body weight (g) of broiler chicks.

Term	Initial weight		1^{st} week		2^{nd} week		3^{rd} week		4^{th} week		5^{th} week	
	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.
Constant	40.4683	***	165.830	***	391.581	***	743.146	***	1195.00	***	1738.92	***
PRO	0.1667	NS	3.500	NS	12.556	**	15.938	NS	27.58	***	33.62	***
PRE	-0.2483	NS	0.585	NS	3.221	NS	2.729	NS	2.96	NS	13.25	NS
PRO*PRE	-0.2767	NS	-0.028	NS	4.472	NS	0.771	NS	-3.96	NS	-5.87	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

Table 5.3: Effect of probiotic, prebiotic and synbiotic on weekly and final body weight gain (g) of broiler chicks (Mean ± standard division).

Time	Treatment			
	CON	PRO	PRE	SYN
1st week	121.44±7.84	127.61±3.26	122.61±6.99	129.78±3.80
2nd week	218.56±22.63	227.67±10.34	214.83±12.67	241.94±2.75
3rd week	344.97±53.84	359.14±15.04	351.39±32.59	350.75±18.03
4th week	435.25±44.53	468.00±5.38	445.17±52.04	459.00±32.63
5th week	525.33±11.36	541.58±26.86	550.42±12.95	558.33±23.77
Final WG	1645.6±16.7	1724.0±34.8	1684.4±3.7 bc	1739.8±13.6

Table 5.4: Estimated coefficients for weekly and final body weight gain (g) of broiler chicks.

Term	1st week		2nd week		3rd week		4th week		5th week		Final WG	
	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.
Constant	125.362	***	225.751	***	351.565	***	451.854	***	543.917	***	1698.45	***
PRO	3.333	NS	9.056	NS	3.382	NS	11.646	NS	6.042	NS	33.46	***
PRE	0.833	NS	2.636	NS	-0.492	NS	0.229	NS	10.458	NS	13.66	**
PRO*PRE	0.248	NS	4.501	NS	-3.702	NS	-4.729	NS	-2.083	NS	-5.77	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

Table 5.5: Effect of probiotic, prebiotic and synbiotic on weekly and accumulative feed intake (g) of broiler chicks (Mean ± standard division).

Time	Treatment			
	CON	PRO	PRE	SYN
1st week	129.44±3.31	123.94±2.66	124.00±4.21	117.61±2.13
2nd week	256.27±4.68	263.61±2.11	245.55±2.71	244.44±3.31
3rd week	538.90±7.09	525.19±9.50	503.05±6.99	495.28±6.81
4th week	953.83±12.63	971.25±10.97	884.08±14.87	888.75±11.03
5th week	1134.3±21.0	1111.3±20.7	1007.0±22.3	994.3±15.6
Final FI	3012.7±35.1	2995.3±27.7	2763.7±30.5	2740.4±24.0

Table 5.6: Estimated coefficients for weekly and accumulative feed intake (g) of broiler chicks.

Term	1st week		2nd week		3rd week		4th week		5th week		Final FI	
	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.
Constant	123.746	***	252.469	***	515.61	***	924.48	***	1061.73	***	2878.0	***
PRO	-2.973	**	1.556	NS	-5.37	**	5.52	NS	-8.90	NS	-10.2	NS
PRE	-2.944	**	-7.472	***	-16.44	***	-38.06	***	-61.06	***	-126.0	***
PRO*PRE	-0.222	NS	-2.112	NS	1.49	NS	-3.19	NS	2.56	NS	-1.5	NS

***= P<0.000, **= P<0.01, *= P<0.05 and NS = Non-significant.

Table 5.7: Effect of probiotic, prebiotic and synbiotic on weekly and feed conversion ratio of broiler chicks (Mean ± standard division).

Time	Treatment			
	CON	PRO	PRE	SYN
1st week	1.06±0.06	0.96±0.01	1.01±0.08	0.90±0.02
2nd week	1.17±0.14	1.15±0.05	1.14±0.08	1.00±0.01
3rd week	1.58±0.25	1.45±0.03	1.43±0.15	1.41±0.09
4th week	2.20±0.20	2.07±0.01	2.00±0.24	1.94±0.12
5th week	2.15±0.06	2.05±0.10	1.82±0.005	1.77±0.08
Final FCR	1.83±0.02	1.73±0.03	1.63±0.02	1.57±0.02

Table 5.8: Estimated coefficients for weekly and final feed conversion ratio of broiler chicks.

Term	1st week		2nd week		3rd week		4th week		5th week		Final FCR	
	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.	Coefficient	P.
Constant	0.985	***	1.119	***	1.472	***	2.052	***	1.950	***	1.692	***
PRO	-0.051	**	-0.039	NS	-0.037	NS	-0.047	NS	-0.037	NS	-0.039	**
PRE	-0.03	NS	-0.045	NS	-0.047	NS	-0.082	NS	-0.150	***	-0.089	***
PRO*PRE	-0.003	NS	-0.027	NS	-0.025	NS	0.017	NS	0.014	NS	0.009	NS

***= P<0.000, **= P<0.01, *= P<0.05 and NS = Non-significant.

Table 5.9: Effect of probiotic, prebiotic and synbiotic on production index and mortality percentage of broiler chicks (Mean ± standard division).

Treatment	EPEF	Mortality %
CON	262.35±5.94	0
PRO	290.82±11.80	0
PRE	300.90±3.86	0
SYN	322.11±7.09	0

EPEF: European Production Efficiency Factor.

Table 5.10: Estimated coefficients for probiotic, prebiotic and synbiotic on production index of broiler chicks.

Term	EPEF	
	Coefficient	P. value
Constant	294.044	***
PRO	12.419	**
PRE	17.461	***
PRO*PRE	-1.817	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.2 The pH value of intestinal tract

Table 5.11 shows the pH values of digesta in the ileum and caecum at 17 and 35 days of age. The pH value of the ileal digesta due to dietary probiotic, prebiotic and synbiotic supplementation were decreased being 6.70, 6.69 and 6.69, respectively compared with the control group 7.34, at 17 days of age. Also, at the end of experiment, the pH value in ileum was reduced compared to the control group. In caeca, all diets treated with probiotic, prebiotic and synbiotic supplementation also had effect on the pH value.

The estimated coefficients table 5.8 showed the coefficients of probiotic, prebiotic, times and interaction between probiotic and prebiotic had a significant effect on pH in ileal and caecal digesta. However, interaction between probiotic with time,

prebiotic with time and all factors together (probiotic with prebiotic and times) not significantly (P>0.05) affected on the pH value in ileum digesta. While, only prebiotic with time had significant effect on pH value in caecum digesta.

Table 5.11: Effect of probiotic, prebiotic and synbiotic on pH value in ileal and caecal digesta of broiler chicks at different days of age (Mean ± standard division).

Time (Days)	Position	Treatment			
		CON	PRO	PRE	SYN
17	Ileum	7.34±0.19	6.70±0.08	6.69±0.15	6.69±0.18
	Caecum	6.13±0.27	5.48±0.12	5.89±0.09	5.73±0.18
35	Ileum	6.13±0.10	5.84±0.07	5.43±0.42	5.36±0.32
	Caecum	5.92±0.06	5.44±0.15	5.48±0.21	5.28±0.17

Table 5.12: Estimated coefficients for probiotic, prebiotic and synbiotic on pH value in ileal and caecal digesta of broiler chicks.

Term	pH ileum		pH Caecum	
	Coefficient	P. value	Coefficient	P. value
Constant	6.276	***	5.672	***
PRO	-0.125	*	-0.186	***
PRE	-0.228	***	-0.075	*
Time	-0.582	***	-0.138	**
PRO*PRE	0.106	*	0.095	*
PRO*Time	0.035	NS	0.017	NS
PRE*Time	-0.062	NS	-0.075	*
PRO*PRE*Time	-0.051	NS	-0.0267	NS

***= P<0.000, **= P<0.01, *= P<0.05 and NS = Non-significant.

5.4.3 Changes in the Short-Chain Fatty Acids and Lactate Concentrations

Table 5.13 showed the effects of probiotic, prebiotic and synbiotic supplementation on the short chain fatty acid in the ileal and caecal digesta of broiler chicks at the end of experiment. All additives supplementations were increased the lactic acid, acetic acid, propionic acid and butyric acid compared with the control group in ileal and caecal digesta, except acetic acid in prebiotic group in caecal digesta.

In ileal digesta, diets treated with probiotic, prebiotic and interaction between probiotic and prebiotic had a highly significant ($P<0.05$) effect on the lactic acid and propionic acid. Only prebiotic had significant ($P<0.05$) effect on acetic acid. Butyric acid was significantly ($P<0.01$) increased in probiotic and prebiotic groups. While, there was no significant interaction observed between the two factors (PRO*PRE) on butyric acid, which indicates that probiotic and prebiotic utilization in the diet had independent effect on butyric acid at the end of the experiment (Table 5.14)

Table 5.14 also showed the coefficient for lactic acid was significantly ($P<0.01$) increased in probiotic and prebiotic in the caecal digesta. While, the interaction between probiotic and prebiotic were not significant ($P>0.05$) in caecal digesta. Only probiotic had highly significant ($P<0.01$) effect on acetic acid. Propionic acid was significantly ($P<0.01$) and ($P<0.05$) increased in probiotic and interaction of prebiotic and probiotic, respectively. While, only prebiotic had highly significant ($P<0.01$) effect on butyric acid in caecum digesta.

Table 5.13: Influence of supplementation of probiotic, prebiotic and synbiotic on the short-chain fatty acid (mmol/L) profile in the ileal and caecal digesta of broilers at the end of the experiment (means ± SD).

Position	Treatment	SCFA			
		Lactic acid	Acetic acid	Propionic acid	Butyric acid
Ileum	CON	6.809±0.38	12.687±1.65	1.931±0.40	2.306±0.23
	PRO	14.188±1.06	15.747±1.95	2.883±0.37	2.886±0.53
	PRE	8.768±0.96	16.506±1.50	1.989±0.26	3.794±0.24
	SYN	18.845±1.17	17.801±1.70	4.300±0.28	4.715±0.27
Caecum	CON	3.594±1.29	12.714±1.74	1.318±0.13	1.905±0.15
	PRO	11.344±1.78	14.652±2.80	2.436±0.27	2.431±0.11
	PRE	5.986±1.12	12.096±1.30	1.775±0.12	2.778±0.27
	SYN	14.929±0.61	17.239±0.75	2.186±0.35	2.860±0.37

Table 5.14: Estimated coefficients for probiotic, prebiotic and synbiotic on the short-chain fatty acid profile in the ileal and caecal digesta of broiler chicks.

item	Term	Lactic acid		Acetic acid		Propionic acid		Butyric acid	
		Coefficient	*P.*	Coefficient	*P.*	Coefficient	*P.*	Coefficient	*P.*
Ileum	Constant	12.152	***	15.685	***	2.775	***	3.4256	***
	PRO	4.364	***	1.088	ND	0.815	***	0.375	**
	PRE	1.654	***	1.468	*	0.368	**	0.829	***
	PRO*PRE	0.674	*	-0.441	ND	0.339	**	0.085	NS
Caecum	Constant	8.963	***	14.175	***	1.929	***	2.493	***
	PRO	4.173	***	1.770	**	0.382	**	0.152	NS
	PRE	1.494	**	0.492	NS	0.051	NS	0.325	**
	PRO*PRE	0.298	NS	0.801	NS	-0.111	*	-0.111	NS

***= $P<0.000$, **= $P<0.01$, *= $P<0.05$ and NS = Non-significant.

5.4.4 Length of digestive tract

Table 5.15 showed the effects of probiotic, prebiotic and synbiotic supplementation on the length of small intestine parts and caecum of broiler chicks at the end of experiment. All additives supplementation increased the length of small intestine and separate parts compared with control group. Synbiotic showed the highest length of small intestine compared with the other groups. Also, the best length of jejunum and ileum was obtained in birds of synbiotic group followed by probiotic, prebiotic and control group.

The estimated coefficients table 5.16 showed the probiotic group only significantly ($P<0.05$) increased the length of duodenum, jejunum and ileum. While prebiotic did not significantly ($P>0.05$) increase the length of these parts of the digestive tract. However, both probiotic and prebiotic significantly ($P<0.01$) and ($P<0.05$) respectively increased the length of small intestine. While, the interaction between probiotic and prebiotic were not significant for the all parameters.

The estimated coefficients table 5.16 also showed the caecum length was significantly ($P<0.05$) increased only in prebiotic group. While, probiotic and interaction between probiotic and prebiotic had no significant ($P>0.05$) effect on caecum length.

Table 5.15: Effect of probiotic, prebiotic and synbiotic on small intestine parts length (cm) of 35 days old broilers chicks (Mean ± standard division).

Treatment	Duodenum[1]	Jejunum	Ileum	Small intestine	Caeca
CON	25.66±1.52	61.00±4.00	59.66±1.52	146.00±7.00	16.66±0.57
PRO	31.33±1.15	70.00±3.60	66.66±3.05	168.00±7.00	17.00±1.00
PRE	29.33±2.30	67.33±4.72	63.66±3.51	160.30±5.77	18.33±0.57
SYN	31.33±2.51	73.66±4.16	69.00±6.55	174.00±7.81	18.33±1.15

[1] Results are mean values from three replications ± standard deviations.

Table 5.16: Estimated coefficients for probiotic, prebiotic and synbiotic on small intestine parts length (cm) of 35 days old broilers chicks.

Term	Duodenum		Jejunum		Ileum		Small intestine		Caeca	
	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value
Constant	29.416	***	68.00	***	64.750	***	162.083	***	17.5833	***
PRO	1.916	***	3.833	*	3.083	*	8.917	**	0.0833	NS
PRE	0.916	NS	2.500	NS	1.583	NS	5.083	*	0.7500	*
PRO*PRE	0.916	NS	-0.666	NS	-0.416	NS	-2.083	NS	-0.0833	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.5 Weight of Caecum

Table 5.17 showed the effect of probiotic, prebiotic and synbiotic on the relative weight of caecum at different age of broiler chicks. The probiotic, prebiotic and synbiotic had a positive effect on relative caecum weight at 17 days old broiler chicks compared to the control group. At 35 day of age, chicks that fed on prebiotic and synbiotic had higher caecal percentages compared to the control group being 0.57, 0.51 and 0.40 % per body weight, respectively.

Table 5.18 showed the estimation of coefficients of probiotic, prebiotic and interaction between probiotic and prebiotic on the caecum weight, only prebiotic significantly increased weight of caecum. While, there were no significant (P>0.05) differences observed for times, probiotic and interactions between probiotic and prebiotic. Also, there were no significant effect between the interaction of probiotic with time, prebiotic with time and synbiotic with time on the caecum weight.

Table 5.17: Effect of probiotic, prebiotic and synbiotic on relative caecum weigh of broiler chicks (Mean ± standard division).

Treatment	Time (Days)	
	17	35
CON	0.37±0.06	0.40±0.07
PRO	0.47±0.02	0.38±0.06
PRE	0.50±0.01	0.57±0.04
SYN	0.51±0.03	0.51±0.08

[1] Results are mean values from three replications ± standard deviations.

Table 5.18: Estimated coefficients for probiotic, prebiotic and synbiotic on relative caecum weigh of broiler chicks.

Term	Relative caecum weigh	
	Coefficient	*P*. value
Constant	0.466	***
PRO	0.004	NS
PRE	0.058	***
Time	-0.0008	NS
PRO*PRE	-0.015	NS
PRO*Time	-0.025	NS
PRE*Time	0.017	NS
PRO*PRE*Time	0.005	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.6 Effects of probiotics, prebiotics and synbiotics on gut microflora

5.4.6.1 Microbial enumeration by conventional based method

Table 5.19 showed the effect of probiotic, prebiotic and synbiotic supplementation on the microflora composition in the ileum digesta of broiler chicks at 17 and 35 days of age. The results showed the all additives supplementation increased the numbers of total anaerobic, *Lactobacillus* spp. and *Bifidobacterium* spp. compared with control group at 17 and 35 days of age. On the other hand, the numbers of total aerobic bacteria and total coliform in all additives supplementation were decreased in the ileum digesta compared with control group at 17 and 35 days of age. At 35 days, the highest number of *Bifidobacterium* spp. was recorded for synbiotic, prebiotic and probiotic supplementation (10.39±0.03, 10.25±0.03 and 10.21±0.12 Log_{10} CFU ml^{-1}) respectively compared with control group (10.00±0.11 Log_{10} CFU ml^{-1}) in the ileum.

The estimated coefficient table 5.20 showed the probiotic, prebiotic and interaction between both significantly ($P<0.01$) increased the number of total anaerobic bacteria and lactobacilli and significantly ($P<0.01$) reduced total coliform bacteria in ileum digesta. While, the interaction between probiotic and prebiotic did not significantly ($P>0.05$) effect total aerobic bacteria and *Bifidobacterium* spp. Also, the time did not significantly ($P<0.05$) effect the number of total anaerobic bacteria. The interaction between probiotic with time had a significant effect on the total aerobic bacteria and lactobacilli. While, there were no significant differences observed between the interactions of probiotic with time on the total anaerobic bacteria, *Bifidobacterium* spp. and coliform bacteria. However, the interaction between prebiotic and time had a significant effect on

the total anaerobic bacteria, *Bifidobacterium* spp. and coliform bacteria. Finally, only total coliform bacteria were significantly ($P<0.05$) influenced by the interaction between probiotic, prebiotic and times.

The composition of caecal microflora of broilers at 17 and 35 days of the experiment is shown in Table 5.21. The results showed the all additives supplementation increased the numbers of total anaerobic, *Lactobacillus* spp. and *Bifidobacterium* spp. compared with control group at 17 and 35 days of age. On the other hand, the numbers of total aerobic bacteria and total coliform in all additives supplementation were decreased in the caecal digesta compared with control group at 17 and 35 days of age.

Table 5.22 showed the coefficients of probiotic and the interaction between probiotic and prebiotic significantly ($P<0.01$) increased the number of total anaerobic bacteria. The total aerobic bacteria were significantly decreased in probiotic, prebiotic, time and interactions between probiotic and prebiotic and probiotic and time in caecal digesta. The probiotic and time were significantly ($P<0.01$) increased the number of lactobacilli and significantly reduced the total coliform bacteria in caecum. While, the prebiotic and the interactions between probiotic and prebiotic, probiotic and time, prebiotic and time and probiotic and prebiotic and time were not significant. The *Bifidobacterium* spp. were significantly ($P<0.01$) increased in probiotic, prebiotic and time in caecal digesta. However, there were no significant observed between all the interactions.

Table 5.19: Bacterial counts (Log_{10} CFU mL^{-1}) at 17 and 35 days of age in ileum digesta of broiler chicks.

Time (Days)	Microbes	Treatments			
		CON	PRO	PRE	SYN
17	Total anaerobic	8.91±0.10	9.76±0.06	9.73±0.10	9.92±0.07
	Total aerobic	9.23±0.36	8.34±0.09	8.67±0.03	8.18±0.12
	Lactobacillus spp.	8.52±0.10	8.99±0.06	8.85±0.11	9.03±0.05
	Bifidobacterium spp.	10.18±0.03	10.34±0.03	10.28±0.07	10.37±0.05
	Total Coliform	7.76±0.08	7.09±0.08	7.07±0.18	7.06±0.10
	Salmonella	n.d.	n.d.	n.d.	n.d.
35	Total anaerobic	9.30±0.44	9.91±0.04	9.68±0.14	9.85±0.06
	Total aerobic	8.22±0.04	7.95±0.08	8.04±0.05	7.75±0.14
	Lactobacillus spp.	9.16±0.04	9.35±0.13	9.28±0.12	9.35±0.07
	Bifidobacterium spp.	10.00±0.11	10.21±0.12	10.25±0.03	10.39±0.03
	Total Coliform	7.15±0.10	6.89±0.14	7.01±0.06	6.94±0.06
	Salmonella spp.	n.d.	n.d.	n.d.	n.d.

n.d. : Not detected

Table 5.20: Estimated coefficients for bacterial counts (Log10 CFU mL-1) at 17 and 35 days of age in ileum digesta of broiler chicks.

Term	Total anaerobic		Total aerobic		*Lactobacillus* spp.		*Bifidobacterium* spp.		Total Coliform	
	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value
Constant	9.637	***	8.301	***	9.068	***	10.255	***	7.124	***
PRO	0.227	***	-0.241	***	0.115	***	0.075	***	-0.125	***
PRE	0.162	***	-0.137	***	0.061	**	0.070	***	-0.100	***
Time	0.052	ND	-0.307	***	0.219	***	-0.040	*	-0.123	***
PRO*PRE	-0.138	**	0.048	ND	-0.050	*	-0.018	ND	0.108	***
PRO*Time	-0.032	ND	0.102	**	-0.047	*	0.011	ND	0.042	ND
PRE*Time	-0.082	*	0.042	ND	-0.030	ND	0.037	*	0.078	**
PRO*PRE*Time	0.027	ND	-0.052	ND	0.020	ND	0.001	ND	-0.057	*

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

Table 5.21: Bacterial counts (Log_{10} CFU mL^{-1}) at 17 and 35 days of age in caecal digesta of broiler chicks.

Time (Days)	Microbes	Treatments			
		CON	PRO	PRE	SYN
17	Total anaerobic	9.85±0.08	10.19±0.08	9.94±0.07	10.05±0.07
	Total aerobic	8.94±0.05	8.37±0.35	7.99±0.04	7.89±0.07
	Lactobacillus spp.	7.98±0.19	8.48±0.44	8.23±0.09	8.45±0.10
	Bifidobacterium spp.	9.90±0.09	10.10±0.06	10.04±0.06	10.15±0.04
	Total Coliform	7.85±0.10	7.39±0.13	7.53±0.12	7.30±0.13
	Salmonella	n.d.	n.d.	n.d.	n.d.
35	Total anaerobic	9.79±0.11	10.23±0.09	9.95±0.10	10.09±0.10
	Total aerobic	8.11±0.15	7.77±0.08	7.84±0.14	7.70±0.15
	Lactobacillus spp.	9.02±0.09	9.32±0.07	9.17±0.15	9.36±0.08
	Bifidobacterium spp.	10.11±0.05	10.39±0.04	10.24±0.04	10.41±0.05
	Total Coliform	7.15±0.05	6.61±0.39	7.09±0.04	6.63±0.34
	Salmonella spp.	n.d.	n.d.	n.d.	n.d.

n.d. : Not detected

Table 5.22: Estimated coefficients for bacterial counts (Log_{10} CFU mL^{-1}) at 17 and 35 days of age in caecum digesta of broilers.

Term	Total anaerobic		Total aerobic		*Lactobacillus* spp.		*Bifidobacterium* spp.		Total Coliform	
	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value
Constant	10.014	***	8.079	***	8.755	***	10.170	***	7.197	***
PRO	0.129	***	-0.143	**	0.152	**	0.096	***	-0.211	***
PRE	-0.002	NS	-0.221	***	0.051	NS	0.041	**	-0.056	NS
Time	0.005	NS	-0.221	***	0.465	***	0.120	***	-0.325	***
PRO*PRE	-0.065	**	0.083	*	-0.048	NS	-0.024	NS	0.039	NS
PRO*Time	0.016	NS	0.023	NS	-0.027	NS	0.016	NS	-0.039	NS
PRE*Time	0.010	NS	0.136	**	-0.005	NS	-0.005	NS	0.045	NS
PRO*PRE*Time	-0.010	NS	0.033	NS	0.021	NS	-0.001	NS	-0.017	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.6.2 Molecular microbiology of caecum and ileum digesta

5.4.6.2.1 Spectrophotometric assay

All the results of DNA concentrations in caeca and ileum samples were more than 20ng/µl, from 17 and 35 days. The protein and humic acid contamination of is higher than 1.7.

5.4.6.2.2 PCR-DGGE analysis

The amplified DNA template from the caecum and ileum samples appeared as single bands by the agarose gel electrophoresis as shown in Figure 5.7. A single band is desirable for successful PCR. Although, some samples in ileum appeared to have double band but at very close proximity.

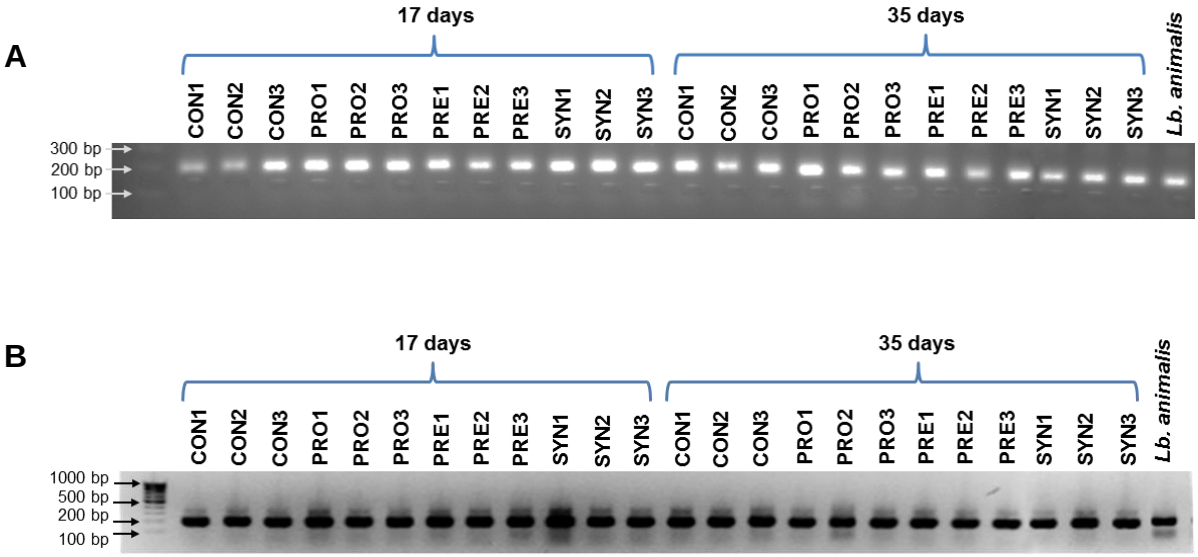


Figure 5.7: PCR amplified product of DNA templates of the Caecum (A) and ileum (B) samples at different days and pure *Lb. animalis* strains.

