The Effects of Lactobacillus plantarum NCIMB 41607 on Salmonella infection and the microbial population in the gastrointestinal tract of the chicken

by

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Abstract

Salmonellosis is a foodborne disease, poultry and poultry products are the main source of the disease. Many countries including Iraq still use antibiotics to control *Salmonella*. The increase in antibiotic resistant bacteria putatively caused by the overuse of antibiotics in agriculture has provided the incentive to look for alternatives to antibiotic to control diseases in livestock. Lactic acid bacteria with probiotic characteristics have the potential to beneficially affect the gut microflora of chickens and help reduce the incidence of *Salmonella* infection.

In this study, *Lactobacillus plantarum* NCIMB 41607 was examined *in vitro* for inhibitory effects against *Salmonella* and *in vivo* for its effects on the gut microflora and on the incidence of *Salmonella* infection. In separate studies *Lb. plantarum* was administered to chicks as fermented moist feed (containing 175 mmol/L lactic acid and $1x10^9$ CFU/g of *Lb. plantarum*), in drinking water or applied to dry feed. Rifampicin resistance was used as a biomarker for the *Lb. plantarum* NCIMB 41607. Traditional and molecular microbiology including DGGE, RISA, and FISH were used with electron microscopy to assess the effect of *Lb. plantarum* on bacterial population in the digestive system of chicks.

Lactobacillus plantarum (NCIMB 41607) was found to reduce the growth of *Salmonella* Typhimurium and *Salmonella* Enteritidis by 4 Log CFU/g in a chicken simulated digestive system *in vitro*.

The presence of rifampicin resistant *Lactobacillus plantarum* in the gut was confirmed by replica plating and fluorescent *in situ* hybridisation with a strain specific probe. In general, the bacterial population in the guts of chicks fed *Lactobacillus plantarum* was more diverse in the guts of chicks fed control feed. There was no significant effect on Salmonella infection in Ross chicks compared with control. However, with specific pathogen free chicks the *Salmonella* infection was reduced. The intestinal villi lengths in FMF group were increased. These studies demonstrate that probiotics can affect the microflora in the chicken gut.

Lactobacillus plantarum NCIMB 41607 may have the potential to control of *Salmonella* Typhimurium infection in chicken and continued research is advised.

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List of abbreviations

µg	Microgram
µl	Microliter
AMF	Acid moist feed
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
CFU	Colony forming unit
CON	Control group
DAPI	4, 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DW	Drinking water
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FCR	Feed conversion rate
FMF	Fermented moist feed
FISH	Fluorescent in situ hybridisation
HPLC	High Performance Liquid Chromatography
Lb. plantarum	*Lactobacillus plantarum*
LAB	Lactic acid bacteria
Mm	Millimetre
mM	Milimolar
MRS	Man, Rogosa and Sharp
ng	Nanogram
NCBI	National Centre for Biotechnology Information
NCIMB	The National Collection of Industrial, Marine and food Bacteria
OD	Optical density
OTU	Operative taxonomy unit

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RISA	Ribosomal Intergenic space area
rRNA	Ribosomal Ribonucleic Acid
S. Typhimurium	*Salmonella* Typhimurium *Sal 1344 nal^r*
S. Enteritidis	*Salmonella* Enteritidis
SCFA	Short chain fatty acid
SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscope
TAE	Tris-acetete-EDTA
TE	Tris-EDTA
WAT	Water group

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Dedication

This thesis is dedicated in memory of my parents and my brave brothers the martyrs of the right way Sameer, Muneer and Ameer.

AUTHOR'S DECLARATION

At no time during the registration for the degree of Master of Philosophy the author has been registered for any other University award without prior agreement of the Graduate Committee. All the investigation reported in this thesis conform to the UK 1986 Animal Scientific Procedures Act under the specific project license # PPL 30/2640 and personal license # PIL 30/9065. This study was financed by The Ministry of Higher Education and Scientific Research (MoHESR), Iraq.

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Signed … Nabil Ali Wali ……..

Date ……

CHAPTER 1

Literature review

Chapter One: Literature review

1.1 Introduction

According to a United Nation report (WHO, 2007) every year worldwide, thousands of people die of septicaemia or dehydration associated with severe forms of Salmonellosis. Consuming poultry or poultry products contaminated with *Salmonella* Enterica subsp enterica serotype Typhimurium (*Salmonella* Typhimurium*)* or *Salmonella* Enteritidis is a major cause of food poisoning which is characterized by diarrhoea, abdominal cramps and fever. However, *Salmonella* infections can be fatal in young, elderly or immunocompromised persons. Both *Salmonella* serotypes are able to cause severe infection in chicks causing high mortality rates, while in older birds they lead to a persistent colonization of the gastrointestinal tract with very low morbidity (Derache *et al*., 2009). Therefore, the persistence of contaminated birds in chicken flocks represents a main source of contamination in the human food chain and subsequent food-borne disease. Reducing the incidence of Salmonellosis in chickens may reduce the disease in humans. There are several ways available to reduce salmonellosis in chickens such as antibiotics, vaccination, feed treatments such as organic acids and probiotics.

One way to reduce *Salmonella* in Chickens is to make the chicken less susceptible to colonization (Heres *et al*., 2004). Probiotics may be one of the solutions. Probiotics can be administered by several methods for example spraying, in drinking water, incorporated into dry feed or as fermented moist food (FMF). FMF has been demonstrated by several researchers as an active way to reduce *Salmonella* in chickens (Heres *et al*., 2003a; Niba, 2008; Savvidou, 2009). However, complete eradication of *Salmonella* is unrealistic (Immerseel *et al*., 2009). Therefore, the aim

of feed interventions is to decrease the incidence of *Salmonella* in chicken and hence reduce the percentage of food-borne diseases.

1.2 Salmonella

Salmonella is a bacterium that can spread to humans through contaminated foods causing an illness called salmonellosis. Poultry and poultry products (meat and eggs) are the main source of the disease (REF). The usual symptoms of human salmonellosis are fever, diarrhoea and abdominal cramps. *Salmonella* is commonly found in the intestines of healthy birds and mammals (EFSA, 2012). Good kitchen hygiene can prevent or reduce the risk posed by contaminated food.

About 100,000 human cases are reported each year in the European Union (99020 reported cases in 2010) and economically the cost could be as high as EUR 3 billion a year (EFSA, 2012). In 2010 in England and Wales 2444 human cases of *Salmonella* Enteritidis were reported compared to 8616 cases in 2000. For *Salmonella* Typhimurium, 1959 cases of human isolates were reported in 2010 and 2688 cases in 2000 (HPA, 2011). In the EU the disease has been reduced by 50% in 5 years (2004-2009), mainly because of the successful implementation of a *Salmonella* control program in poultry (laying hens, breeding hens and broilers) (EFSA, 2012).

Salmonella is a Gram-negative rod bacterium, and is a member of the Enterobacteriaceae family. There are two species of *Salmonella*, namely *S. bongori* and *S. enterica. Salmonella enterica* is subdivided into six subspecies including Enterica, Salem, Arizona, Diarizonae, Houtenae, and Indica (Kauffmann, 1966).

The classification of *Salmonella* is based on both serotype and subspecies names. For example, *Salmonella enterica* subspecies *enterica* serotype Typhimurium is

abbreviated to *Salmonella* Typhimurium *(*Dunkley *et al*., 2009*)*. *Salmonella* can be also subdivided onto biotype, page type and antigen type including somatic O, flagella H, and capsular VI antigens. Antigens have been used to separate and identify more than 2500 serotypes of *Salmonella* (Dunkley *et al.*, 2009). *Salmonella* is oxidase negative, catalase positive, Indole negative, methyl red positive, H_2S producing and urea negative. These characteristics are used for biochemical confirmation of *Salmonella* (FAO & WHO, 2001).

Salmonella causes non-symptomatic intestinal infections in chicken but acute outbreaks showing clinical disease along with high levels of mortality occur in chicks younger than 2 weeks old. *Salmonella* can migrate from the intestine to the liver, spleen and ovaries. There are many reasons to study *Salmonella* instead of other pathogens such as; it causes significant human morbidity worldwide particularly in poor countries, pregnant women, children, old people and people with low immunity. *Salmonella* has ability to establish persistent colonisation in many species that serve as reservoirs for transmission and shedding. It has shown resistance to many antibiotics and causes a continuing threat to consumer health.