5.4.6.2.3 DGGE analysis of caeca bacterial community

Figure 5.8 shows the PCR–DGGE bacterial profiles of the digesta from a chicken caecum at 17 (A) and 35 (B) days of age. Many different bands are shown in the DGGE image and the gel bands which are called operative taxonomy units (OTU) in each sample.

The similarity of bacterial population within and between the treatments were measured by nonmetric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 5.9. The both analyses of caecal bacteria populations showed more similarity within samples from same treatments than those from other groups. The half matrix similarity of caeca DGGE fingerprints is shown in (Table 5.23) indicates the average similarity within the control treatment is 42.4% at day 17 and 63.57% in day 35, probiotic 67.02% at day 17 and 63.75% at day 35, prebiotic 56.31% at day 17 and 58.75% at day 35, synbiotic 56.76% at day 17 and 73.54% at day 35. The average bacterial population similarity between control groups at day 17 and 35 was 52.98%, while the probiotic was 65.38%, prebiotic was 57.53% and synbiotic was 65.15%.

There were 39 DNA bands detectable in synbiotic group increased compared with probiotic, prebiotic and control groups being 32, 29 and 28 bands, respectively at 17 days. The average bands also were increased in all additives supplementation at 35 days old compared with control group (Table 5.24).

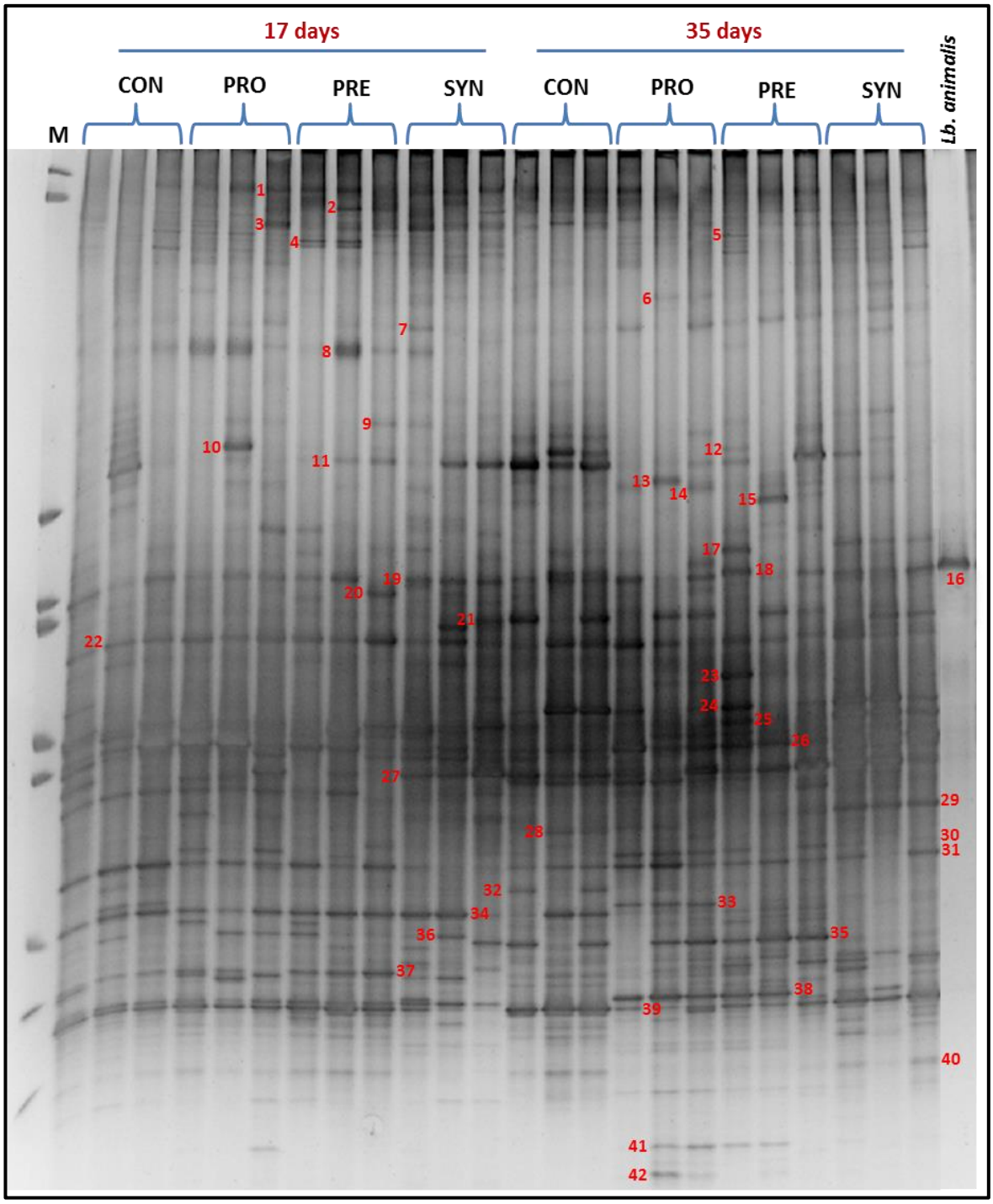


Figure 5.8: DGGE fingerprints of caecum digesta of treated and control group chicks at 17 and 35 days of age. Numbers represent the bands or operative taxonomy unites (OUT) excised and sequenced.

Table 5.23: The half matrix similarity of bacterial population of DGGE fingerprints of caeca showing the similarities between the replicates treatment.

Group	CON A1	CON A2	CON A3	PRO A1	PRO A2	PRO A3	PRE A1	PRE A2	PRE A3	SYN A1	SYN A2	SYN A3	CON B1	CON B2	CON B3	PRO B1	PRO B2	PRO B3	PRE B1	PRE B2	PRE B3	SYN B1	SYN B2	SYN B3
CON A1	100																							
CON A2	40.67	100																						
CON A3	47.27	39.28	100																					
PRO A1	45.61	37.93	62.96	100																				
PRO A2	44.44	46.87	60	67.74	100																			
PRO A3	31.25	46.15	45.9	66.66	66.66	100																		
PRE A1	50	45.61	60.37	54.54	59.01	48.38	100																	
PRE A2	40.67	43.33	46.42	55.17	59.37	64.61	66.66	100																
PRE A3	42.62	41.93	48.27	46.66	57.57	59.7	47.45	54.83	100															
SYN A1	31.88	45.71	30.3	35.29	43.24	50.66	41.79	57.14	58.33	100														
SYN A2	38.8	41.17	37.5	48.48	44.44	49.31	55.38	52.94	51.42	56.41	100													
SYN A3	35.29	28.98	40	41.79	46.57	54.05	48.48	52.17	45.07	53.16	54.54	100												
CON B1	36.66	32.78	45.61	44.06	40	48.48	41.37	52.45	50.79	47.88	43.47	45.71	100											
CON B2	49.23	42.42	54.83	53.12	48.57	50.7	50.79	54.54	47.05	39.47	40.54	42.66	62.68	100										
CON B3	35.48	31.74	40.67	42.62	35.82	38.23	36.66	41.26	46.15	43.83	33.8	33.33	59.37	57.97	100									
PRO B1	38.09	34.37	33.33	48.38	44.11	49.27	45.9	46.87	36.36	51.35	47.22	52.05	40	48.57	44.77	100								
PRO B2	42.85	45.07	35.82	46.37	42.66	44.73	47.05	45.07	52.05	44.44	45.56	50	41.66	41.55	35.13	64	100							
PRO B3	40	39.43	41.79	46.37	48	50	38.23	50.7	46.57	44.44	48.1	50	38.88	49.35	40.54	56	70.73	100						
PRE B1	38.88	43.83	37.68	45.07	46.75	51.28	40	52.05	56	53.01	49.38	48.78	37.83	37.97	34.21	49.35	64.28	66.66	100					
PRE B2	24.61	36.36	32.25	40.62	40	50.7	38.09	48.48	32.35	36.84	43.24	40	38.8	38.88	28.98	42.85	51.94	62.33	60.75	100				
PRE B3	30.3	32.83	34.92	43.07	45.07	41.66	34.37	47.76	37.68	38.96	37.33	36.84	38.23	41.09	42.85	47.88	51.28	51.28	52.5	63.01	100			
SYN B1	43.47	31.42	42.42	52.94	43.24	45.33	38.8	48.57	33.33	30	43.58	48.1	33.8	50	35.61	45.94	44.44	46.91	45.78	44.73	46.75	100		
SYN B2	37.14	28.16	38.8	49.27	45.33	42.1	32.35	36.61	30.13	29.62	40.5	42.5	44.44	46.75	35.13	48	39.02	46.34	40.47	41.55	48.71	71.6	100	
SYN B3	46.37	22.85	36.36	52.94	45.94	42.66	38.8	45.71	30.55	37.5	43.58	48.1	36.61	42.1	32.87	51.35	46.91	49.38	40.96	42.1	46.75	72.5	76.54	100

Note: CON = control, PRO = probiotic, PRE = prebiotic, SYN = synbiotic. A= at day 17, B= at day 35, 1-3 refers to replicate number in each case, (n=24).

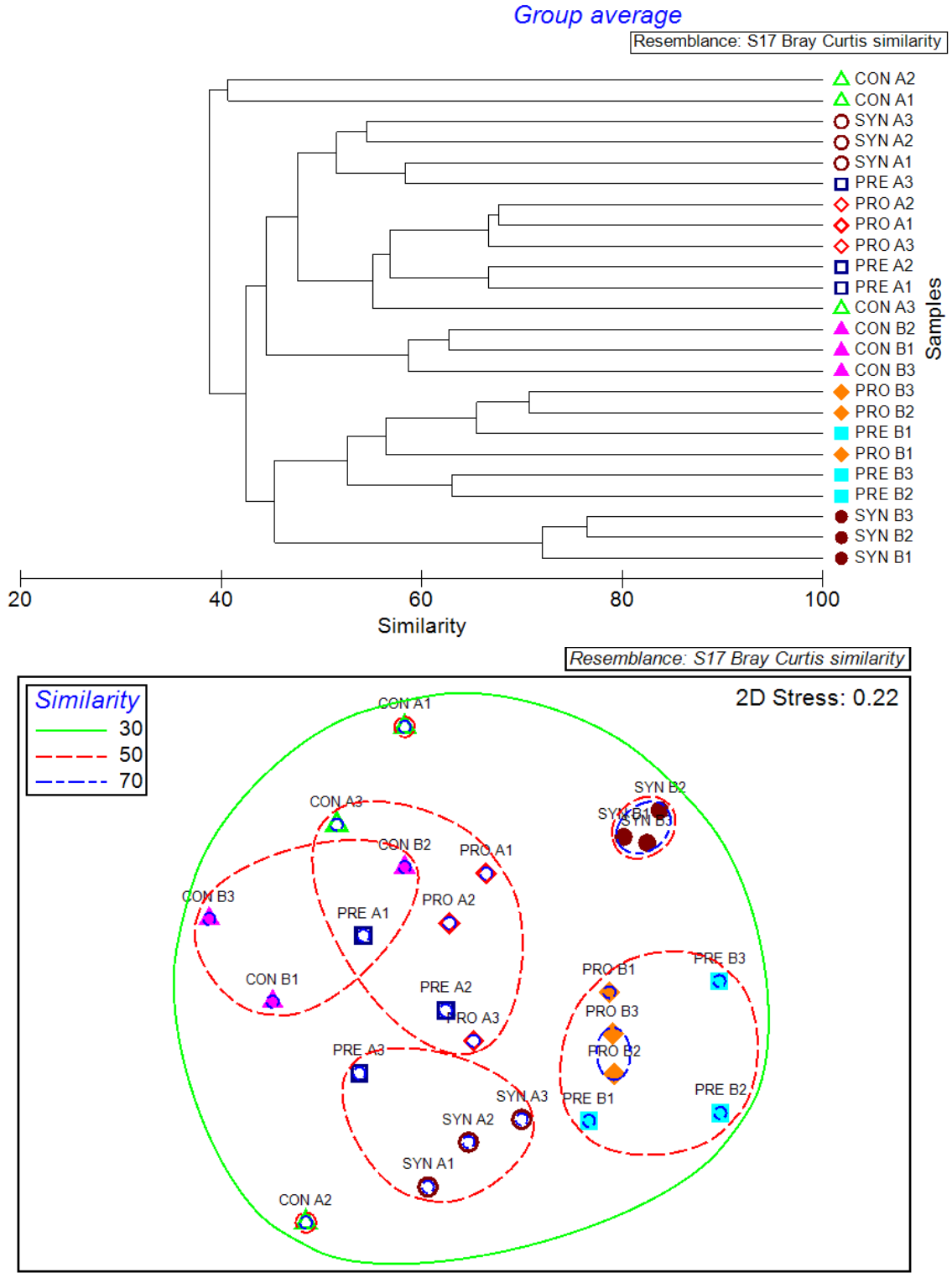


Figure 5.9: (Top) Cluster analysis (Bottom) non-metric multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in poultry caeca. A: 17 days, B: 35 days old of broilers. 1-3 denotes replicate number in each sample.

The estimated coefficient table 5.25 showed the probiotic, prebiotic and time had highly significant effect on the band numbers. While, the interaction between probiotic and prebiotic, probiotic and time, prebiotic and time and probiotic and prebiotic with time had not significant (P>0.05) effect on the band numbers. Table 5.25 also showed that probiotic and time were significantly (P<0.001) increased the similarity of the bacterial profile. Only, the interaction between probiotic and prebiotic and times were significantly (P<0.01) increased the similarity in caecum digesta.

Table 5.24: Band numbers of bacterial community in caecal based on the PCR-DGGE DNA fingerprinting and similarity within treatments (Mean ± SD).

Time (Days)	Treatment	Band number	Similarity
17	CON	28.33±2.08	42.40±4.26
	PRO	32.33±3.78	67.02±0.62
	PRE	29.66±2.5	56.31±9.69
	SYN	39.00±1.00	56.76±1.78
35	CON	33.33±2.5	60.00±2.41
	PRO	38.66±4.04	63.57±7.37
	PRE	38.66±3.78	58.75±5.53
	SYN	40.33±0.57	73.54±2.63

Table 5.25: Estimated coefficients for band numbers of bacterial community in caecal based on the PCR-DGGE DNA fingerprinting and similarity within treatments.

Term	Band number		Similarity	
	Coefficient	*P*. value	Coefficient	*P*. value
Constant	35.042	***	59.799	***
PRO	2.542	***	5.429	***
PRE	1.875	**	1.546	NS
Time	2.708	***	4.172	**
PRO*PRE	0.208	NS	-1.617	NS
PRO*Time	-0.792	NS	-0.838	NS
PRE*Time	-0.125	NS	0.633	NS
PRO*PRE*Time	-1.125	NS	4.423	**

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

Diversity analysis of caecal microflora showed in table 5.26. The Shannon index and Margalef index indicate the diversity and richness of alimentary canal microflora of broilers (Amann *et al*., 1993; Chen *et al*., 2012). These indexes were used to display the microbial population diversity and richness in the caeca. The diversity index of bacterial community based on the PCR-DGGE DNA fingerprinting indicated that; at 17 day of age, birds fed the diets containing probiotic, prebiotic and synbiotic had greater Shannon index and Margalef index than birds fed control group.

The estimated coefficients table 5.27 showed the probiotic, prebiotic and time had highly significant effect on the diversity and richness of bacteria profile in caecum digesta. However, there were no significant (P>0.05) interaction between the probiotic and prebiotic, probiotic with time, prebiotic with time and probiotic and prebiotic with time on the diversity and richness of population of bacteria.

Table 5.26: Diversity index of bacterial community in caecal digesta based on the PCR-DGGE DNA fingerprinting at different day of age (Mean ± SD).

Time (Days)	Treatment	Shannon index[1]	Margalef index[2]
17	CON	3.34±0.07	8.17±0.44
	PRO	3.47±0.12	9.00±0.79
	PRE	3.38±0.08	8.45±0.53
	SYN	3.66±0.02	10.37±0.2
35	CON	3.50±0.07	9.21±0.51
	PRO	3.65±0.10	10.29±0.81
	PRE	3.65±0.09	10.30±0.75
	SYN	3.69±0.01	10.63±0.11

[1] Shannon diversity index: H‟ = -SUM(pi*Log(pi)).
[2] Margalef species richness: d = (S -1)／log (N). (S: Total species, N: Total individuals)

Table 5.27: Estimated Coefficients for diversity index of bacterial community in caecal digesta based on the PCR-DGGE DNA fingerprinting.

Term	Shannon index		Margalef index	
	Coefficient	*P*. value	Coefficient	*P*. value
Constant	3.546	***	9.557	***
PRO	0.074	***	0.521	***
PRE	0.053	**	0.383	**
Time	0.080	***	0.556	***
PRO*PRE	0.005	NS	0.041	NS
PRO*Time	-0.026	NS	-0.167	NS
PRE*Time	-0.005	NS	-0.027	NS
PRO*PRE*Time	-0.031	NS	-0.228	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.6.2.4 Sequence analysis

Forty bands were excised from the PCR-DGGE gel and 23 bands were sequenced but unfortunately only 14 samples were returned and subjected to BLAST analysis and the others were below the required standard and sequencing data was zero. The results of purification were not good to send all the samples for the sequencing as recommended by GATC company around (20-80 ng/µl). The results of the trial sequence analysis shown in Table 5.28. The most family BLAST results in caecum were related to *Clostridium* spp., *Ruminococcus* spp., *Lactobacillus* spp., *Eubacterium* spp., *Coprococcus* spp. and *Anaerostipes* spp. strains.

Table 5.28: Summary results of sequencing analysis bands of PCR-DGGE fingerprints of chicken caecum samples.

Band Number	NCBI Accession number	Max. Identity (%)	NCBI BLAST matches
1	NR 025796.1	100	*Clostridium jejuense* strain HY-35-12
2	NR 044265.1	100	*Ruminococcus gauvreauii* strain CCRI-16110
3	NR 117566.1	99	*Peptoniphilus indolicus* strain ATCC 29427
8	NR 118676.1	97	*Eubacterium xylanophilum* strain ATCC 35991
10	JF709467.1	98	Uncultured bacterium clone EDBAC06G05
16	JX944776.1	95	*Lactobacillus* sp. OR 11
20	NR 118669.1	99	*Clostridium herbivorans strain 54408*
21	KF109414.1	97	Uncultured bacterium clone nck331a03c1
23	JX851714.1	98	Uncultured bacterium clone PCS439
25	NR 104799.1	98	*Anaerostipes hadrus* strain DSM 3319
29	NR 044049.1	99	*Coprococcus elutactus* strain ATCC 27759
30	NR 115502.1	97	*Ruminococcus torques* strain GIFU 12126
39	NR 113319.1	97	*Anaerostipes butyraticus* strain JCM 17466
40	FJ833032.1	97	Uncultured bacterium clone A1Q102

5.4.6.2.5 DGGE analysis of ileum bacterial community

Figure 5.10 shows the PCR–DGGE bacterial profiles of the digesta from a chicken ileum at 17 (A) and 35 (B) days of age. Many different OTU are shown in the DGGE image, but the PCR-DGGE analysis was not revealed complex microbial communities as present in caecum digesta.

The similarity of bacterial population within and between the treatments were measured by nonmetric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 5.11. The both analyses of ileum bacteria populations showed more similarity within samples from same treatments than those from other groups. The average DNA band numbers were detected in synbiotic group (23.33) increased compared with probiotic, prebiotic and control groups being 13, 15 and 12.33 bands, respectively at 17 days. However, the average bands were increased of all additives supplementation compared with control group at 35 days (Table 5.30).

The estimated coefficients table 5.31 showed the probiotic and prebiotic were significantly increased band numbers in ileum digesta. The interactions between probiotic and prebiotic and probiotic and time were not significant. While, the interactions between prebiotic and time and probiotic, prebiotic and time were significantly influenced. Table 5.31 also showed that probiotic, prebiotic, time and all interaction between the three factors (Probiotic, Prebiotic and Time) were not significant on similarity in ileum digesta.

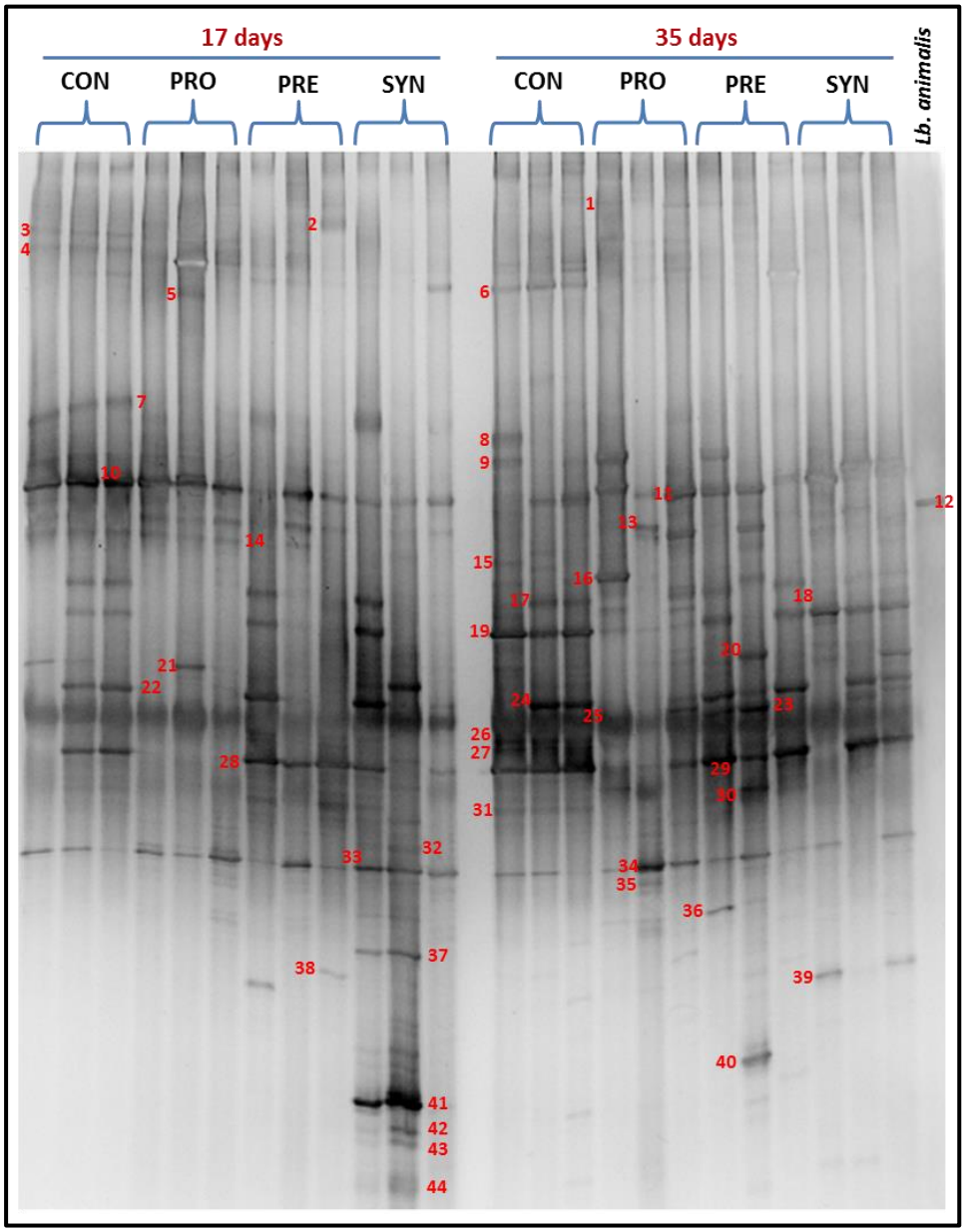


Figure 5.10: DGGE fingerprints of ileum digesta of treated and control group chicks at 17 and 35 days of age. Numbers represent the bands or operative taxonomy unites (OUT) excised and sequenced.

Table 5.29: The half matrix similarity of bacterial population of DGGE fingerprints of ileum showing the similarities between the replicates treatment.