European Food Safety Authority (EFSA) reported in 2007 that *Salmonella* was, as in previous years, the most commonly reported cause of food-borne disease outbreaks in the Europe (EU) and that poultry and eggs were the most frequently implicated foodstuffs in these outbreaks. In 2012 EFSA reported that broilers and eggs contributed to 27% of human salmonellosis cases. *Salmonella* was reported to be present in about 1 in 4 chicken flocks (23.7%). *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most commonly reported *Salmonella* types in human cases in the EU (EFSA, 2007).

1.2.1 Pathogenesis of Salmonellosis in chicken

There are two types of *Salmonella* infection dependant on host species:

- Host restricted

Salmonella serotypes that colonize a particular host species, such as *Salmonella* Pullorum*, Salmonella* Gallinarum*, Salmonella* Hadar*, Salmonella* Virchow and *Salmonella* Heidelberg which are restricted to the chicken and not linked to human infection.

- Unrestricted host

Some *Salmonella* serotypes are associated with a broad range of unrelated host species and can induce systemic disease in them such as *Salmonella* Enteritidis and *Salmonella* Typhimurium.

Salmonella is a facultative, intracellular pathogen capable of infecting a variety of hosts, resulting in several manifestations of disease, including enteric fever, bacteraemia, and gastroenteritis (Henderson, Bounous & Lee, 1999). Following oral ingestion of *Salmonella* Typhimurium, these bacteria adhere to and invade the intestinal epithelial cells and cells of intestinal lymphoid follicles or Peyer's patches. *Salmonella* invasion is very rapid, and bacteria appear within membrane-bound vacuoles in a few minutes of starting host cell contact. Cell surface returns to normal after the bacteria are internalized (Finlay & Falkow, 1997).

The interaction of *Salmonella* to intestinal epithelium induces the recruitment of heterophils and macrophages to the site of infection in the intestine (Henderson, Bounous & Lee., 1999)

In chicks over a few days of age, *Salmonella* Typhimurium colonise the gastrointestinal tract but does not cause clinical disease (Sivula, Bogomolnaya & Andrews-Polymenis, 2008). *Salmonella* Typhimurium invasion produces a strong inflamma-

tory response, that may limit the spread of *Salmonella* largely to the gut, whilst *Salmonella* Gallinarum does not induce an inflammatory response and may not be limited by the immune system, leading to the severe systemic disease fowl typhoid (Sivula, Bogomolnaya & Andrews-Polymenis, 2008).

1.2.2 *Salmonella* diagnosis

Salmonella can be isolated from faecal samples of infected chickens. Salmonellae may be isolated using a variety of techniques that may include the standard method of pre-enrichment, enrichment media and plating onto selective agars to differentiate salmonellae from other Enterobacteriacae. Serological and molecular tests can be applied to the pure culture of *Salmonella* to provide definitive confirmation of an isolated strain or serovar. *Salmonella* species are identified with biochemical tests, and the serovar can be identified by serological tests for the somatic (O), flagella (H) and capsular (Vi) antigens.

Faecal samples would be plated onto xylose lysine deoxycholate (XLD) agar and brilliant green (BGA) agar, which are used as selective media. Any putative *Salmonella* are plated onto nutrient agar for biochemical tests and serology. The biochemical tests for *Salmonella* include oxidase (negative), catalase (positive), Indole (negative), methyl red reduction (positive), H_2S production and urea (negative). These characteristics are used for biochemical confirmation of *Salmonella* (FAO & WHO, 2001). The serological tests including enzyme-linked immunosorbent assays (ELISA) and agglutination tests, the latter is the method of choice for servoar diagnosis of *Salmonella.*

The isolation of *Salmonella* can be difficult particularly if the numbers are low in the faecal samples. So, the standard method is pre-enrichment in buffered peptone water (BPW) for 24 hours at 37 ºC. About 0.1ml of the incubated pre-enrichment can be transferred to 20 ml of *Salmonella* enrichment media of modified semi-solid Rappaport-Vassilliadis (MSRV) medium and incubated for another 48 hours at 42 ºC. A loopful of incubated MSRV media is then streaked onto XLD and/or BGA agar and incubated at 37 ºC for 24 hr. The *Salmonella* colonies on XLD agar are round black or black centred colonies due to the production of hydrogen sulphide (H_2S) and pink colour colonies on BGA with reddening of the media.

1.2.3 *Salmonella* control

The numbers of *Salmonella* contaminated broiler flocks will generally decrease in the future due to the legislation and established action plans as mentioned below, but complete eradication of *Salmonella* is unrealistic (Immerseel *et al.*, 2009). The central issue is to reduce the numbers of bacteria in infected animals to such a low level, that contamination of meat is decreased, and transmission to humans becomes a highly unlikely event. This study will concentrate on using probiotic lactic acid bacteria to reduce *Salmonella* and to investigate its effect on the microbial population in the chicks gut.

Many ways of *Salmonella* control are available:

- Strict hygiene of poultry houses by using automatic modern technology and detergents with a disinfectant like Iodine or formalin.

- Decontaminated feed by ensuring adequate pelleting temperatures and monitoring salmonella in chicken feed by microbiology screening.
- Logistic slaughter particularly in developing countries where there is no regulations governing slaughter of chickens
- Control of rodents and wild birds which may introduce *Salmonella* into chicken houses
- Vaccination, of layer chickens with vaccines for both serotypes of *Salmonella* Enteritidis and *S.* Typhimurium. There are two types of vaccines: live attenuated which is administered via drinking water and inactivated vaccines administered via injection.
- Organic Acid Supplementation (OAS) such as lactic acid and propionic.
- Prebiotics; Non-starch polysaccharides, Oligosaccharides and Fructo-oligosaccharides lead to an increase in short chain fatty acids, lower pH and increasing lactic acid bacteria populations especially Bifidobacterium in the gut. This may decrease colonization of *Salmonella* (Campbell *et al*., 1997).
- Probiotic; Probiotics are live micro-organisms that when administered in adequate amounts confer a benefit to the host (FAO & WHO, 2001).

Many researchers have demonstrated the value of administering normal gastrointestinal flora (aerobic and anaerobic bacteria) from healthy adult chickens to one day old chicks to reduce intestinal colonization of *Salmonella* (Heres, 2002; Niba, 2008; Savvidou, 2009; Vicente et al., 2007a).

1.3 Digestive System of chicken

An understanding of the structure and function of the digestive tract of the bird is important to understand digestion and metabolism of the bird. The digestive system of chickens Figure 1.1 is composed of: Crop, proventiculus, gizzard, small intestine, caeca and rectum. The small intestine is over 130 cm in length and consist of the duodenum, jejunum and ileum. The large intestine is about 10 cm (rectum) and cloaca. The Caeca consist of two pouches that fill and empty from the same direction. Their main function is associated with a breakdown of fibre, storage of undigested waste material and absorption of water.