Group	CON A1	CON A2	CON A3	PRO A1	PRO A2	PRO A3	PRE A1	PRE A2	PRE A3	SYN A1	SYN A2	SYN A3	CON B1	CON B2	CON B3	PRO B1	PRO B2	PRO B3	PRE B1	PRE B2	PRE B3	SYN B1	SYN B2	SYN B3
CON A1	100																							
CON A2	76.92	100																						
CON A3	66.67	81.48	100																					
PRO A1	66.67	50.00	52.63	100																				
PRO A2	86.96	75.86	58.33	66.67	100																			
PRO A3	57.14	64.71	48.28	53.85	64.52	100																		
PRE A1	48.00	77.42	61.54	26.09	57.14	42.42	100																	
PRE A2	50.00	73.33	64.00	36.36	51.85	62.50	62.07	100																
PRE A3	38.46	62.50	51.85	25.00	48.28	47.06	77.42	66.67	100															
SYN A1	33.33	52.38	37.84	29.41	41.03	50.00	58.54	50.00	52.38	100														
SYN A2	27.78	47.62	32.43	17.65	35.90	50.00	53.66	50.00	57.14	88.46	100													
SYN A3	35.71	58.82	48.28	23.08	38.71	61.11	66.67	50.00	58.82	68.18	77.27	100												
CON B1	38.10	66.67	63.64	31.58	41.67	41.38	53.85	64.00	51.85	48.65	48.65	48.28	100											
CON B2	27.27	57.14	52.17	20.00	32.00	33.33	66.67	69.23	64.29	57.89	57.89	53.33	78.26	100										
CON B3	28.57	59.26	63.64	31.58	33.33	34.48	61.54	56.00	51.85	48.65	43.24	41.38	81.82	78.26	100									
PRO B1	60.00	66.67	51.61	57.14	66.67	63.16	57.14	47.06	44.44	52.17	43.48	57.89	38.71	37.50	32.26	100								
PRO B2	50.00	52.63	48.48	40.00	57.14	75.00	48.65	44.44	47.37	50.00	50.00	65.00	36.36	29.41	36.36	71.43	100							
PRO B3	54.55	66.67	47.06	38.71	61.11	63.41	63.16	48.65	46.15	61.22	53.06	53.66	41.18	45.71	47.06	79.07	66.67	100-						
PRE B1	32.00	58.06	46.15	34.78	42.86	54.55	60.00	48.28	51.61	63.41	53.66	60.61	53.85	59.26	61.54	68.57	59.46	63.16	100					
PRE B2	50.00	64.71	48.28	38.46	58.06	55.56	60.61	50.00	64.71	63.64	63.64	66.67	48.28	46.67	55.17	57.89	65.00	58.54	72.73	100				
PRE B3	33.33	60.00	48.00	36.36	44.44	50.00	62.07	57.14	53.33	55.00	50.00	56.25	48.00	61.54	56.00	64.71	50.00	59.46	89.66	68.75	100			
SYN B1	44.44	60.61	42.86	32.00	53.33	62.86	68.75	58.06	66.67	55.81	55.81	68.57	42.86	48.28	42.86	75.68	71.79	70.00	75.00	74.29	70.97	100		
SYN B2	50.00	64.71	55.17	38.46	58.06	61.11	66.67	56.25	58.82	59.09	50.00	55.56	41.38	46.67	55.17	73.68	75.00	73.17	84.85	77.78	75.00	85.71	100	
SYN B3	46.15	62.50	44.44	33.33	48.28	64.71	64.52	60.00	62.50	57.14	57.14	64.71	44.44	57.14	51.85	66.67	63.16	71.79	70.97	70.59	66.67	90.91	82.35	100

Note: CON = control, PRO = probiotic, PRE = prebiotic, SYN = synbiotic. A= at day 17, B= at day 35, 1-3 refers to replicate number in each case, (n=24).

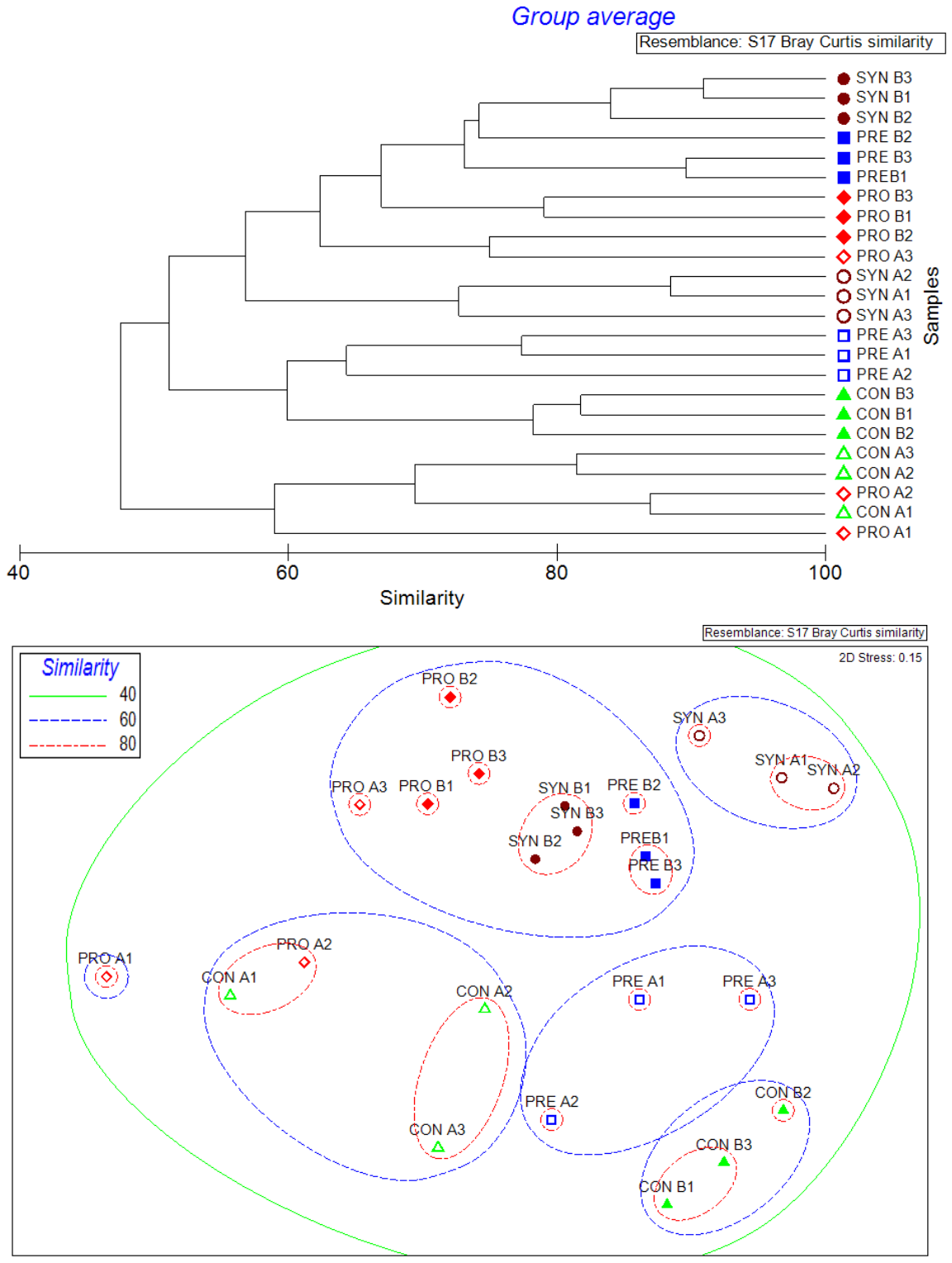


Figure 5.11: (Top) Cluster analysis (Bottom) non-metric multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in ileum digesta. A: 17 days, B: 35 days old of broilers. 1-3 denotes replicate number in each sample.

Table 5.30: Band numbers of bacterial community based on the PCR-DGGE DNA fingerprinting and similarity within treatments (Mean ± SD).

Time (Days)	Treatment	Band number	Similarity
17	CON	12.33±2.21	75.02±7.58
	PRO	13.00±2.04	61.68±6.86
	PRE	15.00±1.00	68.92±7.62
	SYN	23.33±4.61	77.97±10.15
35	CON	11.33±0.57	79.44±2.05
	PRO	21.66±1.52	72.39±6.25
	PRE	15.66±2.08	77.04±11.1
	SYN	17.00±1.00	86.32±4.31

Table 5.31: Estimated coefficients for band numbers of bacterial community in ileum based on the PCR-DGGE DNA fingerprinting and similarity within treatments.

Term	Band number		Similarity	
	Coefficient	*P*. value	Coefficient	*P*. value
Constant	16.167	***	59.798	***
PRO	2.583	***	1.619	NS
PRE	1.583	*	0.272	NS
Time	0.250	NS	4.172	NS
PRO*PRE	-0.167	NS	-0.597	NS
PRO*Time	0.333	NS	0.199	NS
PRE*Time	-1.667	*	-0.200	NS
PRO*PRE*Time	-2.083	**	-0.917	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

Diversity analysis of ileum microflora showed in table 5.32. The diversity index of bacterial community based on the PCR-DGGE DNA fingerprinting indicated that; at 17 and 35 days of age, birds fed the diets containing probiotic, prebiotic and synbiotic had greater Shannon index and Margalef index than birds fed control groups.

Table 5.32: Diversity index of bacterial community in ileum digesta based on the PCR-DGGE DNA fingerprinting at different day of age (Mean ± SD).

Time (Days)	Treatment	Shannon index[1]	Margalef index[2]
17	CON	4.49±0.80	2.49±0.24
	PRO	4.64±0.75	2.51±0.40
	PRE	5.16±0.24	2.70±0.06
	SYN	7.07±1.03	3.13±0.21
35	CON	4.25±0.14	2.42±0.05
	PRO	6.71±0.34	3.07±0.07
	PRE	5.32±0.49	2.74±0.12
	SYN	5.64±0.23	2.83±0.05

[1] Shannon diversity index: H‟ = -SUM(pi*Log(pi)).
[2] Margalef species richness: d = (S -1)/log (N). (S: Total species, N: Total individuals)

The estimated coefficients table 5.33 showed the coefficients of probiotic, prebiotic and the interactions between prebiotic and time and probiotic and prebiotic and time were significantly affected on the Shannon index and Margalef index. However, the time and the interactions between probiotic and prebiotic and probiotic and time were not significant on the Shannon index and Margalef index.

Table 5.33: Estimated coefficients for diversity index of bacterial community in ileum digesta based on the PCR-DGGE DNA fingerprinting.

Term	Shannon index		Margalef index	
	Coefficient	*P*. value	Coefficient	*P*. value
Constant	5.416	***	2.740	***
PRO	0.604	**	0.147	**
PRE	0.388	*	0.114	*
Time	0.070	NS	0.029	NS
PRO*PRE	-0.047	NS	-0.019	NS
PRO*Time	0.091	NS	0.035	NS
PRE*Time	-0.388	*	-0.095	*
PRO*PRE*Time	-0.487	**	-0.121	**

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

A positive sequencing was done for 36 out of the 44 PCR fragments which were analysed. The results of the trial sequence analysis shown in Table 5.34. The other samples sequencing quality were below the required standard and sequencing data was zero.

The band number (No.13) in ileum was uncultured *Lactobacillus* spp. and it was found in treated group with probiotic (*Lb. animalis*). *Lb. animalis* (band No.12) was absent in the control group but some traces of the band existed in an inulin treatment and this could be due to the presence of *Lactobacillus* spp. in the GI tract. This result confirms the survival of *Lactobacillus* spp. in chicken GI tract.

Table 5.34: Summary results of sequencing analysis bands of PCR-DGGE fingerprints of chicken ileum samples at 17 and 35 days old

Band Number	NCBI Accession number	Max. Identity (%)	NCBI BLAST matches
1	HM846969.1	98	Uncultured bacterium
2	AB331843.1	100	Uncultured bacterium
5	JF427735.1	92	Uncultured *Lactobacillus* sp. isolate DGGE gel band A1SB2
6	FJ713030.1	91	Uncultured *Bacillus* sp. clone 28
7	NR_074902.1	99	*Escherichia fergusonii* strain ATCC 35469
8	JF522217.1	97	Uncultured Burkholderia sp. clone AG12P
10	NR_104559.2	100	*Enterococcus gallinarum* strain LMG 13129
11	NR_104559.2	100	*Enterococcus gallinarum* strain LMG 13129
12	AB911530.1	100	*Lactobacillus animalis gene*
13	KF504919.1	99	Uncultured *Lactobacillus* sp.
14	NR_075022.1	100	*Enterococcus hirae* strain ATCC 9790
15	KC113205.1	100	*Enterococcus faecalis* strain P26-24
16	NR_037053.1	100	*Staphylococcus succinus* subsp. casei strain SB72
17	NR_114844.1	97	*Lactobacillus paralimentarius* strain DSM 13238
19	NR_113594.1	97	*Streptococcus equinus* strain NBRC 12553
20	KC164845.1	100	Uncultured bacterium clone TSC1
21	NR_075064.1	96	*Lactobacillus johnsonii* NCC 533 strain
22	JX013453.1	96	Uncultured bacterium
23	FJ875424.1	98	Uncultured bacterium
24	NR_074902.1	98	*Escherichia fergusonii* strain ATCC 35469
25	NR_113999.1	96	*Lactobacillus siliginis* strain NBRC 101315
28	LM995446.1	100	*Escherichia coli*
29	NR_114042.1	100	*Escherichia coli* strain NBRC 102203
31	NR_118568.1	97	*Enterobacter cloacae* strain ATCC 13047
32	FJ837171.1	100	Uncultured bacterium
33	KF323750.1	100	Uncultured bacterium clone GXTJ5A301BRSAS
35	KM499326.1	98	Uncultured bacterium
36	NR_117057.1	95	*Lactobacillus gigeriorum* strain CRBIP 24.85
37	LN568439.1	100	Uncultured bacterium partial
38	NR_041887.1	100	*Clostridium caminithermale* strain DVird3
39	NR_075045.1	98	*Lactobacillus acidophilus* NCFM strain
40	NR_119032.1	100	*Clostridium paraputrificum* strain DSM 2630
41	HQ620538.1	100	Uncultured bacterium
42	KF109483.1	100	Uncultured bacterium
43	NR_075024.1	97	*Lactobacillus brevis* ATCC 367 strain
44	NR_044702.1	98	*Lactobacillus amylophilus* strain DSM 20533

5.4.7 Jejunum Histomorphology

Table (5.35) refers to effects of probiotic, prebiotic and synbiotic on jejunum morphology of the ileum at 17 and 35 days old broiler chickens. The treatments

(PRO, PRE and SYN) had increased villus height and crypt depth of the jejunum in 17 and 35 days old broiler chicks. At 35 days of age, supplementation of probiotic, prebiotic and synbiotic to chicks diet increased the villi length compared to the control group (966.95, 825.2 and 755.92 vs. 681.67 µm) respectively. Figures 5.12 illustrated that clearly the differences between the additive supplementation with control treatment at 17 and 35 days old of broilers.

The estimated coefficient table 5.36 showed the coefficient of probiotic, prebiotic and time had a highly significant ($P<0.001$) effect on villus height. However, the interactions between probiotic and prebiotic and probiotic with time were not significant. However, the interaction of prebiotic with time and synbiotic with time had a significant effect on the villus height. Probiotic and prebiotic in the diet had an effect on the crypt depth. However, all interaction between the three factors had not significantly influenced on the crypt depth.

Table 5.37 showed the probiotic, prebiotic and synbiotic was increased the microvilli density and goblet cell number/ per 100µm of villus height in the jejunum of broiler compared with the control group. Probiotic, prebiotic and synbiotic fed birds had a 33.55%, 24.14% and 37.45%, respectively higher goblet cell number than the control fed birds at 35 days (See figure 5.13).

The estimated coefficient table 5.38 showed the prebiotic only significantly ($P<0.01$) increased the microvilli density. However probiotic and interaction between probiotic and prebiotic had no significant effect on microvilli density. While, all additive supplementation probiotic, prebiotic and interaction between both were significantly increased the goblet cell number.

Table 5.35: Effect of probiotic, prebiotic and synbiotic on the Jejunum villus high (µm) and crypt depth (µm) of broiler chickens (Mean ± standard division).

Time (Days)	Parameters	Treatments			
		CON	PRO	PRE	SYN
17	Villus height	630.81±31.16	750.85±33.79	767.55±37.51	794.17±38.24
	Crypt depth	116.17±16.44	127.62±16.58	137.62±11.35	141.58±19.14
35	Villus height	681.67±38.18	755.92±33.17	825.2±45.31	966.95±44.06
	Crypt depth	97.97±6.05	121.11±.17.5	138.45±17.75	145.08±8.98

Table 5.36: Estimated Coefficients for probiotic, prebiotic and synbiotic on the villus high and crypt depth of broiler chickens.

Term	Villus high		Crypt depth	
	Coefficient	*P*. value	Coefficient	*P*. value
Constant	771.635	***	128.201	***
PRO	45.333	***	5.641	*
PRE	66.829	***	12.489	***
Time	35.794	***	-2.541	NS
PRO*PRE	-3.241	NS	-3.005	NS
PRO*Time	8.667	NS	1.790	NS
PRE*Time	21.813	**	3.635	NS
PRO*PRE*Time	20.115	**	-1.130	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

Table 5.37: Effect of probiotic, prebiotic and synbiotic on the Jejunum microvilli density and Goblet cell of broiler chickens (Mean ± standard division).

Treatment	Microvilli density (Arbitrary unit)	Goblet cell number per (100 µm villus height)
CON	1.08±0.03	7.13±0.91
PRO	1.18±0.09	10.73±0.79
PRE	1.41±0.13	9.40±0.98
SYN	1.53±0.13	11.40±0.73

Table 5.38: Estimated Coefficients for probiotic, prebiotic and synbiotic on the Jejunum microvilli density and Goblet cell of broiler chickens

Term	Microvilli density		Goblet cell	
	Coef.	*P*. value	Coef.	*P*. value
Constant	1.30442	***	9.666	***
PRO	0.05542	NS	1.400	***
PRE	0.16792	**	0.733	**
PRO*PRE	0.00225	NS	-0.400	*

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significan.

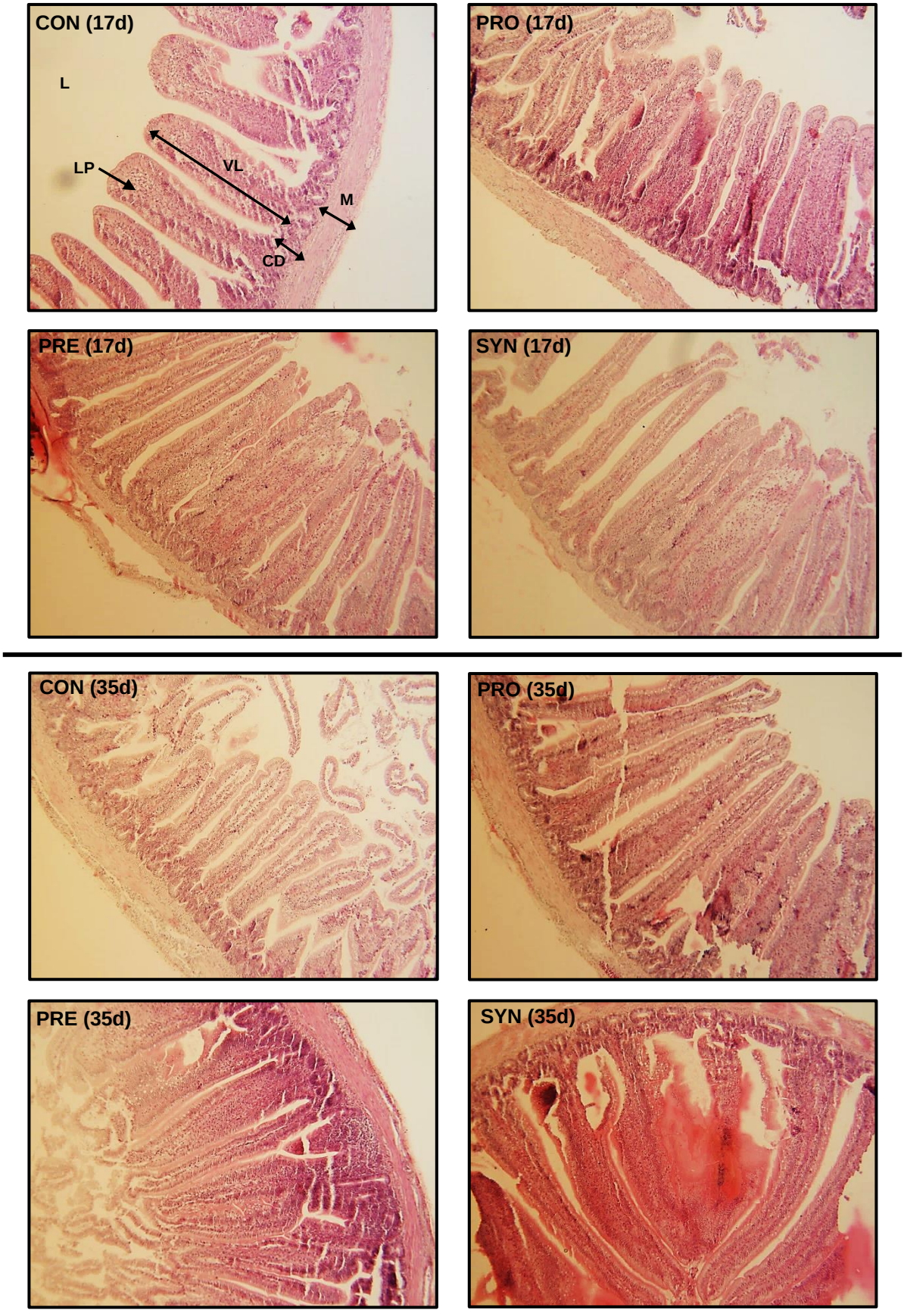
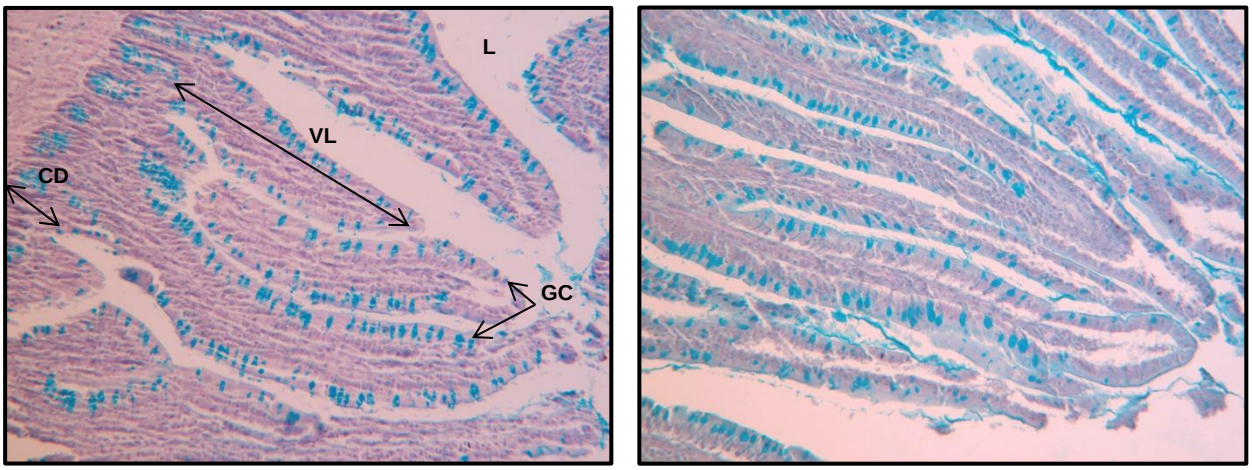
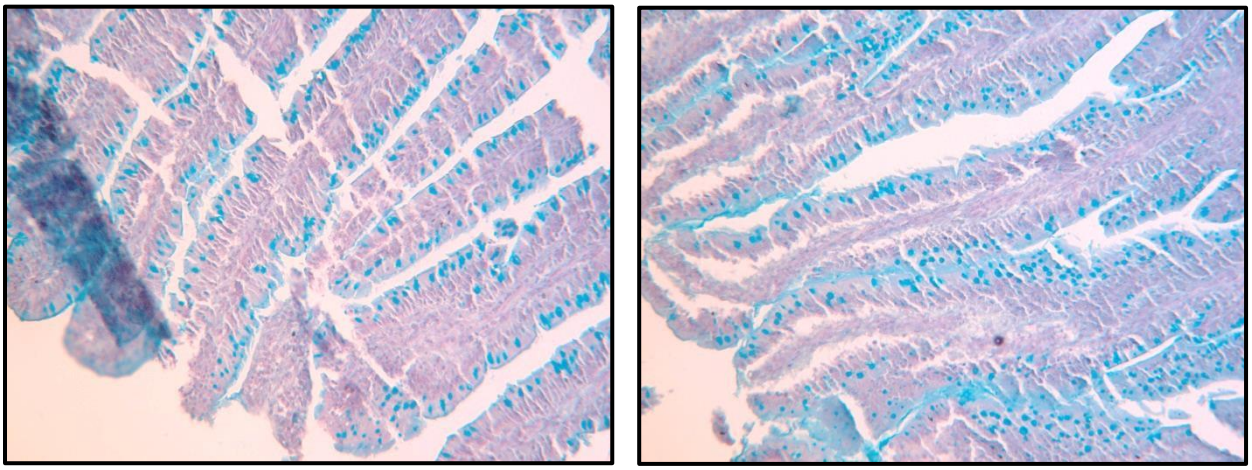


Figure 5.12: Haematoxylin and eosin stained section of jejunum of broilers fed diets containing probiotic, prebiotic and synbiotic at 17 and 35 days of age. L: Lamina, LP: Lamina propria, VL: Villus length, CD: Crypt depth, M: Muscularis. (10X Magnification).



Control

Probiotic



Prebiotic

Synbiotic

Figure 5.13: Alcian blue and PAS stained section of jejunum of broilers fed diets containing probiotic, prebiotic and synbiotic at 35 days of age. L: Lamina, VL: Villus length, CD: Crypt depth, G: Goblet cells. (20X Magnification).

Villi and microvilli morphological of the jejunum were examined by scanning electron microscopy. SEM confirmed a number of rod shape bacteria and some cocci shapes at the top and between the microvilli which cover the villi of the synbiotic group. Figure 5.19 illustrated that these kinds of bacteria were observed around the apical area of villi of jejunum in synbiotic group compared to the other groups. These bacterial populations were not present in control group. Figures 5.16, 5.17 and 5.18 for probiotic, prebiotic and synbiotic, respectively, showed the tongue shapes villi in the jejunum and the villus were arranged as in zigzag, resembling a wave compared with control group which was damaged. Figure 5.22 SEM image control jejunum chicken group showed the deformed and irregular distribution of microvilli on the top of villi. At higher magnification (X2000 to X20000) microvilli can be seen clearly and the density of microvilli increased in additives supplementation treatments compared to control group (Figure 5.24).

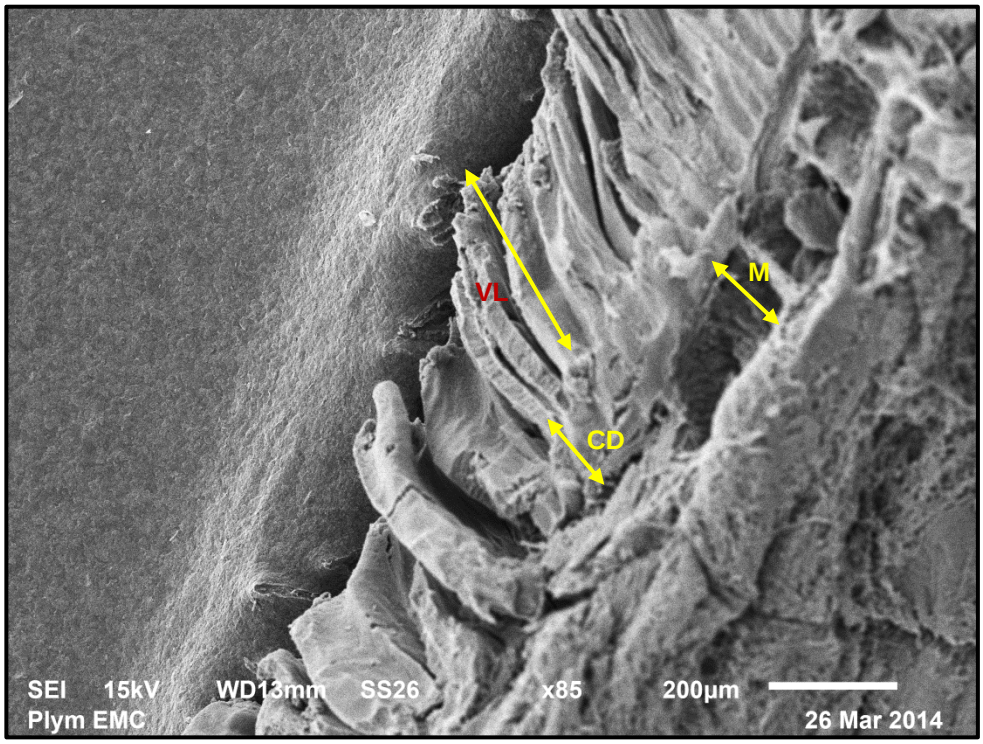


Figure 5.14: SEM micrograph of control chicken jejunum showed the length of villi (VL), crypt depth (CD) and M= Muscularis.

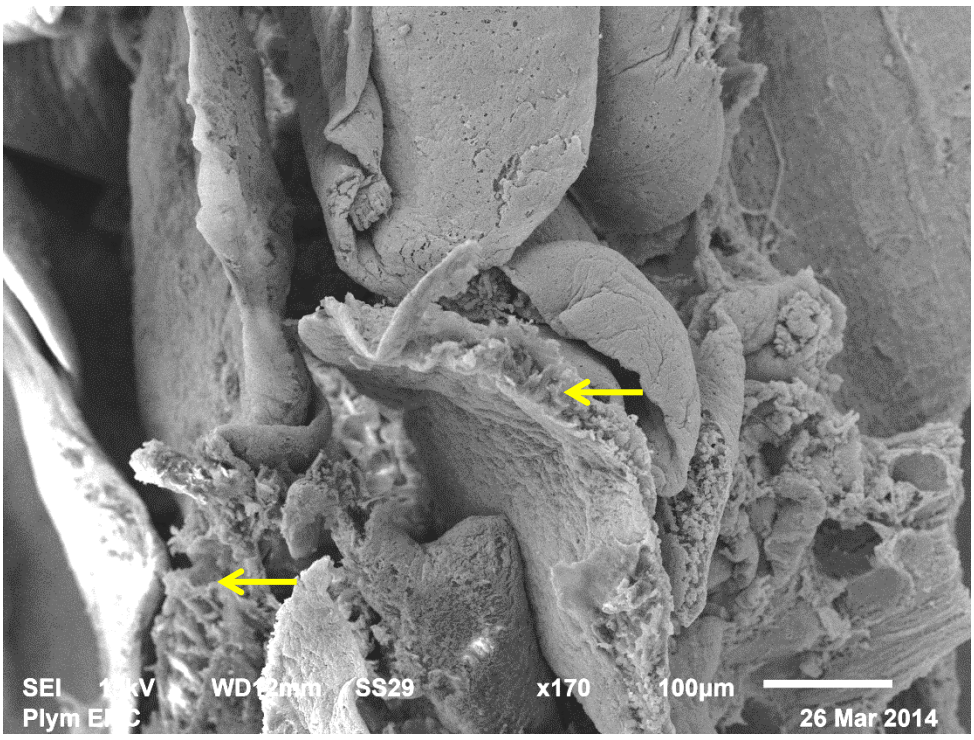


Figure 5.15: SEM micrograph of top side view of the intestinal villi of control group showed the density and damaged shapes of villi.

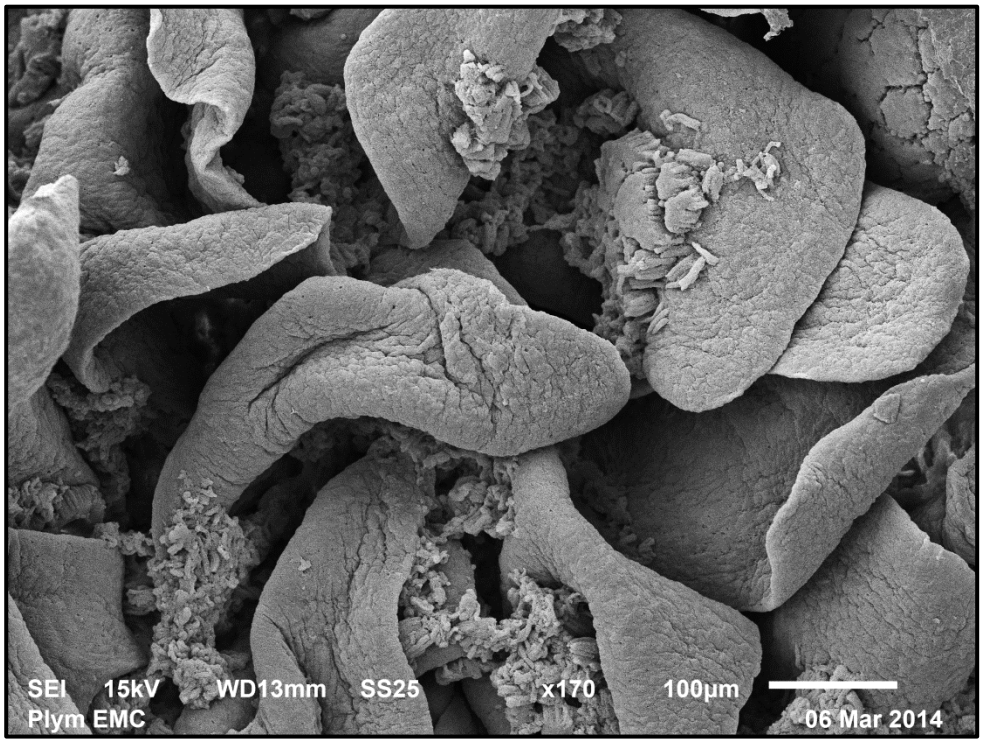


Figure 5.16: SEM micrograph of top side view of the intestinal villi of probiotic group showed the density and tongue shapes of villi.

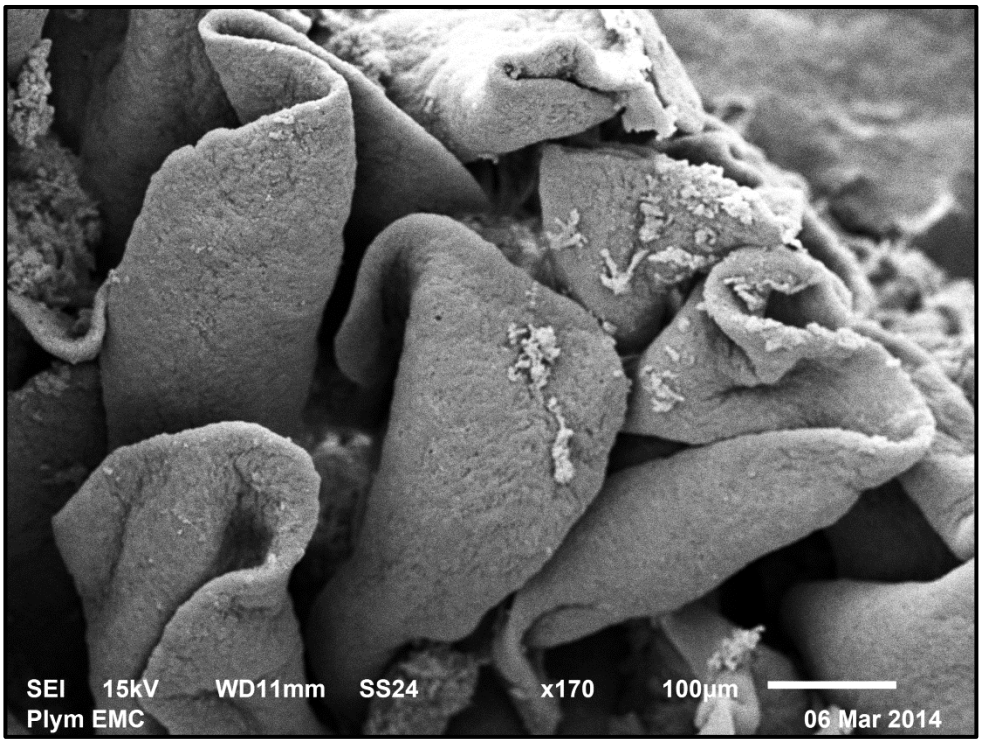


Figure 5.17: SEM micrograph of top side view of the intestinal villi of prebiotic treatment group showed the density and tongue shapes of villi.

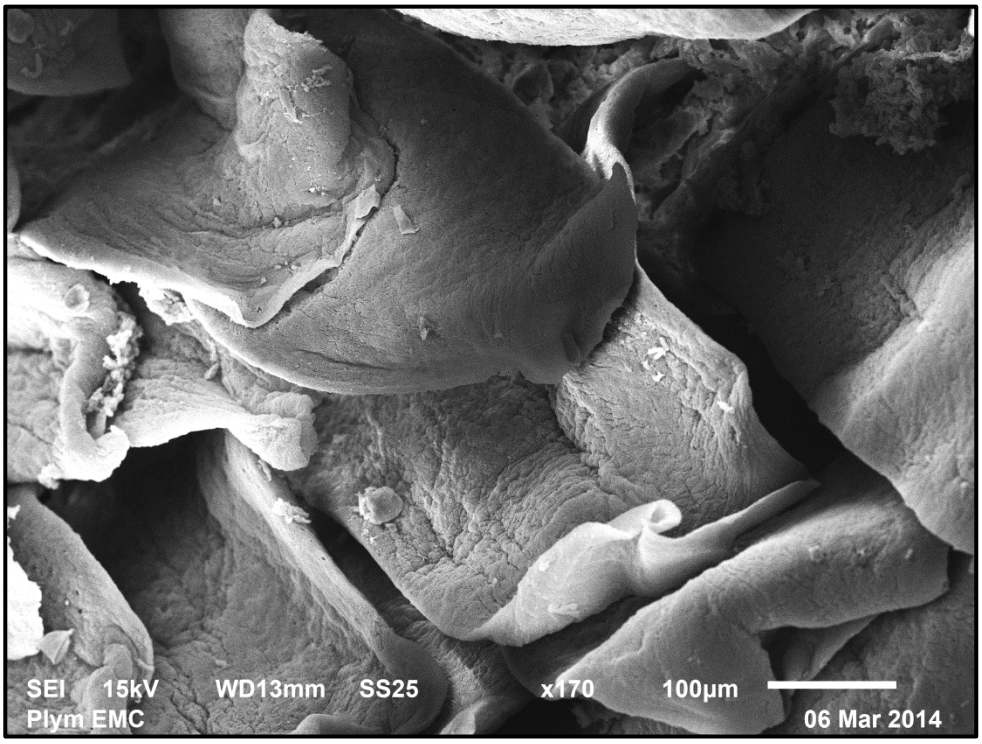


Figure 5.18: SEM micrograph of top side view of the intestinal villi of synbiotic treatment group showed the density and tongue shapes of villi.

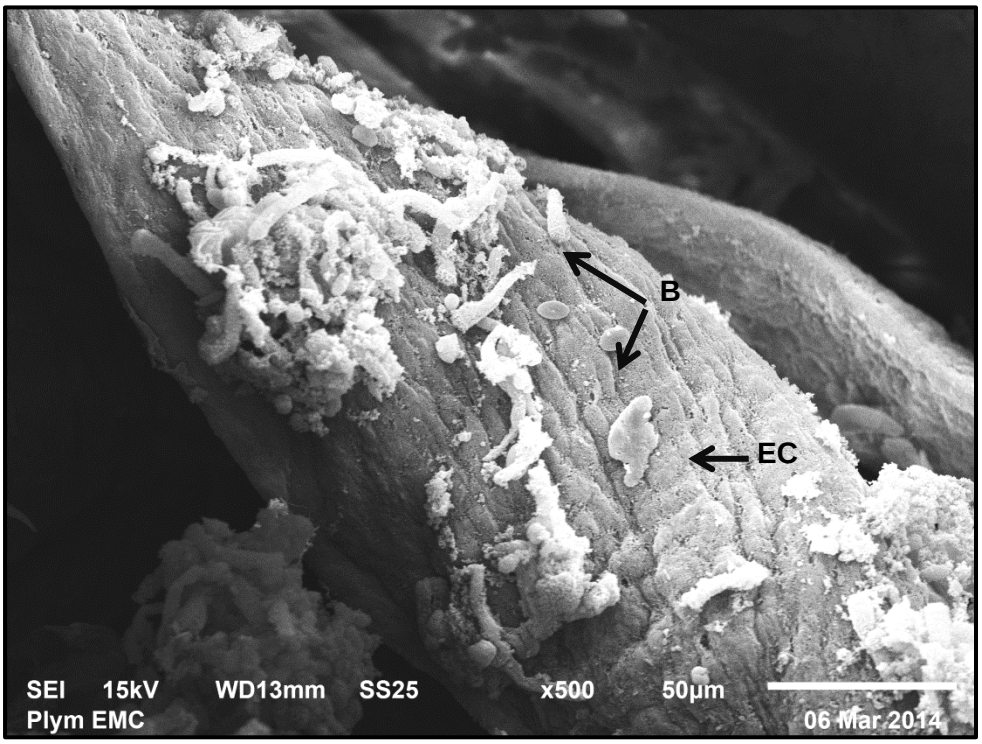


Figure 5.19: SEM micrograph of bacterial colonisation in the jejunum of the chicken fed synbiotic. B= bacteria and EC= epithelial cells.

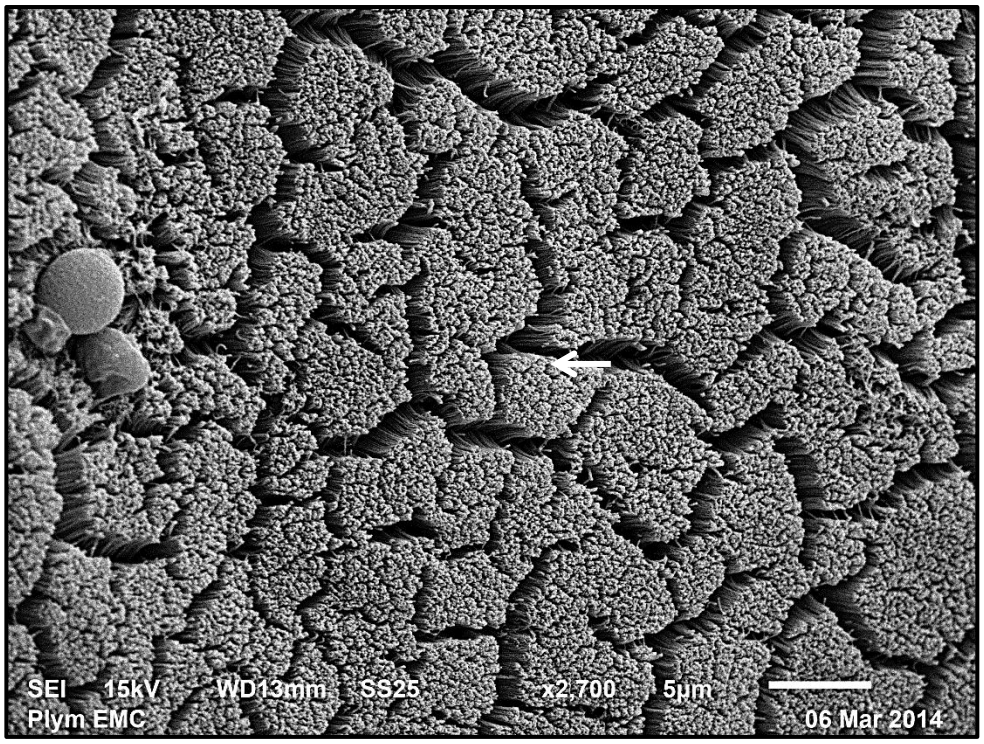


Figure 5.20: SEM image of the jejunum of chicken fed synbiotic showed the microvilli with a regular distribution and the edge of the enterocytes (arrow).

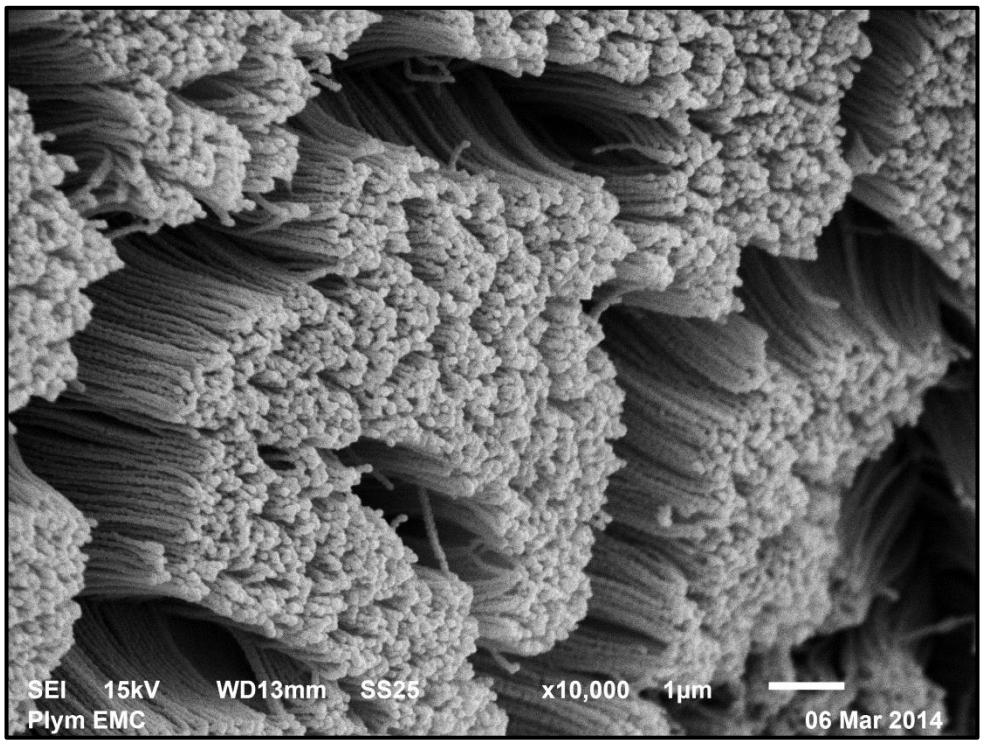


Figure 5.21: SEM image of the jejunum of chicken fed synbiotic showed the top and length of microvilli with a regular distribution and the edge of the enterocytes.

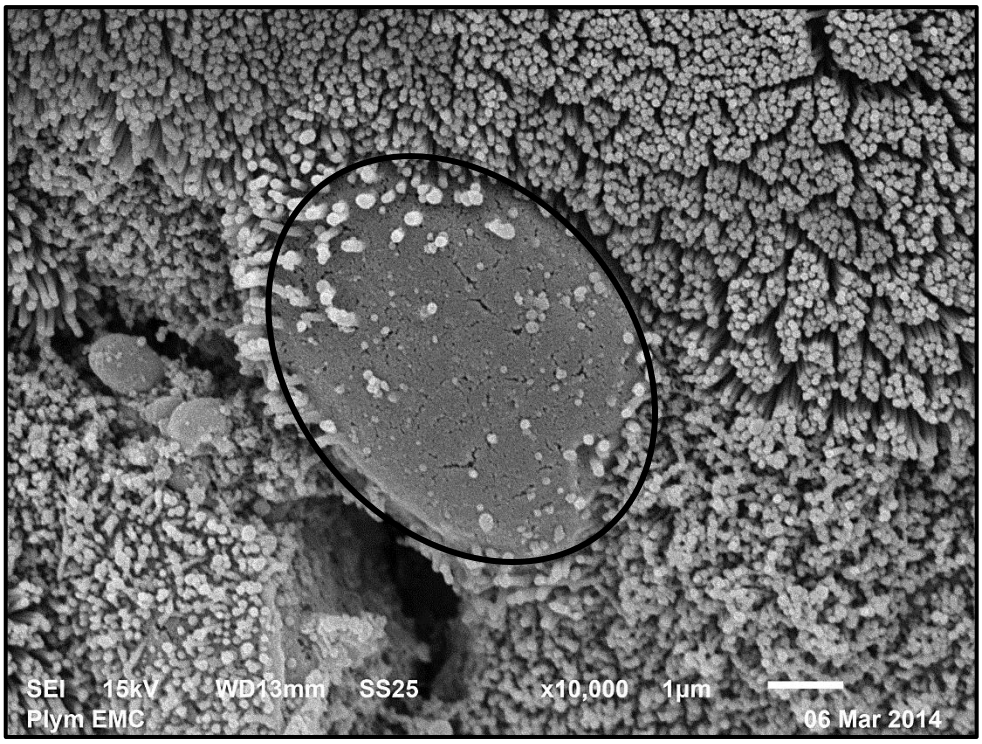


Figure 5.22: SEM image of the control jejunum chicken group showed the deformed and irregular distribution of microvilli (oval mark) on the top of villi.

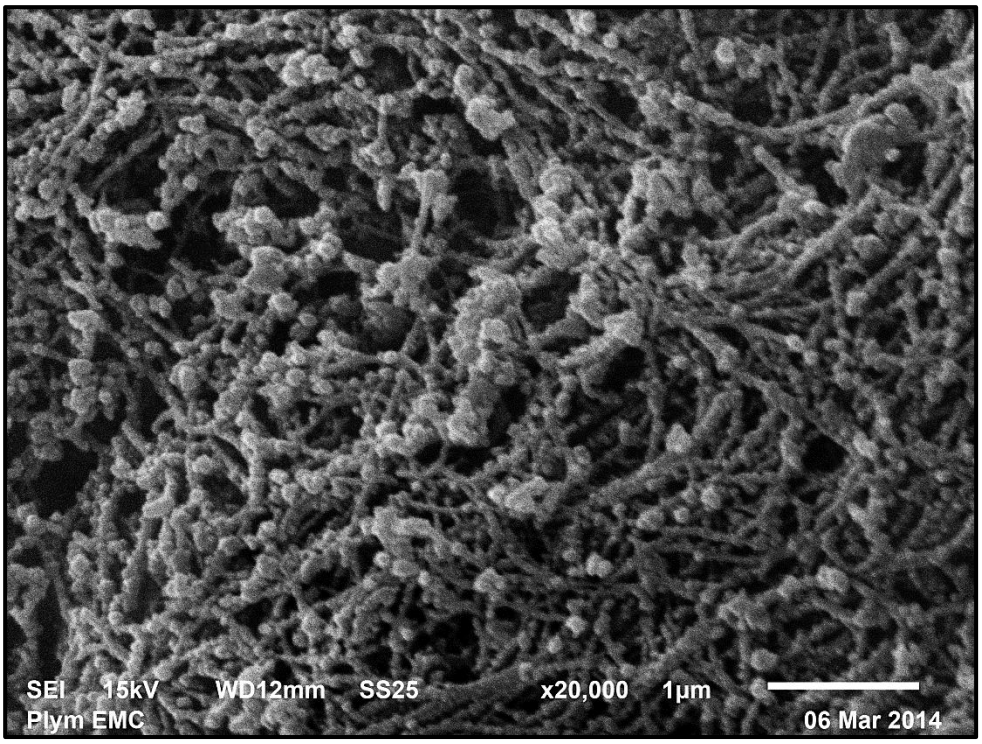
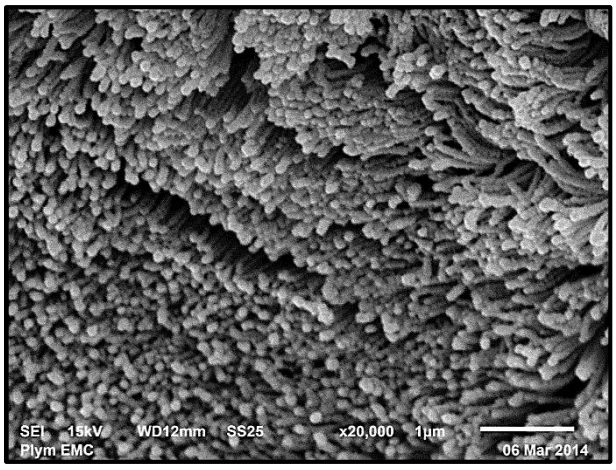
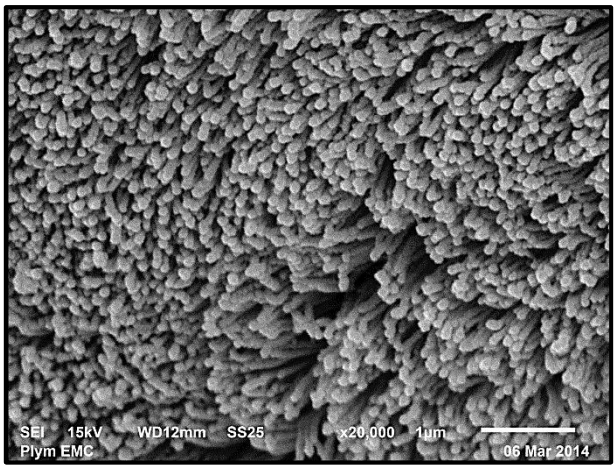


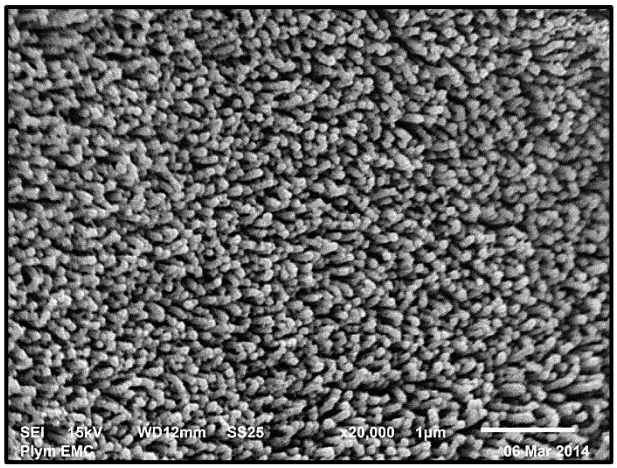
Figure 5.23: SEM image of the control jejunum chicken group showed the microvilli damaged and crashed.