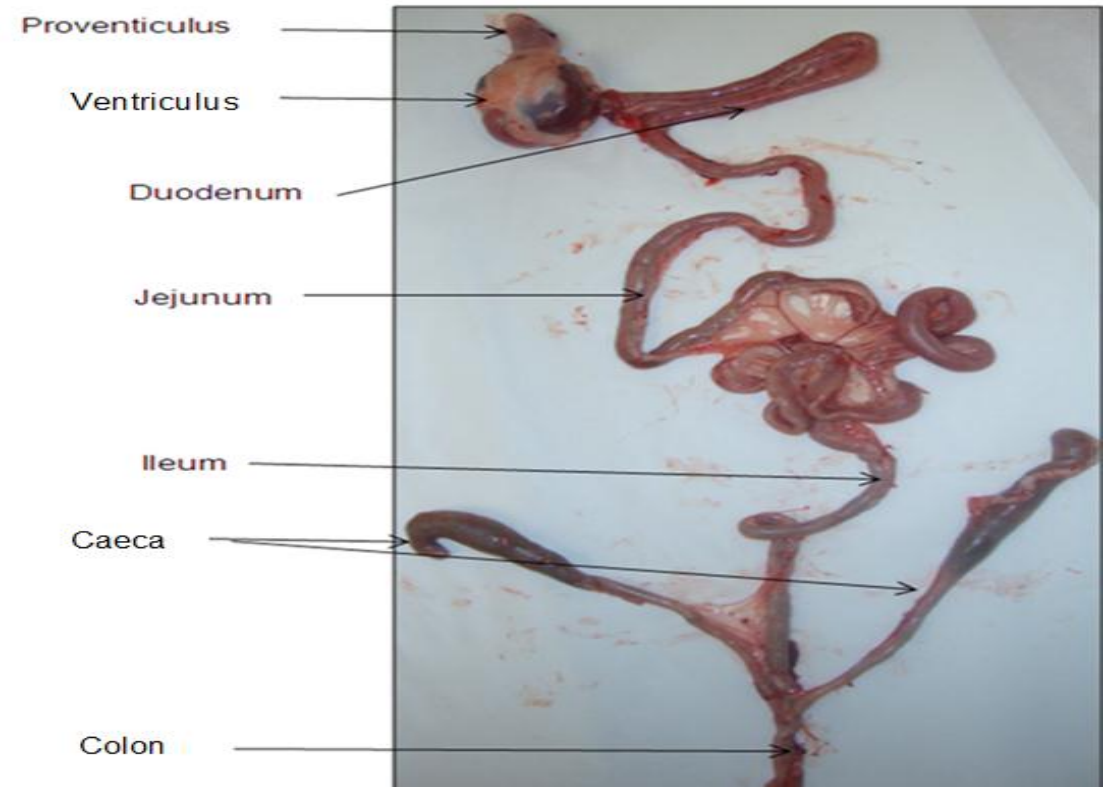

Figure 1. 1 Digestive system of chicken

1.3.1 Histology of avian digestive system

The small intestine is histologically composed of four layers from inside to outside: Mucosa, sub-mucosa, muscularis externa and serosa. The epithelial layer (mucosa) is composed of villi, which are finger-like, have a base (crypt of Lieberkuhn) and apex with epithelial cells called enterocytes (primary absorptive cells of the intestine). The intestinal villi play a vital role in digestion and absorption of nutrients, intestinal villi are immature at hatch, and maximum absorption capacity is achieved by 10 days of age (Uni, Noy & Sklan, 1995) cited In (Tellez *et al*., 2006). The rate of development of the GIT in chicks exceeds the rate of body weight gain physically (relative weight) and morphologically (villus height and perimeter, and villus volume) (ShiHou *et al*., 1998). Increased villus height indicates a greater surface area increasing absorption of available nutrients (Caspary, 1992). The villi are affected by the type of commensal bacteria but the effects of probiotics on intestinal villi are still unclear.

1.3.2 Development and microbial ecology of the chicken

It is generally considered that a balanced intestinal microbial population is characteristic of a healthy and well-functioning gastrointestinal tract. The GIT is more densely populated with microorganisms than any other organ (Tellez *et al.*, 2006). Generally, microflora of the digestive tract can be divided into two groups:

1. Harmful bacteria, which may be involved in the induction of infection, intestinal putrefaction and toxin production.
2. Commensal bacteria, which are involved in vitamin production, stimulation of the immune system and inhibition of harmful bacteria (Koutsos & Arias, 2006)

Chicks establish a protective microflora within the first couple of days after hatching. The digestive flora develop with age (Gabriel *et al*., 2006). In only one day, the previously sterile ileum and caeca contain 10^8 and 10^9 bacteria per gram of content respectively (Apajalahti, Kettunen & Graham, 2004) and 3 days later this increases to 10^9 and 10^{11} per gram of content respectively. Thereafter, the numbers remain relatively stable until 30 days of age (Gabriel *et al.*, 2006). Coliforms and Enterococci dominate the gut of the chicks initially. *Lactobacillus* and lactic acid bacteria generally, colonise much more slowly, but eventually, they become the dominant species in the upper part of the GI tract (Apajalahti, Kettunen & Graham, 2004).