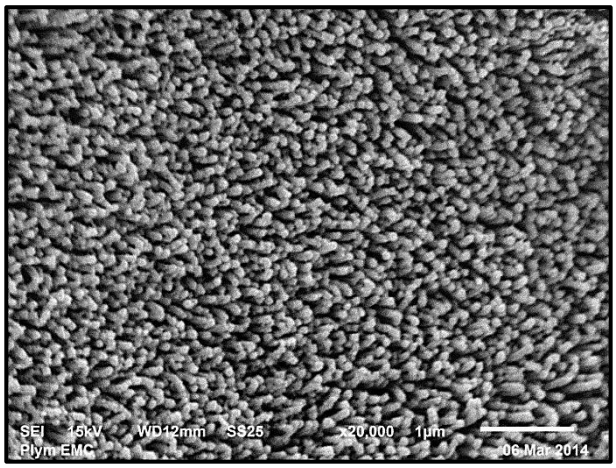
Control



Probiotic



Prebiotic



Synbiotic

Figure 5.24: Comparative SEM micrographs of microvilli density of jejunum intestine of broiler chickens fed additive supplementation at 35 day of age.

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5.4.8 Relative weight of Bursa of Fabricius

Table 5.39 showed the relative weight of Bursa of Fabricius from the chicks treated with dietary probiotic, prebiotic and synbiotic supplementation compared with the control treatment at different age of birds. All additives supplementations were increased the relative weight of BF compared with control group, at different days of age.

The estimated coefficients table 5.40 showed the prebiotic and time had a significant (P<0.01) effect on the relative weight of BF. However, probiotic and all interaction between the three factors (Probiotic, Prebiotic and Time) were not significant.

Table 5.39: Effect of probiotic, prebiotic and synbiotic on relative Bursa of Fabricius weigh of broiler chickens (Mean ± standard division).

Treatment	Time (Days)	
	17	35
CON	0.42±0.02	0.29±0.04
PRO	0.44±0.04	0.37±0.04
PRE	0.49±0.03	0.38±0.05
SYN	0.50±0.02	0.39±0.005

Table 5.40: Estimated Coefficients for probiotic, prebiotic and synbiotic on relative Bursa of Fabricius weigh of broiler chickens.

Term	Relative Bursa of Fabricius weigh	
	Coefficient	*P*. value
Constant	0.412	***
PRO	0.015	NS
PRE	0.029	**
Time	-0.052	***
PRO*PRE	-0.009	NS
PRO*Time	0.006	NS
PRE*Time	-0.002	NS
PRO*PRE*Time	-0.005	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.9 Histology of Bursa of Fabricius

Table 5.41 showed the results of the Bursa Histology measured in broilers at 17 and 35 days of age. Also, the results of tissue sections of the Bursa of Fabricius of four treatments were described in microscopic photos (Figures 5.25). The diameters of follicles of Fabricius were increased in dietary probiotic, prebiotic and synbiotic supplementations compared with control group, at 17 and 35 days of age.

The estimated coefficients table 5.42 showed the probiotic, prebiotic and time were significantly (P<0.01) increased the diameter of Follicles of BF. However, all interaction between the three factors (Probiotic, Prebiotic and Time) was not significant.

Table 5.41: Diameter of Follicles of Bursa of Fabricius in broilers fed diets containing probiotic, prebiotic and synbiotic at 17 and 35 days of age.

Treatment	**Time (Days)**	
	17	**35**
CON	282.39±49.92	337.1±27.95
PRO	344.28±61.33	380.99±45.92
PRE	350.44±63.82	365.31±31.88
SYN	377.11±68.20	404.65±31.16

Table 5.42: Estimated coefficients for Diameter of Follicles of Bursa of Fabricius in broilers fed diets containing probiotic, prebiotic and synbiotic at 17 and 35 days of age.

Term	Diameter of Follicles of Bursa of Fabricius	
	Coefficient	*P*. value
Constant	355.265	***
PRO	21.495	***
PRE	19.115	***
Time	16.706	**
PRO*PRE	-4.992	NS
PRO*Time	-0.645	NS
PRE*Time	-6.104	NS
PRO*PRE*Time	3.814	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

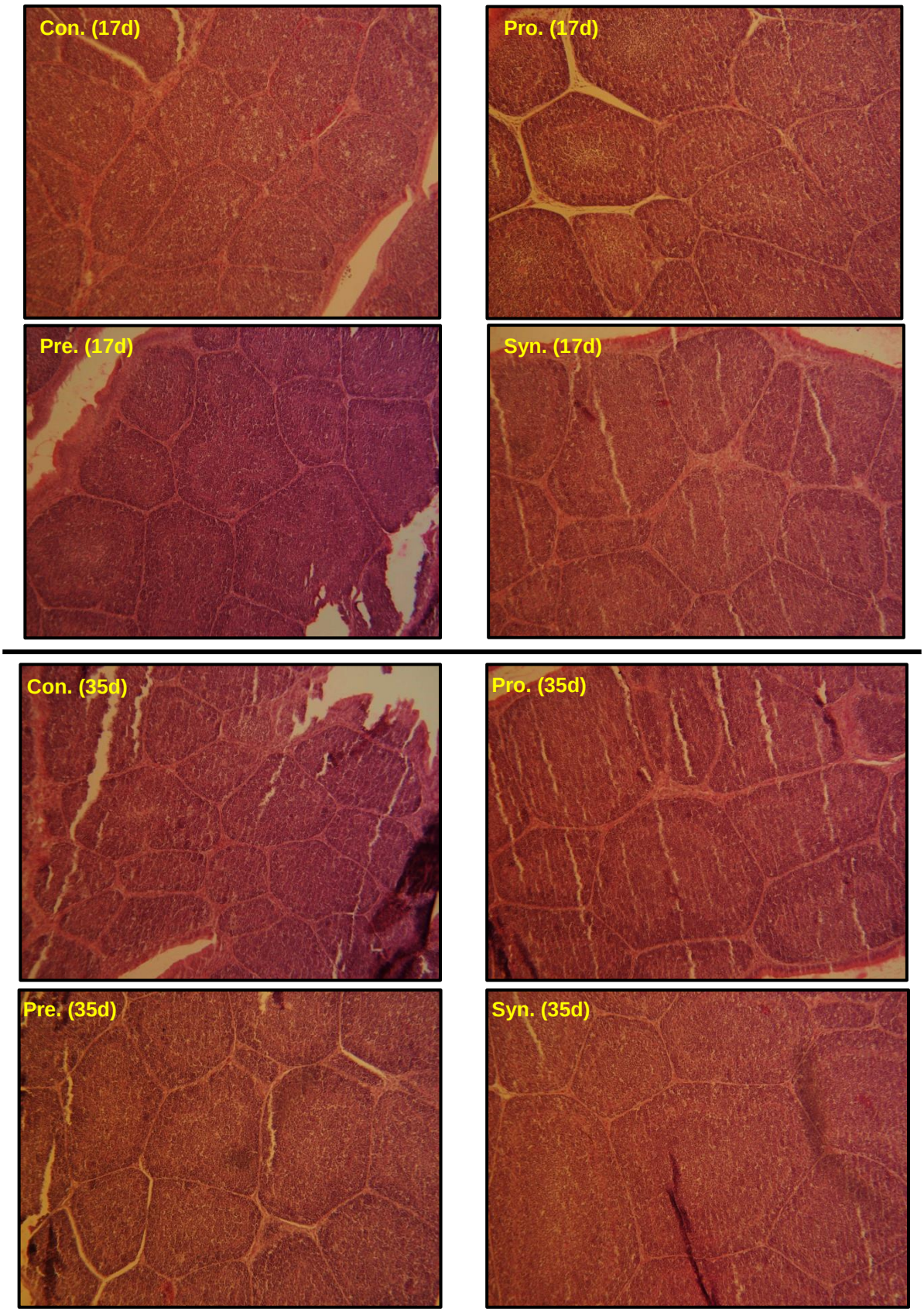

Figure 5.25: Follicles of Bursa of Fabricius in broilers fed diets containing probiotic, prebiotic and synbiotic at 17 and 35 days of age. (10X Magnification).

5.4.10 Haematological and biochemical traits

Table 5.43 showed the result of Haematological and biochemical parameters at the end of the experiment. The highest haematocrit (Hct %) and Haemoglobin were recorded for the probiotic, prebiotic and synbiotic compared with the control group of broilers chicks. The additives also were increased the Lymphocyte count, and decreased Heterophil count and H/L ratio at 35 day of age. The better H/L ratio was observed for chicks fed synbiotic. All the additive supplementation reduced the cholesterol content in the whole blood compared with control group.

The estimated coefficients table 5.44 showed the probiotic and prebiotic were significantly increased the Hct%, and only prebiotic was significantly increased haemoglobin content and Lymphocyte count at 35 days. However, the probiotic and prebiotic were significantly reduced the Heterophils count and H/L ratio. While, the interaction between probiotic and prebiotic were not significant for Hct%, haemoglobin, Lymphocyte, Heterophils and H/L ratio. The chicks fed on probiotic, prebiotic and synbiotic (Interaction between probiotic and prebiotic) had significantly ($P<0.01$) lower blood cholesterol at 35 days. The coefficient of interaction between probiotic and prebiotic were increased but still was a highly significant on cholesterol content.

Table 5.43: Haematological and biochemical parameters of broiler chicks at 35 days of age (Mean ± standard division).

Parameters	Treatment			
	CON	**PRO**	**PRE**	**SYN**
Hct (%)	28.99±0.30	29.59±0.48	30.11±0.48	31.03±0.50
Haemoglobin (g/dl)	10.62±0.25	11.15±0.85	11.74±0.36	11.96±0.22
Lymphocyte	59.66±1.52	61.00±2.64	63.00±3.00	65.66±3.51
Heterophils	37.66±2.08	31.33±2.08	31.33±2.51	27.00±4.00
H/L ratio	0.62±0.04	0.51±0.03	0.50±0.02	0.40±0.03
Cholesterol (mg/dl)	160.60±1.56	152.87±1.57	151.84±0.59	150.94±0.59

Table 5.44: Estimated coefficients for haematological and biochemical parameters of broiler chicks.

Term	Hct (%)		Haemoglobin (g/dl)		Lymphocyte		Heterophils		H/L ratio		Cholesterol (mg/dl)	
	Coefficient	*P*.	Coefficient	*P*.	Coefficient	*P*.	Coefficient	*P*.	Coefficient	*P*.	Coefficient	*P*.
Constant	29.931	***	11.370	***	62.333	***	31.833	***	0.510	***	154.062	***
PRO	0.638	**	0.187	NS	1.000	NS	-2.667	*	-0.052	**	-2.158	***
PRE	0.378	*	0.484	**	2.000	*	-2.667	*	-0.059	***	-2.675	***
PRO*PRE	0.081	NS	-0.075	NS	0.333	NS	0.500	NS	0.081	NS	1.708	**

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.11 Chemical composition of breast and leg

Table 5.45 showed the result of breast and leg meat chemical composition at the end of the experiment. The protein and ash percentage were increased in dietary probiotic, prebiotic and synbiotic supplementations compared with control group. Otherwise, the fat content was reduced in all additives supplementations.

The estimated coefficients table 5.46 showed the probiotic, prebiotic and interaction between both in combinations were not significant on moisture and dry matter content in breast and legs. However, the probiotic, prebiotic and interaction between probiotic and prebiotic were significantly increased the protein content and decreased the fat content in breast and leg. While, the ash content was significantly increased in probiotic and prebiotic group but in interaction between probiotic and prebiotic were not significant in breast and leg of broiler chickens.

Table 5.45: Effect of probiotic, prebiotic and synbiotic on chemical composition of breast and thigh of broiler chicks at the end of experiment (Mean ± standard division).

Item	Parameters	Treatment			
		CON	PRO	PRE	SYN
Breast	Moisture%	64.06±2.37	62.63±1.71	63.01±1.56	61.67±2.06
	Dry Mater%	35.93±2.57	37.36±0.68	36.98±0.02	38.32±0.51
	Protein%*	33.34±0.13	35.78±0.30	35.28±0.17	36.70±0.18
	Fat%*	2.05±0.07	1.01±0.14	1.06±0.09	1.09±0.06
	Ash%*	1.75±0.05	2.08±0.21	1.94±0.08	2.26±0.06
Leg	Moisture%	64.69±1.58	64.82±1.83	64.90±2.67	62.96±4.42
	Dry Mater%	35.30±1.43	35.17±1.30	35.09±2.01	37.03±2.56
	Protein%*	22.97±0.09	25.16±0.16	25.36±1.02	26.00±0.10
	Fat%*	12.65±0.10	9.94±0.17	9.85±1.02	9.55±0.30
	Ash%*	1.22±0.02	1.35±0.02	1.40±0.02	1.47±0.02

* Freeze dry matter basis.

Table 5.46: Estimated coefficients for probiotic, prebiotic and synbiotic on chemical composition of breast and leg of broiler chicks at the end of experiment.

Item	Term	Moisture%		Dry Mater%		Protein%		Fat%		Ash%	
		Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value	Coefficient	*P*. value
Breast	Constant	62.842	***	37.153	***	35.278	***	1.309	***	2.012	***
	PRO	-0.692	NS	0.692	NS	0.965	***	-0.252	***	0.163	**
	PRE	-0.504	NS	0.503	NS	0.716	***	-0.226	***	0.094	*
	PRO*PRE	0.024	NS	-0.023	NS	-0.252	**	0.266	***	-0.001	NS
Leg	Constant	64.345	***	35.651	***	24.876	***	10.501	***	1.363	***
	PRO	-0.452	NS	0.452	NS	0.707	**	-0.754	**	0.051	***
	PRE	-0.412	NS	0.412	NS	0.807	**	-0.796	**	0.075	***
	PRO*PRE	-0.517	NS	0.517	NS	-0.388	*	0.602	**	-0.014	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.4.12 Colour and pH of meat

The results of the L*, a* and b* values determined in this study were shown in Table 5.47. The colour parameters lightness (L* value) and yellowness (b* value) of the breast and leg meat were increased in all additives supplementation compared with control group. However, the colour parameter redness (a* value) of the breast and leg meat were reduced in probiotic, prebiotic and synbiotic compared with control group. Also, the pH value was reduced in all additives supplementation compared with control in breast and leg of broiler chickens.

The estimated coefficients table 5.48 showed the prebiotic only was significantly increased the L* value in breast and legs. While, the probiotic and interaction between probiotic and prebiotic were not significant. The redness (a* value) only in probiotic was significantly ($P<0.01$) reduced in breast meat. While the prebiotic and interaction between probiotic and prebiotic were not significant. In the leg meat, probiotic, prebiotic and interactions between both in combination were significantly decreased the redness (a* value). In breast and leg meat, probiotic and prebiotic were significantly increased b* value. However, the interaction between probiotic and prebiotic were not significant.

Also, table 5.48 showed the coefficient of additives supplementation on the pH value in the breast and leg meat. Probiotic and prebiotic were significantly decreased the pH value in breast and leg meat. However, the interaction between probiotic and prebiotic in the diet were not significant on the pH values in breast and leg meat of broiler chickens.

Table 5.47: Effect of probiotic, prebiotic and synbiotic on breast and thigh color and pH value of broiler chicks at the end of experiment (Mean ± standard division).

Item	Parameters	Treatment			
		CON	PRO	PRE	SYN
Breast	L*	48.58±2.34	50.57±2.22	52.08±2.10	52.77±2.62
	a*	2.02±0.35	1.66±0.21	1.92±0.26	1.60±0.15
	b*	3.51±0.39	4.47±0.53	4.82±0.72	5.41±0.74
	pH	6.21±0.20	5.97±0.06	5.94±0.05	5.80±0.04
Leg	L*	46.76±1.44	49.31±4.20	51.70±2.77	52.25±1.78
	a*	3.35±0.55	2.26±0.22	2.01±0.21	1.77±0.26
	b*	4.46±0.64	4.97±0.61	4.89±0.92	5.76±0.44
	pH	6.04±0.04	5.94±0.09	5.93±0.09	5.75±0.1

Table 5.48: Estimated coefficients for probiotic, prebiotic and synbiotic on breast and leg color and pH value of broiler chicks at the end of experiment.

position	Term	L*		a*		b*		pH	
		Coeff.	*P*.	Coeff.	*P*.	Coeff.	*P*.	Coeff.	*P*.
Breast	Constant	51.000	***	1.801	***	4.554	***	5.985	***
	PRO	0.669	NS	-0.168	**	0.387	*	-0.094	*
	PRE	1.427	**	-0.043	NS	0.562	**	-0.110	**
	PRO*PRE	-0.324	NS	0.006	NS	-0.094	NS	0.025	NS
Leg	Constant	50.035	***	2.352	***	5.020	***	5.920	***
	PRO	0.750	NS	-0.332	***	0.348	*	-0.070	*
	PRE	1.943	**	-0.455	***	0.348	*	-0.075	*
	PRO*PRE	-0.471	NS	0.212	**	0.090	NS	-0.021	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

6.4.13 Cooking losses and shearing force

Table (5.49) showed the effect of probiotic, prebiotic and synbiotic on the values of cooking loss and shearing force in the breast meat at 35 days of broiler age.

Table (5.50) showed the probiotic, prebiotic and the interaction between probiotic and prebiotic were not significant differences (P>0.05) on the values of cooking loss and shearing force in the breast meat at the end of experiment.

Table 5.49: Cooking losses and shearing force of breast muscle in broilers fed diets containing probiotics, prebiotics and synbiotics (Mean ± standard division).

Treatment	Cooking loss (%)	Shearing force (kgf/kg)
CON	8.19±0.76	2.12±0.23
PRO	8.52±0.46	2.01±0.28
PRE	9.34±0.57	2.05±0.18
SYN	7.95±0.96	2.03±0.32

Table 5.50: Estimated coefficients for cooking losses and shearing force of breast muscle in broilers fed diets containing probiotics, prebiotics and synbiotics.

Term	Cooking loss (%)		Shearing force (kgf/kg)	
	Coefficient	*P*. value	Coefficient	*P*. value
Constant	8.5067	***	2.05517	***
PRO	-0.268	NS	-0.035	NS
PRE	0.145	NS	-0.014	NS
PRO*PRE	-0.426	NS	0.0208	NS

***= *P*<0.000, **= *P*<0.01, *= *P*<0.05 and NS = Non-significant.

5.5 Discussion

The microbial populations in the gastrointestinal tracts of poultry play an important role in normal digestive processes and in maintaining animal health. Consumption of a probiotic, prebiotic and synbiotic can result in synergistic effects which improves the functions and shelf life of probiotic (Awat *et al*., 2008; El-Banna *et al*., 2010; Abdel-Raheem *et al*., 2012). The aim of this study was to investigate the influence of dietary supplementation of a probiotic (*Lactobacillus animalis*), a prebiotic Jerusalem artichoke tuber (*Helianthus tuberosus*) and a combination of both (Synbiotic) on the growth performance, organ weights, length measurements of small intestine, ileum and caecal microflora, jejunum histology, immune organ and meat quality of Hubbard broiler chickens.

The key production parameters of broiler growth promotion are weight gain and feed intake. The effect of probiotic, prebiotic and synbiotic supplementation on broiler chicken performance in the current study revealed that the parameters studied were significantly ($p<0.05$) affected by the treatments. The results of the present study demonstrated that average weight gain was increased in probiotic, prebiotic and synbiotic treatments (1724.0 g, 1684.4 g and 1739.8 g) compared with the control (1645.6 g). Feed conversion ratio was improved by the dietary supplementation of the probiotic, prebiotic and synbiotic compared with the control (1.73, 1.63, 1.57 and 1.83) respectively. European production efficiency factor was increased in probiotic, prebiotic and synbiotic compared with control (290.8±11.8, 300.9±3.86, 322.1±7.09 and 262.3±5.94) respectively. The higher performance production observed in broilers fed probiotic, prebiotic and synbiotic may be due to the fact that additives suppress pathogenic bacteria which lead to improved health status and ultimately improved growth and overall performance.

This investigation found that the synbiotic had a greater effect on broiler performance compared with control group. While, there were no significant interaction observed between the two factors, which indicates that probiotic and prebiotic utilization in the diet had independent effect on growth performance at the end of the experiment.

Broiler performance, expressed by EPEF formula, (which depends on number and weight of birds at the end of rearing period, slaughter age, and the amount of feed consumption), ultimately reflects the effect of any factor that could play a role in final production profile. The results of the present study demonstrated that all additive supplementations increased this factor.

The results were in agreement with the findings of Zhang *et al*. (2005), Kalavathy *et al*. (2008) Awat *et al.* (2008) and Falaki *et al.* (2011) whom demonstrated that dietary supplementation of broilers with probiotics and synbiotics significantly increased live body weight when compared with control. Awad *et al*. (2009) showed that synbiotic (1 kg of Biomin IMBO/ton of the starter diets and 0.5 kg/ton of the grower diets) significantly ($P<0.05$) increased the BW, average daily weight gain, and feed conversion ratio compared with the control and probiotic fed broilers. Moreover, a slight improvement in growth performance was observed in broilers fed the probiotic compared with control group. The results studied by Mookiah *et al*. (2014) showed that use of prebiotic IMO (Wako, Osaka, Japan), probiotic 11 *Lactobacillus* strains (*Lb. reuteri* C 1, C 10 and C 16; *Lb. gallinarum* I 16 and I 26; *Lb. brevis* I 12, I 23, I 25, I 218 and I 211, and *Lb. salivarius* I 24) and combination of both (synbiotic) in poultry feed significantly ($P<0.05$) improved weight gain of broiler chickens at 22-42 and 1-42 days of age, and feed conversion ratio from 1 to 21, 22-42 and 1-42 days of age compared with control

group. Also, some researchers showed that probiotics supplementation in the feed of chickens improve the feed conversion ratio (Ayanwale *et al*., 2006; Silva *et al*., 2008). Zhang *et al*., (2003) showed that prebiotic IMO enhanced growth performance of broiler chickens during the initial 3 weeks, but no further effects were detected during the latter 4 weeks of the experiment.

Nevertheless, the results were in contrast with the finding of Murry *et al*. (2006), Celik *et al*. (2007), and Al-Kassi and Mohssen (2009) whom found that the probiotic and synbiotic had no significant effect on live body weight compared with control group. Yousefi and Karkoodi (2007) also reported that feed consumption and feed conversion ratio were not affected by the dietary probiotic and yeast supplementation. In addition, Ahmad (2004) could not detect any difference in the feed conversion ratio of the broilers as compared to the control. Biggs *et al*. (2007) reported that 4 or 8 g kg^{-1} of various prebiotic oligosaccharides (MOS, short-chain FOS, oligofructose, transgalacto-oligosaccharide) had no significant effects on growth performance of young broiler chickens. Jung *et al*. (2008) also reported that the oral administration of prebiotic GOS singly or in combination with a *Biffidobacterium lactis*-based probiotic (synbiotic) did not have any significant effect on broiler growth, feed consumption and feed conversion ratio (FCR). In another study, Midilli *et al.* (2008) reported that dietary probiotic (Bio-Plus 2B®), prebiotic (Bio-Mos®) and synbiotic (Bio- Plus2B®+Bio-Mos®) supplementation did not significantly ($P>0.05$) affect body weight gain and feed intake but improved feed conversion ratio.

The reason for the variable effect of additive supplementations in literature may be due to dissimilarity in gut microflora, environmental conditions, also dose rate, basal diet and strain of probiotic (Mahdavi *et al*., 2005). Several researchers

reported that when chicks were housed in a clean environment a probiotic had an affect on performance (Gunal *et al*., 2006).

In this study, birds fed additives supplementation showed lower feed intake and higher weight gain compared to control group. These results may be due to the elimination of undesirable bacteria from the gastrointestinal tract. Alternatively, it may be due to an improvement in the health of the intestinal mucosa and reduction of the stress on the mucosa by the presence of additive supplementations to the diet. Because, one reason for decreased nutrient absorption is the presence of pathogenic bacteria, which can increase the rate of passage of the digesta, and interfere with intestinal cell well turnover rate and the thickness of intestinal mucosa.

Probiotic microbes and pathogenic bacteria compete for nutrients in the intestines. This suppresses the growth of pathogenic bacteria in the intestines and limits the bioavailability of dietary minerals, such that growth rate and feed efficiency is increased. Lactic acid bacteria ferment lactose to sugars acid which reduces the pH to a level that harmful bacteria cannot tolerate and which favours increased activity for intestinal enzymes and digestibility of nutrients (Choudhari *et al*., 2008). The findings of the present study showed that pH in the ileum and caecum digesta were reduced and by this inhibit colonization of pathogenic microorganisms in the intestine.

Two methods of standard-based microbiology techniques culture based and molecular were used to detect the bacterial populations in the GI tract of broiler chickens. The growth of intestinal microflora may be affected by feed additives and can be used to investigate good gut health. In the present study, probiotic, prebiotic and synbiotic supplementation increased the number of *Lactobacillus*

spp*., Bifidobacterium* spp*.* and total anaerobic bacteria compared with control group in the ileum and caecum digesta, at 17 and 35 days of age. On the other hand, the number of total aerobic bacteria and coliform bacteria in probiotic, prebiotic and synbiotic supplementation were decreased compared with control group. This result are in agreement with findings of Dibaji *et al.* (2014) demonstrated that the addition of the synbiotic (Biomin Imbo) reduced *Escherichia coli* and total coliform populations in the intestines of broiler chickens. On the contrary, different levels of synbiotic increased the numbers of *Lactobacillus* in the intestine of broiler chickens. Mookiah *et al*. (2014) showed that use of prebiotic IMO, probiotic 11 *Lactobacillus* strains and combination of both (synbiotic) in poultry diet significantly ($P<0.05$) increased the caecal populations of lactobacilli and bifidobacteria, and decreased the caecal *Escherichia coli* compared with control group. Mountzouris *et al*. (2010) showed that probiotic (PoultryStar ME, Biomin GmbH, Herzogenburg Austria) at 10^{10} cfu /kg of diet were effective at beneficially modulating caecal microflora composition, they found that the caecal coliform bacteria was decreased at 42 day old broilers compared with control group. However, the numbers of *Lactobacillus* spp. and *Biffidobacterium* spp. in 10^{10} cfu probiotic/ kg of diet were significantly increased compared with the control group.

Erdogan *et al*. (2010) showed that the addition of synbiotics to the diet resulted in a decrease of caecal coliform organism counts, which could be because of the positive effects of probiotics and prebiotics on gut microbial ecology. It is possible that probiotics and prebiotics could balance the intestinal microecosystem by controlling pathogenic bacteria via a competitive exclusion which improves the count of beneficial bacteria. Previous studies have indicated that probiotics and

prebiotics could regulate the intestinal microecological environment in different ways (Li *et al*., 2007; Mountzouris *et al*., 2007; Xu *et al*., 2003).

This molecular fingerprinting technique has been used successfully to describe the intestinal microbial community of broilers (Van der Wielen *et al*., 2002; Knarreborg *et al*., 2002; Hume *et al*., 2006, Rahman *et al*., 2008). However, no reports in broilers are available using this technique to demonstrate if the synbiotic (*Lb. animalis* with inulin) induced changes in the intestinal microbial community. Cluster analysis and multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints were used to displayed percentage and relative similarity of bacterial communities composition between control and treatment groups in ileum and caeca of broiler chickens. MDS is the method that can reduce complex DGGE patterns to points into a second dimensional scale (Fromin *et al*., 2002). The higher the distance between points that means the higher differences in community compositions.

The DGGE profile band numbers in the probiotic, prebiotic and synbiotic were higher than the control group in ileal and caecal digesta. The high species richness in gut microflora is associated with decreased ability of pathogens to colonize the gut (Dillon *et al.*, 2005). Bacteria species may facilitate each other's growth may be due to more effective resource use when more species are present. This means less space for the invader pathogen to colonize. A number of key issues could arise from these trial results. Adding *Lactobacillus animalis,* inulin and combination between both increased the microbial diversity in the treated broiler chicks, which leads to decrease the possibility for colonisation of pathogens.

The sequence analysis of DGGE bands was helpful to know the types of the bacterial population profile inside the GI tract. Each sample has different numbers and species of bacteria. The band sequencing results confirm the survival of *Lactobacillus animalis* via the conditions of chick's GI tract. Future work requires more bands be sequenced to confirm all types of present bacteria inside the chicken GI tract by using this method because cheap or using new techniques of next generation sequencing method.

Bacterial fermentation in the caeca leads to the formation of short-chain fatty acids, which are necessary metabolism of the intestinal epithelial cells and also decrease luminal pH and create an environment less favorable for pathogenic species in the GI tract (Topping and Clifton, 2001). In the present study, the birds fed synbiotic increased the SCFA compared with control group. Butyrate is a major source of energy for enterocytes and colonocytes (Chapman *et al*., 1995), and has a fundamental role in maintaining a healthy GI tract. Lawhon *et al*. (2002) reported that butyrate and propionate were more efficient compared to other types of SCFA in inhibiting *Salmonella typhimurium*, whereas other researchers observed that acetic acid was more effective (Van der Weilem *et al*., 2000).

The use of molecular techniques has several advantages compared with the culture-dependent techniques for enumerating bacteria, and does not introduce the bias of traditional methods. One major advantage is the rapidity, more accurate and sensitivity of the determination compared with culture technique. Sequencing that returned with an ideal result for caecum digesta showed that four of 14 bands detected from DGGE belonged to *Clostridium* spp. and *Ruminococcus* spp. In poultry caeca the highest viable bacterial count and most complex microbiota exist (Huyghebaert, 2003). Based on 16S rDNA analysis,

Apajalahti *et al*. (2004) determined that approximately 7 % of caecal bacteria belong to the *Clostridaceae*. Both Zhu *et al*. (2002) and Lu *et al*. (2003) reported *Clostridaceae* as the major component of the caecum making up between 50 % and 65 % of the population. In the other research, Amit-Romach *et al*. (2004) showed that almost one-third of the bacteria in the chicken caeca at three day consisted of *E. coli* and *Clostridium* species. At 25 day, proportions of *E. coli* and *Clostridium* also remained approximately 30% in the chicken caeca using 16S ribosomal DNA. However, chicken caeca also contain cellulolytic bacteria that are capable of producing acetate. Based on 16S rDNA analysis, 19% of the caecal bacteria were *Ruminococcus* spp. which produce acetic and formic as their primary products (Apajalahti *et al*., 1998).

The gastrointestinal tract development and health is the key to productivity in all farm animals and poultry. The small intestine considered as the most important part in the GI tract, because majority of the enzymatic digestion occurs and that will remain the food mass for a long time and for more than eight hours in this part of GI tract. The small intestine is also the most important centre for the presence of microorganisms inside the digestive tract. Results suggested that the longer small intestine length for all additive supplementations, the better in nutrient absorption which resulted in a heavier body weight and improved the FCR. Yusrizal and Chen (2003) have supported the idea that the use of prebiotics can lengthen villi within the gut and also influence the length of the gut.

The results of the present study showed that probiotic, prebiotic and synbiotic increased length of small intestine (SI). The increases of length of SI might reflect to improve production performance of broiler chickens. Denli *et al*. (2003) who found that the addition of 0.1% probiotic to the broiler diet had no significant effect

on the intestinal length at 42 days compared with the control being 197.1 and 180 cm/bird, respectively. Also, Sato *et al*. (2002) did not observe any effect of probiotics addition to the diet of broilers on the length of the intestine. Also, Beski (2010) showed that supplementation of probiotic (2.5 and 5 g/kg diet) and synbiotic (2.5 and 5 g/kg diet) to the broiler diet had no significant effect on length of small intestine at 42 days compared to the control being 178.7, 183.7, 179.2, 184.9 and 173.8 cm, respectively. Elrayeh and Yildiz (2012) showed that supplementation of prebiotic (0.7 % inulin) to the broiler diet had no significant effect on length of small intestine at 42 days compared to the control being 158.1 and 150 cm respectively. While, the results were in contrast with the finding of Parviz and Ali (2007) who reported that the addition of different levels (0, 1, 3, and 5%) of probiotic to the broiler diet caused significant reduction in the length of small intestine at 42 days. Pelicano *et al*. (2005) showed that administration of a probiotic and prebiotic to poultry increased the length of small intestine and the height of villi that lead to increase residence time of digesta and opportunity to digest and absorb nutrients, also, increase the surface area available for nutrient absorption.

A shortening of the villi and crypts may lead to poor nutrient absorption in the gastrointestinal tract and lower performance (Xu *et al*., 2003). Results of the present study demonstrated that supplementation of probiotic, prebiotic and synbiotic to broiler chicks increased the villi length compared with the control group (966.95, 825.2 and 755.92 vs. 681.67 µm) respectively, at 35 days of age. Xu *et al*., (2003) found that feeding on FOS as prebiotic (0.4%) has been reported to increase the ileal villus height and crypt depth in broilers. Similarly, MOS has been found to increase the villus length of the small intestine in broilers (Iji *et al*.,

2001). Rehman *et al*. (2007) demonstrated that supplementation of dietary inulin increased the jejunal villus length and crypt depth in broilers, at 35 days old. Hassanpour *et al.* (2013) indicated that 0.1% synbiotic (Biomin IMBO) significantly increased villus height, which increased overall villus surface area. This effect of 0.1% synbiotic, probably provide evidence of improved intestinal function including nutrient absorption.

Dietary supplementation of probiotics, prebiotics and synbiotics has been reported to decrease colonization of pathogens on the intestinal wall, thus preventing damage to the epithelial cells (Sherief *et al*., 2012, Wali, 2012, Abdelqader *et al*., 2013). Scanning electron microscopy (SEM) showed that the gut of all additive supplementations had normal morphology without signs of cell damage compared with control group, but varied in the density of microvilli. Chickens fed probiotic, prebiotic and synbiotic had much more microvilli than the control group. This result was in agreement with the finding of Luo *et al*. (2013) who showed that the dietary supplementation of broiler with probiotic (*Enterococcus faecium*) had much more microvilli than the control group.

The integral function of the epithelial layer and release of brush border membrane (microvilli) enzymes are fundamental to the digestion and absorption of nutrients from the intestinal lumen. The epithelium is covered by a layer of mucus composed of mucin glycoproteins that are synthesised by goblet cells. Goblet cells are responsible for the secretion of mucin that is used for the mucinous lining of the intestinal epithelium (Schneeman, 1982). Thus, a higher density of goblet cells may result in an increase in the secretion of mucin. Intestinal microbes might influence goblet cell dynamics via release of bioactive compounds or indirect activation of the immune system (Bienenstock and Befus, 1980).

The mucous layer acts as a layer of protection, lubrication and transport between luminal contents and epithelial cells (Uni *et al.*, 1998). Changes in the properties of this barrier could affect the absorption of both dietary and endogenous macromolecules and ions. On the other hand, increasing the thickness of this layer will lead to a narrow gut and this in turn means would slow the speed of the passage of the food mass and thus will provide a greater opportunity to digest and absorb nutrients. Another role of the mucous layer is to bind pathogenic microorganisms and reduce their colonization of the gut mucosa (Blomberg *et al*., 1993).

Dietary probiotic, prebiotic and synbiotic resulted significantly ($P<0.05$) increased proliferation of goblet cells on the surface of the villus membrane. This is indicative of an increased host dependence on mucus secretion for protection. Decreasing numbers of viable Gram-positive bacteria, such as Lactobacilli and Bifidobacteria, may increase the presence of Gram-negative species. An increase in these types of microbes may actually require the need for more mucus production and hence more goblet cells (Edens *et al*., 1997).

The present study did not show any significant effect by addition of prebiotic, probiotic and synbiotic on the relative weight of BF between groups. In agreement with these findings, it's reported that weight of Bursa did not show any significant differences using dietary supplementation of probiotic, prebiotic and synbiotic (Awad *et al.*, 2009; Dizaji *et al*., 2012). Dizaji *et al*. (2013) also reported that addition of probiotic, prebiotic and synbiotic to broilers diet did not show any significant effect on Bursa weight compared with control group.

The broiler industry is constantly searching for ways to improve its product and quality in order to meet the demands of an increasingly discriminating consuming

public. In this regard, numerous references exist on increasing poultry meat yields and improving carcass quality. For this reason, many ingredients have been used in broiler diets, in recent years. It is reported that additional benefits can be gained by supplementing broiler diets, particularly use of probiotics as feed additives. Probiotic, prebiotic and synbiotic are used to eliminate abnormalities in the gastrointestinal tract produced by stress and reduction of pathogenic bacteria and therefore normalize the gut activity.

In the present study, the results showed that chemical breast and leg composition were indicated that there were no significant effects ($P>0.05$) between additive supplementations and control group on the moisture of breast and leg meat at the end of experiment. This result agreed with Abaza *et al*. (2008), who found that chemical analysis of breast meat indicated that moisture determination was not significantly affected by additive supplementations.

Fat percentage significantly ($P<0.01$) decreased in probiotic, prebiotic and synbiotic group in breast and leg meat compared with control group. Pietras (2001) also reported that meat of chickens given probiotic (*Lactobacillus acidophilus* and *Streptococcus faecium* bacteria) on the whole rearing period had significantly higher protein content, while crude fat and total cholesterol contents tended to decrease. Khaksefidi and Rahimi (2005) demonstrated that addition of probiotic contained similar proportions of six strains of variable organisms namely *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Aspergillus oryzae*, *Streptococcus faecium* and *Torulopsis sps* and was fed at 100 mg/kg diet, proximate composition (moisture%, protein% and ash%) of leg and breast meat were significantly ($p<0.05$) increased in probiotic fed chickens, whereas, the fat% of leg and breast meat was decreased ($p<0.05$) in probiotic fed

chickens compared with control group. Nevertheless, the findings of this current study are in contrast with some findings. Joy and Samuel (1997) noted that implication of *Lactobacillus sporogenes* in broiler diets did not influence carcass protein, carcass fat and fat pad thickness. Zhou *et al*. (2010) did not observe any significant improvement (P>0.05) in contents of breast chemical composition including moisture, crude protein, crude fat, and crude ash between probiotic (*Bacillus coagulans* ZJU0616) and control group.

The results of the present study showed that probiotic, prebiotic and synbiotic supplementations decreased pH of breast and leg meat can improve shelf life of meat, because high pH value associated with higher bacterial growth and subsequent shorter shelf life (Allen *et al*., 1997). On the other hand, the pH of raw meat had significant negative correlation with lightness. A low ultimate pH of meat reduces the importance of myoglobin in selectively absorbing green light, resulting in meat that appears less red and more yellow. When the pH of meat is above the isoelectric point of myofibrillar proteins, water molecules are tightly bound, causing more light to be absorbed by the muscle, and meat appears darker in colour (Castellini *et al*., 2002). As resulted in the present study pH of breast and leg meat was lower in probiotic, prebiotic and synbiotic compared with control group, by this a* value (redness) of meat was reduced in additives supplementation compared with control group. Salakova *et al*. (2009) also found correlations between indicators of raw meat. Lightness (L*) and yellowness (b*) were found to correlate negatively to pH, whereas redness (a*) had a positive correlation.

The results were in agreement with the findings of Aksu *et al*. (2005) who observed that the use of probiotic in broiler diets improved meat quality during

storage. Also, Karaoglu *et al*. (2004) showed that the use of 0.1% probiotic (*Saccharomyces cerevisiae*) in broiler diets for 49 days decreased pH of carcass during the 24 hour period after slaughter compared with control group.

The colour and variations in colour are important quality attributes that affect selection and acceptability of many foods. The colour of carcass skin affects acceptability of broiler carcasses and its products. Broiler skin and meat colour are also affected by numerous factors such as live production, slaughter, processing, handling, and packaging (Froning, 1995; Fletcher, 1999; Petracci and Fletcher, 2002).

There were no significant differences for the values of cooking loss and shearing force values in the breast meat when different additives were used. These findings agreement with the results reported by Pelicano *et al*. (2003), who found no differences in SF and CL in the meat of chicken fed with probiotics. Pelicano *et al*. (2005) added two types of probiotics (*Bacillus subtilis*) based probiotics at 150 g/ton and probiotics based on *Lactobacillus acidophilus* and *Lb. casei*, *Streptococcus lactis* and *Streptococcus faecium*, *Bifidobacterium bifidum* and *Aspergillus oryzae* at 1 kg/ton from 1 to 42 days of age, prebiotic (Mannanoligosaccharids) and combination between both (Synbiotic) were no significant affected by the use of different supplementation on the pH value, color (L*- lightness, a*- redness, and b*- yellowness), cooking losses and shearing force at 42 days of age compared with control group.

According to Lyon & Lyon (1990), shear force values up to 7.5 kgf might be considered tender; nevertheless, Simpson & Goodwin (1974) suggested that values of up to 8 kgf. In regard to these reference values for shear force, it can be inferred that the use of probiotics and prebiotics in the present study had no effect

on breast meat tenderness. All these parameters together water holding capacity, CL and SF are quality parameters intimately related with the process of meat tenderness, which is a determining qualitative factor and one of the most important sensory characteristics of the meat quality (Koohmaraie *et al*., 1990).

Haematological and biochemical parameters of animal are determined as an index of their health status. At the end of experiment (35 day), the haematocrit (Hct%) was increased for birds supplemented with probiotic, prebiotic and synbiotic (29.59%, 30.11% and 31.03%) respectively compared with control group (28.99%). The higher Hct in the chicks fed on probiotic, prebiotic and synbiotic may be due to the acidic condition of the GI tract caused by additives supplementation which resulted in better iron salt absorption from the small intestine. This may also cause better vitamins B complex production by beneficial bacteria which may results in positively affecting blood-forming processes (Kander, 2004).

The results of the present study showed that only prebiotic significantly ($P<0.01$) increased haemoglobin compared with the control, while there were no significant differences between probiotic and synbiotic groups compared with the control group. Agawane and Lonkar (2004) who found that when the probiotic added to the diet of broiler at a rate of 10mg/kg, there was no significant effect in Hb concentration at 6 week compared with the control. In the present study also showed that the H/L ratio was decreased in all additives supplementation compared with control group. The results in agreement with finding of AL-Kassie *et al*. (2008) who found a significant decrease in H/L ratio of broiler fed on the diet supplemented with 10g/kg of probiotic (*Aspergillus niger*) and the prebiotic (*Taraxacum officiale*) at 42 days compared with control being 0.28, 0.26 and 0.31,

respectively. Also, Paryad and Mahmoudi (2008) who reported that addition of different levels of probiotic 0.5, 1.5 and 2 % to the broiler diet significantly decreased the H/L ratio at 42 days compared with control group.

Haematocrit is the volume percentage (%) of red blood cells in blood. It is normally is about 22-35% (Jian, 1993). Because the purpose of red blood cells is to transfer oxygen from the lungs to body tissues, haematocrit of a blood sample (the red blood cell volume percentage) can become a point of reference of its capability of delivering oxygen to the tissues. Physiological and pathological stress in avian species affected neuro-endocrine system (glucocorticoids, catecholamins, epinephrine, norepinephrine, prolactin and growth hormones) and reduced the lymphocyte production (Marketon and Glaser, 2008). When birds are stressed, glucocorticoid hormones are secreted and the physiological stress is response (Dhabhar *et al*., 1996). Stress could cause an increase in the stimulation of the adrenal gland to produce hormones which has a direct effect to analyses a lymphatic cell which causes an increase in H/L ratio (Gross and Siegel, 1983). Thus H/L ratio could be used as an indicator for the health of animals and any increase of H/L ratio refers to an increase in stress case (James and Stanley, 1989).

In this study, the supplementation of probiotic, prebiotic and synbiotic applied to the diet for broiler chickens significantly reduced ($P < 0.05$) the cholesterol concentration content in chicken blood compared with the control group. The results were in agreement with the findings of Alkhalf *et al*. (2010) who found that Chicken fed a diet containing various levels of commercial probiotic supplementation (Bactocell®) in the diet of broilers showed a significant decrease ($P<0.05$) in cholesterol concentration compared with control group. Also, Karimi *et*

al. (2010) who found a significant decrease in plasma cholesterol of broiler as a result of probiotic supplementation in drinking water for 40 days of age. Paryad and Mahmoudi (2008) who found a significant decrease in serum cholesterol as a result of different levels 0, 0.5, 1.5 and 2 % of probiotic addition to the broiler diet at 42 days of age. Panda *et al*. (2006) who reported that there was a significant reduction in serum cholesterol at 42 due to the dietary supplementation of different levels 0, 100, and 200 mg/kg of probiotic to the broiler diet. Also, Kalavathy *et al*. (2003) reported that when the diet of male broiler chicks was supplemented with 0.1%, probiotic there was significant reduction in serum cholesterol at 42 days compared with the control being 132.52 and 143.10 mg/dl, respectively. However, the results were in contrast with the findings of Capcarova *et al*., (2010) who found that probiotic (*Lactobacillus fermentum* and *Enterococcus faecium*) supplementation in the drinking water of broilers did not have any effect of cholesterol concentration. Also, Safalaoh (2006) who reported that the probiotic was added to the drinking water of broiler chicks did not cause any significant effect on serum cholesterol at 42 days of age.

The significant reduction in serum cholesterol of broiler chickens fed probiotic supplemented diet could be attributed to reduced absorption and/or synthesis of cholesterol in the gastrointestinal tract by probiotic supplementation (Mohan *et al*., 1995, 1996). Furthermore, some probiotic bacteria may interfere with cholesterol absorption in the gut by deconjugating bile salts (Li *et al*., 2007; Liong and Shah, 2006). Also, it was showed that *Lactobacillus acidophillus* reduces the cholesterol in the blood by deconjugating bile salts in the intestine, thereby preventing them from acting as precursors in cholesterol synthesis (Abdulrahim *et al*., 1996).

Lactobacillus has found to have a high bile salt hydrolytic activity, which is responsible for deconjugation of bile salts (Surono, 2003).

The effects of probiotic and prebiotic on serum cholesterol concentrations are inconsistent among previous studies. Some studies have shown that probiotic and prebiotic exhibited lipid-lowering properties which might be related to the changes in the intestinal bacterial flora composition, which ferments prebiotics to produce short-chain fatty acids in the gut and then causes a decrease in the systemic levels of blood lipids and cholesterol. Another explanation to these inconsistent results might be because of the level of dose used and the period of time administered as well as the species of probiotic bacteria and type of prebiotic (Angel et al., 2005; O'Dea et al., 2006; Patterson and Burkholder, 2003).

Deconjugated bile acids are less soluble at low pH and less absorbed in the intestine and is more likely to excrete in faeces (Klaver and van der Meer, 1993). This could be the case in the present study as the probiotic and prebiotic utilized in the study is acidophilic and it lowers the pH of the environment it occupies.

The lower concentration of cholesterol in the groups fed on probiotic and synbiotic may be due to that some microorganisms present in the probiotic had the ability of cholesterol utilization for their metabolism and depressed the cholesterol absorption from gastrointestinal tract (Nelson and Gilland, 1984; Mohan *et al*., 1995). In addition probiotic microorganism had the ability to inhibit the activity of hydroxymthyl-glutaryl-coenzymeA which involved in the cholesterol synthesis (Fukashima and Nakon, 1995). Also prebiotic had hypocholesterolemic effects through reducing lipid absorption in intestine by binding bile acids, which resulted in increased cholesterol elimination and hepatic synthesis of new bile acid (Zhang *et al*., 2003).

The recent researches have revealed that probiotics affect gene expression of carrier proteins which are responsible for cholesterol absorption. The protein called Niemann-Pick C1-like 1 (NPC1L1) which is abundantly expressed on the surface of enterocytes, plays a key role on the absorption of cholesterol from intestines. Reduction or inhibition of expression levels of this protein leads to a decrease in plasma cholesterol levels. The probiotic *Lactobacillus acidophilus* ATCC 4356 reduced NPCIL-1 gene expression and inhibited the cellular uptake of micellar cholesterol in Caco-2 cells.

5.6 Conclusion

In conclusion, the present study indicates that inulin extracted from Jerusalem artichoke had a positive prebiotic effect as demonstrated by increases in the beneficial bacteria population in broiler chickens. Additionally, the strain *Lactobacillus animalis* as probiotic also had a positive effects on gut microflora and intestinal histology. On the other hand, the combination of *Lactobacillus animalis* and inulin extracted from Jerusalem artichoke (synbiotic) also resulted to improve and produce a good performance compared with control group. While, there were no significant interaction observed between these two factors in some parameters, which indicates that probiotic and prebiotic utilization in the diet had independent effect on some parameters at the end of the experiment. Greater prebiotic and probiotic effects and represents an important dietary strategy that could potentially improve the growth performance, the gut microbial ecology and morphology and the overall health of broiler chickens compared with control group. The higher production performance observed in broilers fed all of these

additives supplements may be due to suppression of pathogenic bacteria which leads to increased intestinal length and villus height and allowed for increased intestinal absorptive area and ultimately improved growth and overall performance.

CHAPTER SIX: General conclusion and future work

6.1 General conclusion

For the past four decades, antibiotics have been used as growth promoters (AGP) and as prophylactic treatments in poultry feed to control disease and subsequently, to enhance the growth performance, improved feed efficiency and protect birds from the negative consequences of pathogenic and non-pathogenic enteric microorganisms. Antibiotic feed additives were banned by the European Union in 2006 due to concerns over the rise of widespread antibiotic resistance in human pathogens. Consequently, poultry producers are seeking alternatives to AGP to maintain efficient poultry production. Probiotic, prebiotic and synbiotic can be used as an attempt to reduce the chances of infection in poultry.

A number of probiotics are available commercially for use in poultry production, such as *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus and Pediococcus* species, these bacteria are used alone or in combination. Additionally, yeast species such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii* have been used as probiotics in poultry diets. Prebiotics such as fructooligosaccharide, mannan-oligosaccharides and inulin enhance the growth of intestinal bacteria and may affect the intestinal histology. Synbiotics may work in one of two ways, they may promote the growth of the co-administered probiotic or they may promote the growth of other beneficial organisms in the gut that in turn benefit the co-administered probiotic.

This study includes four experiments, one *in vitro* and three *in vivo* field studies were designed to investigate the influence of dietary supplementation of a probiotic (*Lactobacillus animalis*), a prebiotic Jerusalem artichoke tuber (*Helianthus tuberosus*) and a combination of both (Synbiotic) in dry feed on the

production performance, microbial population in ileum and caecum digesta, jejunum histology, immune organ and meat quality of broiler chickens.

The *in vitro* work (Chapter 2), the aim of this chapter was to isolate and screen for the best lactic acid bacteria with antimicrobial activity against enteric pathogenic bacteria. The examinations confirmed that C4 strain is gram-positive, catalase-negative, and a rod-shaped bacterium. Molecular methods are known to be important for bacterial identification (Drancourt *et al*., 2000; Taheri *et al*., 2009). Hence, the amplification of the 16S rRNA gene of the C4 strain by the PCR technique followed by sequence analysis and homology search via BLAST identified (100%) the strain as *Lactobacillus animalis*. The finally selected bacterial strain of this research is different from those that have been isolated by other researchers. Garriga *et al*. (1998); Ehrmann *et al*. (2002) and Savvidou (2009) were selected *Lactobacillus salivarius* and Taheri *et al*. (2009) selected *Lactobacillus crispatus* at the end of their screening procedures. This strain had potential probiotic properties, rapid auto-aggregation and co-aggregation ability, resistance to acidotic pH and bile salts, strong suppression of pathogens and very good adhesive capacity to chicken epithelial cells. Also, the results from this chapter showed that both types of Inulin (Commercial inulin Frutafit® HD and inulin extracted from Jerusalem artichoke) supported good growth of this strain of LAB and could be a suitable candidate for synbiotic production in broiler diet. This organism was selected as a candidate that could be used *in vivo* experiment as a chicken probiotic.

The second part of this study was done by three *in vivo* experiments (Chapter 3, 4 and 5). The first *in vivo* experiment (Chapter 3) was with 27 specific pathogen free (SPF) chicks, to investigate the influence of dietary inulin supplementation from

different source of commercial inulin (Frutafit® HD) and inulin extracted from Jerusalem artichoke on intestinal microflora, immune functions and blood characteristics of SPF chicks. The results confirmed the significant increases of *Lactobacillus* spp. and *Bifidobacterium* spp. at 18 and 21 days of age. The microbial population diversity in the gastrointestinal tract of chickens changed due to inulin addition to feed treatment. The culturable species identified in this study from caecum part of GI tract included those from the genera *Clostridium*, *Lactobacillus*, *Ruminococcus*, *Sedimentibacter*, *Lachnospiraceae*, *Lactonifactor*, *Coprococcus*, *Amphibacillus*, *Oribacterium*, and *Arthrobacter*. The both types of inulin significantly increased the villus length compared to the control treatment.

The second *in vivo* experiment (Chapter 4) was conducted with 102 Hubbard broiler chicks. This study was designed to investigate the influence of dietary probiotic supplementation on intestinal microflora, histology and immune functions of broiler chickens. The chicks were divided into three treatments; control group (without additive), control diet supplemented with *Pediococcus acidolactici* and *Lactobacillus animalis* as probiotics (PRO1 and PRO2), respectively. This study showed beneficial effects of dietary inclusion of *Lactobacillus animalis* based probiotic. This strain which was isolated from a chicken caecum had potential probiotic properties as ability to improve growth performance of broilers compared with the control. Final weight gain was significantly ($P<0.01$) higher in PRO1 (7.62%) and PRO2 (7.84%) groups compared with the control group. Intestinal microbiota profiles based on the PCR-DGGE DNA fingerprinting indicated that; at 14 day of age, both types of probiotic significantly ($P<0.01$) increased diversity and richness of microbiota compared with birds fed control group. However, only PRO2 increased significantly ($P<0.01$) diversity and richness of ileal microflora

compared with control group at the end of experiment. Villus height and crypt death of jejunum were increased, reduction of pH in ileum, increase the size of follicle of Fabricius were observed by supplementation of PRO1 and PRO2 in the diet of broilers at the end of the experiment. The results from this study showed that both types of probiotics supported good growth of healthy of chicks and could be a suitable candidate as a source of probiotic in broiler diet. There is not difference between both types of probiotics when compared to each other.

The third *in vivo* experiment (Chapter 5) was conducted with 72 Hubbard broiler chicks, to investigate the influence of dietary probiotics, prebiotics and the interaction between both in a combination (synbiotics) supplementation on performance production, intestinal microflora, and jejunum histology including scanning electron microscopy, immune functions and meat quality of broiler chickens. The intestinal microflora was analysed by conventional culture-based techniques and the molecular techniques. The dietary treatments were: control group (standard broiler diet), control diet supplemented with 100 mg *Lactobacillus animalis* / kg of diet, containing 1.72×10^{10} CFU/kg (Probiotic); 1% Jerusalem artichoke inulin (Prebiotic); 100mg Probiotic (1.72×10^{10}) + 1% Prebiotic / kg diet (Synbiotic). The results of this experiment revealed the body weight gain and feed conversion ratio were increased in all treated birds compared with control group. The bird treated with probiotic, prebiotic and synbiotic had highly significant effect on the European production efficiency factor (EPEF) at the end of the experiment. The chicks in probiotic, prebiotic and synbiotic group were improved EPEF compared with control group. The higher performance production observed in broilers fed probiotic, prebiotic and synbiotic may be due to the fact that additives

suppress pathogenic bacteria which lead to improved health status and ultimately improved growth and overall performance.

Probiotic, prebiotic and synbiotic supplementation increased the number of *Lactobacillus* spp. and *Bifidobacteria* spp. compared to control group in the ileum and caecum digesta. On the other hand, the decreases of total coliform and aerobic bacteria numbers were observed in all additive supplementations in ileum and caecum of chickens. This change of intestinal microflora composition of broiler chickens might have made the chickens more resistant to pathogen colonisation to the GI tract. The dietary probiotic, prebiotic and synbiotic added to the diet significantly decreased the level of pH in caecum digesta and only in synbiotic group in ileum digesta compared with control group at 35 days of age. This reduction may be due to the increasing of beneficial bacteria in gut chickens to produce the SCFA from ferment of nutrients during metabolism. The length of small intestine was increased by all additive supplementations compared with control. Morphology data for the jejunum showed that all the additive supplementations increased villus length compared to the control group at 17 and 35 days of age. In addition, Probiotic, prebiotic and synbiotic supplementation decreased the Heterophil/Lymphocyte ratios compared with control group, which is important indicator of stress reduction on birds.

DGGE is very helpful tool to understand the very complex bacterial populations in the gut and detect the changes in the intestinal microbial populations. The sequence analysis of DGGE bands was helpful to know the types of the bacteria in the population. The most family BLAST results in caecum were related to *Clostridium* spp., *Lactobacillus* spp., *Ruminococcus* spp., *Eubacterium* spp., *Coprococcus* spp., *Anaerostipes* spp., *Stomatobaculum* spp., *Enterococcus* spp.

and *Roseburia* spp. strains. However, the results in ileum were related to *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Ruminococcus* spp., *Escherichia* spp., *Clostridium* spp., *Acidaminobacter* spp. and *Enterobacter* spp. strains. The sequencing revealed the variety of bacteria in the chicken gut. Some species were common between all groups and other is single for subjected group. Intestinal microbiota based on the PCR-DGGE profiles indicated that; at the end of the experiment, probiotic, prebiotic and synbiotic groups increased diversity of microbiota compared with birds fed control group, in ileal and caecal microflora.

This investigation found that the probiotic, prebiotic and synbiotics which was a combination of *Lactobacillus animalis* and inulin extracted from Jerusalem artichoke had a positive effect on broiler performance, intestinal microflora, intestinal histology, blood characteristics compared with control group. While, there were no significant interaction observed between the two factors (PRO*PRE), which indicates that probiotic and prebiotic utilization in the diet had independent effect on some parameters.

6.2 Future work

The following areas can be studied further:

1- The research is need to increase knowledge regarding the effect of various levels of probiotics, prebiotics and synbiotics to evaluate their effects on the growth performance, modulate the composition of gut microflora and histology of small intestine.

2- There is some knowledge accumulated on the application of probiotic, prebiotic and synbiotic in poultry production and health status but this is still limited and the research should continue. For example, little is known about the immunological response of the chicken to these additives supplementation.

3- The action of *Lb. animalis* need to using with multi-bacterial species as probiotic instead of single bacteria may have more effectiveness on the poultry production. This will help to increase the mode of action of probiotics.

4- This application should be carried out to study the effect of the diet supplementation with probiotic, prebiotic and synbiotic on commercial layer and broiler breeder's performance.

5- Based on these benefits of *Lb animalis*, inulin from Jerusalem artichoke and synbiotic, poultry producers may interested to use in their farms. However, in order to deliver as adequate amount of product to the poultry farms, the product must be increased and protected in a special product commercially.

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Appendices

Appendix 1

Calculation of inulin in the tubers of Jerusalem artichoke:

The amount of fructan present in the sample was calculated according to the following equations:

The absorbance was determined by differences between A2 and A1 for both "sugars" and "fructan + sugars" and calculated values for ΔA_{sugars} and $\Delta A_{fructan + sugars}$ as described below.

Determination of D-fructose + D-glucose in the "sugars" sample:

ΔA_{sugars} = (A2-A1) (from the "sugars" sample).

Determination of D-fructose + D-glucose in the "fructan +sugars"sample:

$\Delta A_{fructan + sugars}$ = (A2-A1) (from the "fructan + sugars" sample).

The concentration of "sugars" and "fructan + sugars" was calculated as follows:

$$C = \frac{V \times MW}{\varepsilon \times MW \times v} \times \frac{0.9}{0.2} \times \Delta A \qquad [g/L]$$

Where:

V = final volume [mL]

MW = molecular weight of D-glucose or D-fructose [g/mol]

Ɛ = extinction coefficient of NADPH at 340 nm = 6300 [l x mol^{-1} x cm^{-1}]

d = light path [cm]

v = sample volume [mL]

0.9/0.2 = 0.2 mL of sample was incubated with 0.2 mL sucrase / maltase enzyme and 0.5 mL acetate buffer added (total 0.9 mL); 0.2 mL of this was taken for incubation with fructanase enzymes (i.e. 0.2 mL removed from 0.9 mL).

The equation for ‘’sugar’’ as follows:

$$C = \frac{2.62 \times 180.16}{6300 \times 1 \times 0.2} \times \frac{0.9}{0.2} \times \Delta A_{sugars} \quad [g/L]$$

$C = 1.6858 \times \Delta A_{sugars}$ ..... [g/L]

The equation for ‘’fructan + sugar’’ as follows:

$$C = \frac{2.62 \times 180.16}{6300 \times 1 \times 0.2} \times \frac{0.9}{0.2} \times \Delta A_{fructan + sugars} \quad [g/L]$$

$C = 1.6858 \times \Delta A_{fructan + sugars}$ ..... [g/L]

For ‘’fructan’’ : $C_{(fructan)} = C_{(fructan+sugars)} - C_{(sugars)}$ .....[g/L]

Content of fructan as g/100g was calculated as follows:

$$\text{Fructan} = \frac{C_{fructan}\,[\text{g/L sample solution}]}{\text{Weight}_{sample}\,[\text{g/L sample solution}]} \times 100 \times \frac{162}{180} \quad [g/100g]$$

Where:

162/180 = factor to convert from free fructose and glucose as determined, to anhydrofructose and anhydroglucose as occurs in fructan.

Appendix 2:

The LAB DNA sequence resulted from chicken caecum. The sequencing result was *Lactobacillus animalis*.

CTTCTTTATCACCGAGTGCTTGCACTCACCGATAAAGAGTTGAGTGGCGAA
CGGGTGAGTAACACGTGGGCAACCTGCCCAAAAGAGGGGGATAACACTT
GGAAACAGGTGCTAATACCGCATAACCATAGTTACCGCATGGTAACTATGT
AAAAGGTGGCTATGCTACCGCTTTTGGATGGGCCCGCGGCGCATTAGCTA
GTTGGTGAGGTAAAGGCTTACCAAGGCAATGATGCGTAGCCGAACTGAGA
GGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGC
CGCGTGGGTGAAGAAGGTCTTCGGATCGTAAAACCCTGTTGTTAGAGAAG
AAAGTGCGTGAGAGTAACTGTTCACGTTTCGACGGTATCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TATCCGGATTTATTGGGCGTAAAGGGAACGCAGGCGGTCTTTTAAGTCTGA
TGTGAAAGCCTTCGGCTTAACCGGAGTAGTGCATTGGAAACTGGGAGACT
TGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAAGAACACCAGTGGCGAAAGCGGCTCTCTGGTCTGTAACTG
ACGCTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCA
GTGCTGCAGCTAACGCAATAAGCATTCCGCCTGGGGAGTACGACCGCAAG
GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTCTG
ACAATCCTAGAGATAGGACTTTCCCTTCGGGGACAGAATGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTG (1031 nt)

Sequencing analysis result of LAB which isolated from chicken caecum.

Sample name	NCBI Accession number	NCBI BLAST match	Maximum Identity	E value	Query cover
C4	AB911530.1	*Lactobacillus animalis* strain: JCM 8692	100%	0.0	100%

Appendix 3:

Buffers

Buffer solutions were used during the samples analyses as described in chapter 3, 4, 5 and 6.

TE Buffer
10 mM (1.57 g/l) Tris/Cl, 1 mM EDTA (0.37 g/l)
Adjusted to pH 8.0 with concentrated HCl

50 x TAE buffer
2 M Trizma base, 30 mM EDTA, 250 mM sodium acetate
pH 7.8 with concentrated acetic acid

Stock 0% denaturant Acrylamide solution
26.7 ml 30% acrylamide solution
2 ml 50 x TAE
71.3 ml water

Stock 80% denaturant acrylamide solution
26.7 ml 30% acrylamide solution
2 ml 50 x TAE
32 ml molecular grade formamide
5.6 M (34 g) molecular grade urea
To 100 ml with distilled water
Store refrigerated in the dark

Sodium maleate buffer (100 mM, pH 6.5).
Dissolve maleic acid (11.6 g, Sigma cat. no. M-0375) in 900 mL of distilled water and adjust the pH to 6.5 with sodium hydroxide solution (2 M). Adjust volume to 1 L. Store at 4°C.

Sodium acetate buffer (100 mM, pH 4.5).
Add glacial acetic acid (5.8 mL) to 900 mL of distilled water. Adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 litre. Store at 4°C.

Appendix 4

Research training and development

1. Postgraduate Research Skills and Training Sessions

NO	Date	Training skills	Facilitator	Venue
1	3/5/2012	Research Owning and Using	Graham Titley	Portland square – Plymouth University
2	9/5/2012	Project Management	Jonathan Moizer	Babbage building - Plymouth University
3	10/5/2012	Developing Professional Writing Skill	John Hilsdon and Joe Allison	Rolle building - Plymouth University
4	16/5/2012	Careers in Academia	--	Roland Levinsky - Plymouth University
5	23/5/2012	Overview to Searching and Accessing Information Resources	Nicola Cockarill	Rolle building- Plymouth University
6	29/5/2012	SPSS	Luciana Dalla Valle	Babbage building - Plymouth University
7	22/6/2012	Transfer Process	Mick Fuller	Roland Levinsky- Plymouth University
8	25/10/2012	Work Place Health & Safety Risk Management for Research Students	David Morton	Babbage building - Plymouth University
9	31/10/2012	Keeping Laboratory Records	Dr Rich Boden	Rolle building - Plymouth University
10	15/11/2012	Overview to Searching and Accessing Information Resources	--	Babbage building - Plymouth University
11	12/12/2012	Excel 2012: Essential Features	--	Babbage building - Plymouth University
12	04/03/2014	Preparing for the Viva	Mick Fuller	Portland square - Plymouth University

2. Modules training and development

2.1 Bio 5124 (Postgraduate Research Skills and Methods) 6th Oct. - 14th Dec. 2011.

2.2 Bio 5102 (Principles and Applications of Electron Microscopy) 6th Oct. - 14th Dec. 2011.

2.3 Home Office License Training

Animals (Scientific Procedures) Act 1986 (PIL 30/10067) 2-3rd July 2012.

- Small Animal Module1, 2 and 3 (2/07 -03/07/2012), Personal license
- Chicken Module1, 2 (02/07 - 03/07/2012), Personal license.

2.4 Animals (Scientific Procedures) Act 1986 (PIL 30/10067) 10th August 2013 Updated.

3. Session and Conferences Attended

No.	Date	Event	Venue
1	17/03/2011	The post graduate Society Conference Series	Plymouth University - United Kingdom
2	04/04/2011	1st Annual Conference	Plymouth University - United Kingdom
3	05/04/2011	Annual Research Day	Plymouth University - United Kingdom
4	06/06/2012	Postgraduate conference for computing: Application and theory	Plymouth University - United Kingdom
5	07/06/2012	Marine Institute Annual Research Centre Conference	Plymouth University - United Kingdom
6	26/06/2012	The post graduate Society Annual Conference	Plymouth University - United Kingdom
7	04/07/2012	Centre for research in translational biomedicine Annual research day	Plymouth University - United Kingdom
8	10-11/09/2012	Prebiotics and Probiotics in medicine, veterinary sciences and aquaculture: the future	Keele University - United Kingdom
10	21/11/2012	The post graduate Society Conference Series	Plymouth University - United Kingdom
11	10/12/2012	CARS Postgraduate Symposium	Plymouth University - United Kingdom
12	21/03/2013	Plymouth PG conference, Plymouth – UK, PG society	Plymouth University - United Kingdom
13	11-13/06/2013	An international scientific conference on probiotics and prebiotics (IPC 2013)	Kosice, Slovakia
14	02/07 – 04/07/2013	SFAM international conference, , ISAPP	Cardiff-Wales
15	11/11/2013	4th CARS Postgraduate Symposium	Duchy College, Cornwall- UK
16	01/03/2014	Iraqi post graduate conference	Plymouth University - United Kingdom
17	19/03/2014	Plymouth PG conference, Plymouth – UK, PG society.	Plymouth University - United Kingdom
18	06/06/2014	5th CARS Postgraduate Symposium	Experimental Station – Rothamsted Research, Okehampton - UK
19	17/06/2014	The Postgraduate Society Conference Series	Plymouth University - United Kingdom
20	24-26/06/2014	An international scientific conference on probiotics and prebiotics (IPC 2014)	Budapest, Hungary
21	30/06-03/7/2014	SFAM international conference	Brighton - United Kingdom
22	19/11/2014	6th CARS Postgraduate Symposium	The Eden Project, Boldeva, Cornwall, UK
23	24/03/2015	The Postgraduate Society Conference Series	Plymouth University - United Kingdom

Membership of Scientific Societies:

- Wold Poultry Science Association (WPSA).
- Society of Applied Microbiology (s*f*am).
- Society of Experimental Biology (SEB).

Appendix 5

Home office personal licence

Home Office

No. PIL 30/10067

ANIMALS (SCIENTIFIC PROCEDURES) ACT 1986

PERSONAL LICENCE

to

carry out regulated procedures on living animals.

In pursuance of the powers vested in him by the above Act, the Secretary of State hereby licenses

Mr R A Akoy
Sch of Biomedical & Biological Sciences
University of Plymouth
Room 409, Portland Square Building
PLYMOUTH
PL4 8AA

to apply the techniques specified in column a of paragraph 15 of the attached Schedule to the kinds of animals in column b of the same paragraph at the place or places specified in paragraph 14 of this Schedule, subject to the restrictions and provisions contained in the Act, and subject also to the limitations and conditions contained in this licence and to such other conditions as the Secretary of State may from time to time prescribe.

This licence shall be in force until revoked by the Secretary of State and shall be periodically reviewed by him.

Home Office
2 Marsham Street
London SW1P 4DF

For the Secretary of State

14 December 2012

NB. This licence does not authorise the licensee to perform any of the procedures specified in it unless they are carried out in the course of a project for which there is a project licence in force under the Act.

WORKING TOGETHER TO PROTECT THE PUBLIC

Appendix 6

Posters

Effect of Inulin Prebiotic on Growth of Lactic Acid Bacteria Probiotic Used as Synbiotic for Broilers diet

Rebin Akoy, Simon Davies, Daniel Merrifield and Jane Beal
School of Biomedical and Biological Science, Plymouth University, PL4 8AA, UK
rebin.akoy@plymouth.ac.uk

RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

Prebiotic supplementation can significantly modulate the colonic microbiota by increasing the number of specific probiotic bacteria such as lactobacilli and bifidobacteria (1). A combination of probiotic and prebiotic, which is called synbiotic, could have a synergistic impact in promoting growth of existing strains of beneficial bacteria in the lower part of gastrointestinal tract as well as improving the survival and growth of newly added probiotic strains to the diets (2).

The aim of this study was to investigate the influence of inulin as an effective prebiotic on eleven strains of LAB, for the future preparation of an inulin synbiotic containing a mixture of inulin and the best strain of LAB.

Methods:

1. The commercial inulin and inulin extract from Jerusalem artichoke (JA) were screened for their effects on the growth of the 11 Lactic acid bacteria.
2. 0.1 ml of an overnight culture of LAB (10^5 CFU ml^{-1}) were inoculated into 0.7 ml of MRS broth containing 2% glucose as a control and 2% inulin and JA extract in 48-well micro-plate.
3. The micro-plate was incubated in a plate reader (TECAN, Germany) at 41-42 °C for 24 h.
4. The growth of each strain was determined every 3h by measuring the optical density at 620 nm. Three replications were made for the experiment.

Results

The results showed that both types of Inulin supported good growth of six strains of LAB after incubation at 41-42 °C for 24 h. This investigation has found that C4 strain which was isolated from a chicken caecum had a shorter lag-phase and much better growth than other strains and could be a suitable candidate for an inulin synbiotic in broiler diet.

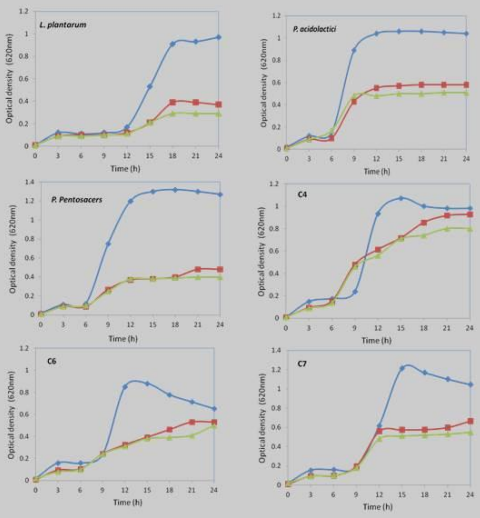

Figure: Growth curves of 6 probiotic LAB in basal MRS medium supplemented with glucose (control) (♦) commercial Inulin (■) and inulin from Jerusalem artichoke extract (▲) per every three hour by Tecan plate reader. Results are the means from three replications.

Table 1: The lag time period (h) of LAB on commercial and JA extract inulin at 41-42 °C by Tecan plate reader.

strains	Time [1] (h)		
	Glucose	C. Inulin	JA extract Inulin
L. plantarum	12	12	12
P. acidolactici	6	6	6
P. pentosacers	6	6	6
C4	8	6	6
C6	8	6	6
C7	8	6	6

[1]Results are mean values from three replications ± standard deviations.

Table 2: Cells densities (OD_{620} ± SD) of LAB 10^5 CFU ml^{-1} on commercial and JA extract inulin at 41-42 °C after 24h of incubation by (Tecan plate reader).

strains	Cell densities [1]		
	Glucose	C. Inulin	JA extract Inulin
L. plantarum	0.97±0.09	0.37±0.01	0.29±0.01
P. acidolactici	1.04±0.05	0.58±0.03	0.51±0.02
P. pentosacers	1.27±0.02	0.48±0.03	0.40±0.01
C4	0.98±0.09	0.92±0.02	0.80±0.05
C6	0.65±0.06	0.53±0.07	0.50±0.09
C7	1.04±0.04	0.66±0.02	0.55±0.05

[1]Results are mean values from three replications ± standard deviations.

Conclusions:

The results of the present study showed that inulin supports the growth of strain C4 which was isolated from a chicken caecum more than the other strains of LAB.

Future work:

- Preparation of synbiotic from inulin extract in Jerusalem artichoke and C4 (Lactic Acid Bacteria).
- This new product will be focus on for further identification using PCR technique then use it in broiler nutrition to support good growth and better health.

Acknowledgment:

Special thanks to my supervisor Dr Jane Beal

Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government.

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2. Schrezenmeir J, de Vrese M (2001). 'Probiotics, prebiotics, and synbiotics—approaching a definition. *American Journal of Clinical Nutrition*., 73: 361-364.

Effect of dietary inulin supplementation on intestinal histology and immune functions of broiler chicks

RESEARCH WITH PLYMOUTH UNIVERSITY

Rebin Akoy, Simon Davies, Daniel Merrifield and Jane Beal
School of Biomedical and Biological Science, Plymouth University, PL4 8AA, UK
rebin.akoy@plymouth.ac.uk

Introduction:

This study dealt with the effects of inulin from commercial and Jerusalem artichoke tubers (*Helianthus tuberosus*) supplementation on the jejunum histology and immune organ of the broiler chicks. The use of prebiotics as alternatives to of antibiotics have become popular in birds to improve the population of beneficial microbes and morphological changes in the GI tract (Zsolt *et al*., 2011; Park and Park, 2012). The bursa of Fabricius is an immunological organ that plays an essential role in the poultry immunity (Alloui *et al*., 2005).

Objectives

The aim of this study was to investigate the influence of inulin from Jerusalem artichoke as prebiotic source on broiler chickens.

Methods:

Experimental Design:

A total of twenty seven newly hatched chicks were randomly divided into three treatments, nine chicks per treatment with three replications.

Treatments:

T1= control group feed standard broiler diets.
T2= standard boiler diets + 1% commercial inulin.
T3= standard boiler diets + 1% Jerusalem artichoke inulin.

Characteristics studied:

- Live body weight (LBW) at the end of the experiment.
- Histology of Jejunum (Villus length and Crypt depth).
- Histology of Bursa of Fabricius (BF).

Figure 1: Chick housing facility.

Figure 2: The bursa of Fabricius in chickens.

Results Discussion

1- Both types of inulin significantly (P<0.000) increased villus length compared to the control treatment. The body weight was not significantly (P>0.05) affected by adding inulin in the diets. Morphological changes in the gastrointestinal tract by increasing villi length may have a positive effect on digestibility of nutrients (Hajati and Rezei, 2010).

Table: Effects of dietary inulin supplementation on LBW (gm), villus height (µm) and crypt depth (µm) in the Jejunum of SPF broiler chicks at 14 and 21 d of age.

Treatments	Parameters				LBW at d 21 (gm)
	14 d		21 d		
	Villus height	Crypt depth	Villus height	Crypt depth	
T1	471.22±31.91^{b}	71.91±11.38^{a}	533.20±20.84^{b}	67.42±17.41^{b}	472.00±27.30^{a}
T2	597.93±66.92^{a}	74.75±11.64^{a}	690.09±70.62^{a}	74.61±8.53^{ab}	504.70±20.02^{a}
T3	615.11±89.24^{a}	78.63±14.71^{a}	696.13±96.66^{a}	84.61±13.47^{a}	511.96±17.34^{a}
P. values	0.000	0.35	0.000	0.005	0.13

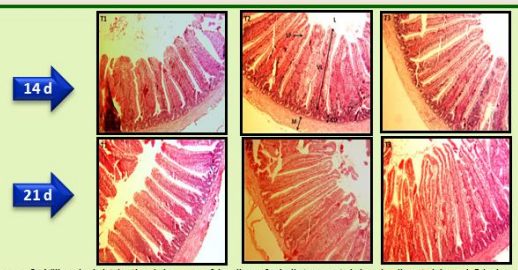

Figure 3: Villus height in the jejunum of broilers fed diets containing inulin at 14 and 21 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

2- The diameters of follicles of Fabricius were increased significantly (P<0.05) in dietary inulin supplementation (10 g of inulin/kg) compared to the control treatment at 14 and 21 days of age. The results showed no significant difference between both types of inulin treatment for growth of follicles of Fabricius at different days of age. The Bursa of Fabricius is an organ responsible for immunity and its evolution in the chicken body is responsible for maturation of lymphocytes B (Alloui *et al*., 2005). The size and weight of the bursa is an indication of the immune response to live body weight. Therefore, the body weight was improved by adding inulin in the diet.

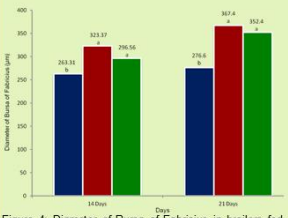

Figure 4: Diameter of Bursa of Fabricius in broilers fed diets containing inulin at d 14 and 21 of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA.

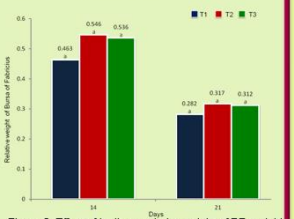

Figure 5: Effect of inulin on relative weight of BF at d 14 and 21 of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA.

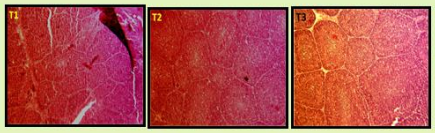

Figure 6: Bursa of Fabricius in broilers fed diets containing inulin at 21 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

Conclusions:

Dietary supplementation by inulin from JA has a significant effect on increasing the villus length and diameter of follicles of Fabricius which are responsible on immunity of broiler chicks.

Acknowledgment:

Special thanks to my supervisor Dr Jane Beal

Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government.

References

1- Alloui M. N., Sellaouil S. and Djaaba S. (2005). 'Morphometrical and Anatomopathological survey of the Bursa of Fabricius in Broiler Chickens'. ISAH.; 2b:52-55.
2- Hajati, H. and Rezaei M. (2010) 'The Application of Prebiotics in Poultry Production', *International Journal of Poultry Science*, 9 (3): 298-304.
3- Park, SO. and Park BS. (2012) 'Effect of feeding inulin oligosaccharides on cecum bacteria, egg quality and egg production in laying hens'. *African Journal of Biotechnology*, 11(39): 9516-9521.
4- Zsolt Z, Jaroslav H, Marianna TM, Eva H, Kateřina S, Ferenc H, Milada P, Jana C, Anna H (2011). Sensorically and antimicrobially active metabolite production of *Lactobacillus* strains on Jerusalem artichoke juice. *Journal of Science and Food Agriculture*, 91: 672–679.

Effect of inulin on the growth of Lactic acid bacteria as prebiotic and screening of LAB toward their selection for chicken probiotic

Rebin Akoy, Simon Davies, Daniel Merrifield and Jane Beal
School of Biomedical and Biological Science, Plymouth University, PL4 8AA, UK
rebin.akoy@plymouth.ac.uk

RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

The development and use of probiotics for poultry is based on the knowledge that the microflora in the gut is participates in the resistance to enteric infections and is suppresses the growth of pathogenic bacteria. The gut microflora protects against a variety of pathogen bacteria including *Escherichia coli, Salmonella, Campylobacter, Clostridium* (Jin *et al*., 1997; Kalavathy *et al*., 2005, 2009; Murry *et al*., 2006). A combination of probiotic and prebiotic (synbiotic) could have a synergistic impact in promoting growth of existing strains of beneficial bacteria in the lower part of gastrointestinal tract as well as improving the survival and growth of newly added probiotic strains (Schrezenmeir and Vrese, 2001).

Objectives

The aim of this study was to investigate the influence of inulin as an effective prebiotic on lactic acid bacteria (LAB) strains, and to screen LAB for selection as a source of chicken probiotic. A synbiotic was produced from the best strain of LAB and inulin for use in poultry diets.

Methods:

The Eleven strains of lactic acid bacteria were screened for potential probiotic properties for growth in inulin from Jerusalem artichoke and commercial inulin (Frutafit® HD, Netherlands). Eight strains of LAB were isolated from chicken caeca and three strains were obtained from the Plymouth University culture collection, (*Pediococcus acidolactici, Lactobacillus plantarum* and *Pediococcus pentosaceus*).

The steps used in the bacterial identification and chosen as a probiotic that were followed are presented schematically in the following tests:

Figure 1: Layout of the screening and selection process.



Results:

The results showed that seven strains of LAB promoted growth on both types of inulin. Inulin supported good growth for C4 strain compared to other strains of LAB.

Table 1: Cells density (OD_{620} ± SD) of LAB strains on commercial inulin and JA inulin after 24h of incubation at 37°C.

Strains	Cell densities [1]		
	Glucose	Frutafit inulin	JA extract inulin
L. plantarum	0.81±0.05	0.85±0.02	0.34±0.05
P. acidolactici	0.72±0.04	0.69± 0.03	0.57±0.04
P. pentosaceus	0.69±0.09	0.58±0.07	0.49±0.01
C2	1.20±0.19	0.98±0.06	0.52±0.06
C4	1.53±0.03	1.43±0.01	0.92±0.03
C6	1.43±0.12	0.78±0.01	0.56±0.03
C7	1.12±0.01	0.84±0.04	0.71±0.04

[1] Results are mean values from three replications and standard deviations.

Table 2: Different test activities of some selected bacterial strains for potential probiotic properties.

Strains	C2	C4	C6	C7	*P. acidolactici*	*L. plantarum*
Co-aggregation activity[1]	++	+++	+	+	++	+
Antagonistic activity[2]	1.62^{ab}	1.92^{a}	1.21^{abc}	0.99^{bc}	1.16^{abc}	0.91^{c}
Survival in acidic conditions[3]	7.317±0.03^{a}	7.337±0.02^{a}	6.565±0.01^{b}	----	6.711±0.09^{b}	6.645±0.02^{b}
Survival in bile salt conditions 0.3%	7.340±0.01^{a}	7.339±0.03^{a}	7.307±0.01^{ab}	----	7.265±0.03^{ab}	7.204±0.03^{b}
Mucous binding assay	0.55±0.09^{b}	0.56±0.03^{b}	0.56±0.03^{b}	----	0.57±0.11^{b}	0.49±0.04^{b}
Adhesion chicken intestinal epithelial cells	Very good	Very good	Good	----	Good	Good
Cell surface hydrophobicity	92.29±0.99^{a}	91.23±0.49^{a}	89.52±1.51^{a}	----	88.32±2.62^{a}	----

[1] The average of co-aggregation properties with three different indicators strains (*Salmonella Enteritidis, Escherichia coli* and *Clostridium perfringens*).
+ = < 45 min, ++ = 45 min and +++ = >120 min.
[2] The average of antagonistic activity with three different indicators strains (*Salmonella Enteritidis, Escherichia coli* and *Clostridium perfringens*).
[3] pH 2 was used to determine survival under acidic condition for 90 min.

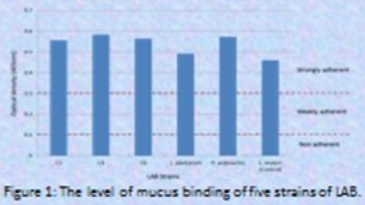

Figure 1: The level of mucus binding of five strains of LAB.

Figure 4: Adhesion of five strains of LAB to the mucus epithelial cells of chicken GI tract observed using light microscopy after Gram-staining. A) C2, B) C4, C) C6, D) *Pediococcus acidolactici* and E) *Lactobacillus plantarum*.

The results showed that C4 was *Lactobacillus animalis* KCTC 3501 strain, identified by PCR technique, had potential probiotic properties. This strain showed the highest auto-aggregation and co-aggregation ability, resistance to acidity and bile salts, strong suppression of pathogens and ability to adhere epithelial cells to the other isolated strains. Also, the results from this study showed that both types of inulin supported good growth of this strain of LAB and could be a suitable candidate for synbiotic production in broiler diet.

Conclusions:

One strain of LAB was isolated from chicken caecum exhibited strong potential as probiotic properties. Also, the results indicated that inulin from Jerusalem artichoke supports growth of this strain as prebiotic source.

Future work:

Preparation of synbiotic from inulin extraction from Jerusalem artichoke and *Lactobacillus animalis* KCTC 3501 strain and then use in broiler diets.

Acknowledgment

Special thanks to my supervisors Dr Jane Beal, Prof Simon Davies and Dr Daniel Merrifield

Special thanks to my sponsor Ministry of Higher Education, Kurdistan Regional Government.

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Schrezenmeir J, de Vrese M (2001). 'Probiotics, prebiotics, and synbiotics—approaching a definition. Am. J. Clin. Nutr, 73:361-364.

The influence of feeding inulin supplementation on intestinal histology and immune functions of broiler chickens

Rebin Akoy, Simon Davies, Daniel Merrifield and Jane Beal
School of Biomedical and Biological Science, Plymouth University, PL4 8AA, UK
rebin.akoy@plymouth.ac.uk

RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

This study dealt with the effects of inulin from commercial and Jerusalem artichoke tubers (*Helianthus tuberosus*) supplementation on the jejunum histology and immune organ of the broiler chicks. The use of prebiotics as alternatives to of antibiotics have become popular in birds to improve the population of beneficial microbes and morphological changes in the GI tract (Zsolt *et al*., 2011; Park and Park, 2012). The bursa of Fabricius is an immunological organ that plays an essential role in the poultry immunity (Alloui *et al*., 2005).

Objectives

The aim of this study was to investigate the influence of inulin from Jerusalem artichoke as prebiotic source on broiler chickens.

Methods:

Experimental Design:

A total of twenty seven newly hatched chicks were randomly divided into three treatments, nine chicks per treatment with three replications.

Treatments:

T1= control group feed standard broiler diets.
T2= standard boiler diets + 1% commercial inulin.
T3= standard boiler diets + 1% Jerusalem artichoke inulin.

Characteristics studied:

- Live body weight (LBW) at the end of the experiment.
- Histology of Jejunum (Villus length and Crypt depth).
- Histology of Bursa of Fabricius (BF).

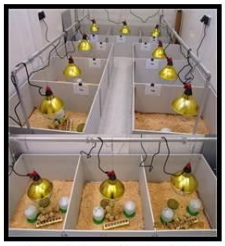

Figure 1: Chick housing facility.

Figure 2: The bursa of Fabricius in chickens.

Results Discussion

1- Both types of inulin significantly (P<0.000) increased villus length compared to the control treatment. The body weight was not significantly (P>0.05) affected by adding inulin in the diets. Morphological changes in the gastrointestinal tract by increasing villi length may have a positive effect on digestibility of nutrients (Hajati and Rezei, 2010).

Table: Effects of dietary inulin supplementation on LBW (gm), villus height (µm) and crypt depth (µm) in the Jejunum of SPF broiler chicks at 14 and 21 d of age.

Treatments	Parameters				LBW at d 21 (gm)
	14 d		21 d		
	Villus height	Crypt depth	Villus height	Crypt depth	
T1	471.22±31.91^{b}	71.91±11.38^{a}	533.20±20.84^{b}	67.42±17.41^{b}	472.00±27.30^{a}
T2	597.93±66.92^{a}	74.75±11.64^{a}	690.09±70.62^{a}	74.61±8.53^{ab}	504.70±20.02^{a}
T3	615.11±89.24^{a}	78.63±14.71^{a}	696.13±96.66^{a}	84.61±13.47^{a}	511.96±17.34^{a}
P. values	0.000	0.35	0.000	0.005	0.13

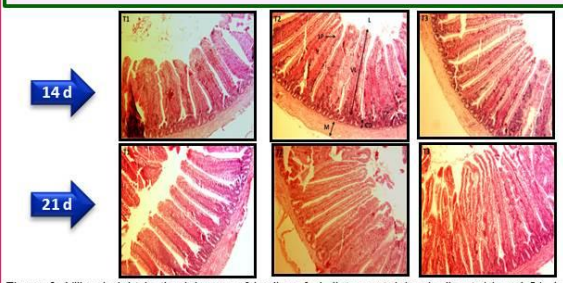

Figure 3: Villus height in the jejunum of broilers fed diets containing inulin at 14 and 21 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

2- The diameters of follicles of Fabricius were increased significantly (P<0.05) in dietary inulin supplementation (10 g of inulin/kg) compared to the control treatment at 14 and 21 days of age. The results showed no significant difference between both types of inulin treatment for growth of follicles of Fabricius at different days of age. The Bursa of Fabricius is an organ responsible for immunity and its evolution in the chicken body is responsible for maturation of lymphocytes B (Alloui *et al*., 2005). The size and weight of the bursa is an indication of the immune response to live body weight. Therefore, the body weight was improved by adding inulin in the diet.

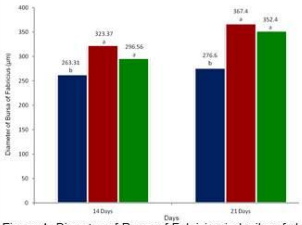

Figure 4: Diameter of Bursa of Fabricius in broilers fed diets containing inulin at d 14 and 21 of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA.

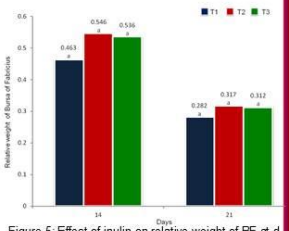

Figure 5: Effect of inulin on relative weight of BF at d 14 and 21 of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA.

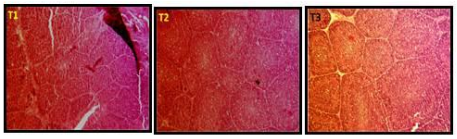

Figure 5: Bursa of Fabricius in broilers fed diets containing inulin at 21 days of age. T1: Control; T2: Commercial inulin and T3: Inulin from JA, (10X Magnification).

Conclusions:

Dietary supplementation by inulin from JA has a significant effect on increasing the villus length and diameter of follicles of Fabricius which are responsible on immunity of broiler chicks.

Acknowledgment:

Special thanks to my supervisors Dr Jane Beal, Simon Davies, Daniel Merrifield

Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government.

References

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The effects of probiotics, prebiotics and synbiotics on the performance, intestinal microflora and histology of broiler chickens

Rebin Aloy, Simon Davies, Daniel Merrifield and Jane Beal
School of Biological Science, Plymouth University, PL4 8AA, UK
rebin.aloy@plymouth.ac.uk

RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

The development and use of probiotic and prebiotic for poultry is based on the knowledge that the microflora in the gut is participates in the resistance to enteric infections and is suppresses the growth of pathogenic bacteria. The gut microflora protects against a variety of pathogen bacteria including *Escherichia coli*, *Salmonella*, *Campylobacter*, *Clostridium* (Jin *et al.*, 1997; Kalavathy *et al.*, 2005, 2009; Murry *et al.*, 2006). The combination of probiotic and prebiotic is called synbiotics. Synbiotic include both beneficial microorganisms and substrates, which may have synergetic effects on the intestinal tract of animals. Synbiotic beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract (Schrezenmeir and Vrese, 2001).

Objectives:

The aim of this study was to investigate the influence of dietary supplementation of a probiotic (*Lactobacillus animalis*), a prebiotic Jerusalem artichoke tuber (*Helianthus tuberosus*) and a combination of both (Synbiotic) on the performance, intestinal microflora and jejunum histology of Hubbard broiler chickens.

Methods:

Seventy two 1-d-old male broiler chicks were randomly distributed into four dietary treatments for 35 d. The design of the field experiment were:

72 one-day old male broiler chicks
CONTROL – CON: Standard broiler diet
PROBIOTIC – PRO
PREBIOTIC – PRE
SYNBIOTIC – SYN
Characteristics studied
Production performance: - Live body weight - Weight gain - Feed intake - Feed conversion ratio - EPEF
Study of intestinal microflora: - Ileum - Caecum
Histological: - Villi length - Crypt depth

Figure 1: layout of the field experiment.

Figure 2: Chick housing facility.

Figure 3: Major stage of the tissues sectioning for histological studies using different instruments.

Results:

The results showed that the average weight gain was significantly (P<0.01) increased in synbiotic and probiotic compared with prebiotic and control. Feed conversion ratio and European production efficiency factor were significantly (P<0.01) improved by the dietary supplementation of the synbiotic, prebiotic and probiotic compared with the control.

Table 1: The growth performance of chicken after five weeks feeding on experimental diets (mean±SD).

Growth performance	Treatment				P. value
	CON	PRO	PRE	SYN	
Initial weight (g)	40.3±2.60 a	41.2±2.81 a	40.3±2.80 a	40.1±2.51 a	0.646
Final weight (g)	1686.2±52.0 b	1765.2±66.8 a	1724.4±44.3 ab	1779.9±50.9 a	0.001
Weight gain (g/bird)	1645.6±16.7 a	1724.0±54.8 ab	1684.4±5.7 ba	1739.8±13.6 a	0.002
Feed intake (g/bird)	3012.7±55.1 a	2995.3±27.7 a	2765.7±50.5 b	2740.4±24.0 b	0.000
Feed convention ratio	1.83±0.02 a	1.73±0.05 b	1.63±0.02 a	1.57±0.02 a	0.000
(EPEF) [1]	262.35±5.94 a	290.82±11.80 b	300.90±5.86 b	322.11±7.09 a	0.000

[abc] Data in the same row with different superscript are significantly different (P<0.05).
[1] EPEF = liveability (%) × live weight (kg) × 100/ age (d) × FCR.

Figures 4, showed that synbiotic and prebiotic supplementation significantly (P<0.01) increased the number of *Bifidobacteria* compared to control group in the ileum. While, in the caecal digesta, the numbers of total anaerobic and *Lactobacillus spp.* were significantly (P<0.01) increased in probiotic and synbiotic compared to the control group at 35 days of age.

Total anaerobic, Total aerobic, Lactobacilli, Bifidobacteria, Total Coliform — Bacteria

Figure 3. Bacteria counts (Log10 CFU mL-1) at 35 days of age in ileum (A) and caecal (B) digesta of broiler chicks.

All the additives supplementation increased villus length and crypt depth compared to the control treatment at different days of age.

Table 2. Effect of probiotic, prebiotic and synbiotic on the Jejunum Histomorphology of broiler chickens (Mean ± standard division).

Parameters	day	Treatment				P. values
		CON	PRO	PRE	SYN	
Villus height	17	650.81±51.36 b	750.85±53.79 ab	767.35±57.51 a	794.17±56.24 a	0.000
	35	681.67±56.35 b	755.92±55.17 a	825.2±45.31 b	966.95±44.06 a	0.000
Crypt depth	17	116.17±16.44 b	127.62±16.58 ab	137.62±11.35 a	141.58±19.14 a	0.000
	35	97.97±6.05 b	121.11±17.5 b	138.45±17.75 a	145.08±8.98 a	0.000

17 days
35 days
CON PRO PRE SYN

Figure 4: Villus height in the jejunum of broilers fed diets containing probiotic, prebiotic and synbiotic at 17 and 35 days of age.

Discussion:

The higher performance production observed in broilers fed probiotic, prebiotic and synbiotic may be due to the fact that additives suppress pathogenic bacteria which leads to improved health status and ultimately improved growth and overall performance. This investigation found that the synbiotics which was a combination of *Lactobacillus animalis* and inulin extracted from Jerusalem artichoke had a greater effect on broiler performance compared with control group. Although differences between the synbiotic, probiotic and prebiotic did not reach statistical significance. The broilers performance was better with the synbiotic.

Conclusions:

The results show that additive supplementation in broiler feed not only caused improvement in performance, but also increased the beneficial bacteria and villi length in the intestinal tract.

Acknowledgment:
Special thanks to my supervisors Dr Jane Beal, Simon Davies, Daniel Merrifield
Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government.

References:

Effects of dietary inulin supplementation on caecal microflora and haematology of broiler chickens

RESEARCH WITH PLYMOUTH UNIVERSITY

Rebin Akoy, Simon Davies, Daniel Merrifield and Jane Beal
School of Biological Science, Plymouth University, PL4 8AA, UK
rebin.akoy@plymouth.ac.uk

Introduction:

This study dealt with the effects of inulin from commercial and Jerusalem artichoke tubers (*Helianthus tuberosus*) supplementation on the caecal microflora and immune function of the broiler chickens. Inulin can stimulate the growth of the intestinal bacteria as well as affect the immune system. The use of prebiotics instead of antibiotics is going to be popular in birds in order to improve the useful microbial population and morphological changes in the GI tract (Zsolt *et al.*, 2011; Park and Park, 2012). The reliability of H/L ratio as a biological index of stress in avian species has been comprehensively reviewed (Maxwell, 1993).

Objectives

The purpose of the present study was to determine the effect of inulin from Jerusalem artichoke as prebiotic source on the caecal microflora and heterophil to lymphocyte ratio of broiler chickens.

Methods:

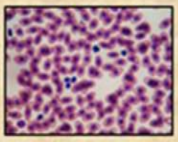
Experimental Design:
A total of twenty seven newly hatched chicks were randomly divided into three treatments, nine chicks per treatment with three replications.

Treatments:
T1= control group feed standard broiler diets.
T2= standard boiler diets + 1% commercial inulin (Frutafit® HD)
T3= standard boiler diets + 1% Jerusalem artichoke inulin.

Characteristics studied:
- Caecal removed and examined by conventional culture-based techniques.
- Short chain fatty acid and lactic acid concentration in caecum contents.
- Haematology

Chick House facility.

Jerusalem artichoke tuber



Blood smear

Results

The results showed that there were no statistically significant differences among all treatments for total aerobics, *Lactobacillus sp.*, *Bifidobacteria sp.*, total coliform. While, the numbers of total anaerobic bacteria, *Lactobacilli* and *Bifidobacteria* were significantly ($P<0.05$) increased in the caecal digesta of commercial and JA inulin at 21 days of age compared to control group.

Table 1: Bacteria counts of microflora at 14, 18 and 21 days of age in caecum digesta of broiler chicks (Log_{10} CFU mL^{-1}).

Microbes	Treatments [1]					
	14 days			21 days		
	T1	T2	T3	T1	T2	T3
Total anaerobic	8.22±0.07	9.29±0.07	9.36±0.06	8.90±0.05	9.30±0.09	9.26±0.08
Total aerobic	8.14±0.05	7.93±0.24	8.02±0.04	7.94±0.05	7.74±0.11	7.81±0.19
Lactobacilli	8.15±0.12	8.64±0.42	8.78±0.37	8.09±0.08	9.29±0.10	9.22±0.13
Bifidobacteria	8.37±0.05	8.43±0.02	8.44±0.01	8.72±0.08	9.34±0.09	9.36±0.09
Total Coliform	7.75±0.18	7.62±0.08	7.69±0.16	7.72±0.17	7.67±0.17	7.64±0.15
Salmonella	ND	ND	ND	ND	ND	ND

[1] T1: control, T2: commercial inulin, T3: inulin from JA [illegible]

The concentrations of short-chain fatty acids and lactate, at the end of experiments are presented in Tables (3). The concentration of lactic acid and butyrate significantly ($P<0.05$) increased in inulin from Jerusalem artichoke compared with control group. While, there is no significant differences observed among all treatments about acetic acid and propionic acid.

Table 3: Concentration of short-chain fatty acids and Lactic acid at the end of the experiment[1] (d 21).

Groups	Treatment		
	T1	T2	T3
Lactic acid	0.204±0.11[b]	0.109±0.028[b]	0.463±0.01[a]
Acetic acid	9.126±2.715[a]	10.268±2.38[a]	13.105±3.68[a]
Propionic acid	0.984±0.69[a]	1.773±1.46[a]	2.276±1.44[a]
Butyric acid	2.248±0.67[b]	10.289±2.54[a]	14.413±3.74[a]
pH	6.21±0.10[a]	5.77±0.13[b]	5.89±0.06[b]

a,b Means within same row with different superscripts are significantly different ($P<0.05$).
[1] Values are (mmol/L) means ± SD of triplicate determination.
[2] T1: control, T2: commercial inulin (1%), T3 inulin from JA (1%).

The treatments had highly significant effects on Lymphocyte count and H/L ratio at 21 day of age. The lower H/L ratio in the experimental treatments may be due to that the inulin addition to the diet could inhibit the nutritional stress or any stress which causes an increase in H/L ratio (Ghareeb *et al.*, 2008). Thus, H/L ratio could be used as an indicator for the health of animals and any increase of H/L ratio refers to an increase in stress case (McFarland and Curtis, 1989).

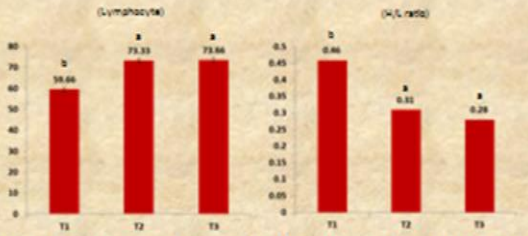


Figure 2: Results of lymphocyte counts and heterophil/lymphocyte ratio in all treatment groups on day 21 of trial.

Conclusions:

Dietary supplementation by inulin from JA has a high effect on the caecal microflora, increase SCFA, decreasing the heterophil/Lymphocyte ratio which are used as an indicator for the health of broiler chicks.

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References
1- Ghareeb, K. [illegible]
[illegible]