Bacterial activity takes place mainly in the caeca and to a lesser degree in the small intestine. The bacterial population is diverse, especially in the caeca where the slow turnover of the contents (1 to 2 times a day) results in the development of more and different types of bacteria. The ileum contains 10^9 bacteria per gram of contents and this consists mainly of facultative anaerobes, predominantly *Lactobacilli* (Apajalahti, Kettunen & Graham, 2004). Recent molecular studies for the characterization of the composition of the chicken microbial ecology (using 16S ribosomal DNA for phylogenetic analysis) has shown that there are a large variety of uncultured bacteria, therefore, these cannot be identified by traditional culture depended methods. Bacteria are normal residents of the animal's body including the gastrointestinal tract, where more than 50 genera and at least 500 to 1000 different species are distributed in the length of the digestive system (Xu & Gordon, 2003). A recent evaluation of the ecology of the microflora of the chicken intestine using 16s rRNA confirms that *Lactobacilli* are the predominant organism in young

birds, while in older chicken Bifidobacteria are dominant (Amit-Romach, Sklan & Uni, 2004).

The poultry production industry has now been intensified globally and chickens are kept in incubators and hatching rooms which are as sterile as possible. This has a negative effect on the development of the chicken intestinal microflora (Apajalahti, Kettunen & Graham, 2004). Additionally, stressful conditions such as high temperature, high humidity, change of feed and transportation, administration of antibiotics may upset the balance of gut microflora. Moreover, opportunistic bacterial invaders from the environment may cause a continual challenge to the chicken. The protective mechanisms of the host, such as the low gastric pH (2.4 in gizzard for 90 minutes), may result in a 10 to 100 fold reduction of bacterial numbers in ingested food or water (Edens, 2003; Jin *et al*., 1997). The production of volatile fatty acids, that are known to suppress the population of enteropathogens, can partly protect the host. Immune protection of poultry starts with the egg as the yolk, releases the maternal antibodies straight into the gut lumen of the developing embryo and protects against pathogens for the first four days of the hatched chick's life (Mahajan & Sahoo, 1998). Supplementation at an early age with probiotics and competitive exclusion products can be very beneficial (Edens, 2003; Ghadban, 2002; Jin *et al.*, 1997; Mahajan & Sahoo, 1998; Nava *et al*., 2009; Savvidou, 2009). Administration of probiotics as early as possible increases the likelihood of colonization of their gastrointestinal tract with beneficial bacteria and enables the beneficial bacteria to attach to available receptor sites on the gut epithelium and exclude pathogens.

The feed requires about 10 hours to pass through the avian GI tract (Larbier & Leclercq, 1994). The pH of the chicken gizzard could be as acidic as pH 2, which

may be an obstacle to the survival of *Lactobacilli* (Adamberg, Kask & Paalme, 2003). The exposure to the extreme acidic environment of the GI tract maybe reduced, as the food particles and/or carrier substances that are consumed by the chicken act as a buffer and protect the *Lactobacilli*.

Chicken bile contains bile salts and lipids (cholesterol and phospholipids). Bile secreted into the duodenum section of the small intestine has been found to reduce the survival of bacteria. This is believed to be due to the cell membrane of the bacteria consisting of lipids and fatty acids, which are broken down by the bile salts (Jin *et al*., 1998).

According to Jin *et al*. (1997) and Ghadban (2002) the Nurmi and Rantala's technique in 1973 is considered the basis of the competitive exclusion (CE) in poultry. One to two-day old chicks orally administered with the adult gut microflora of a healthy chicken, showed a significant resistance to *Salmonella* Infantis, supplied by feeding one day later. The CE bacteria preferentially establish in the gastrointestinal tract and become antagonistic to opportunistic pathogens. Several commercial CE products, have been developed such as AviFree, Aviguard, Broilact, lypho-Lac, MSC and Preempt, or CF-3 (Doyle & Erickson, 2006; Edens, 2003). The Scientific Committee on Animal Nutrition (European commission, 2003) stated that the microbial products are safe for use as feed additives and growth promoters in poultry production, when used according to manufacturer's instructions.

1.4 Probiotics

Some *Lactobacilli* have probiotic properties. *Lactobacilli* are gram-positive, non-spore forming bacilli. They grow best under anaerobic conditions and their major product of glucose fermentation is lactic acid (Kimura *et al*., 1997). *Lactobacilli* can

be found in the chicken digestive tract.

Fuller at (1971) was the first one approved that *Lactobacilli* adhere to the surface of the crop wall. Jin *et al*., in (1998) mentioned that the ileum contains a larger population of *Lactobacilli* than other parts of the small intestine.

There are two groups of LAB:

Homofermentative LAB, which convert glucose to lactic acid.

Heterofermentative LAB, which produce lactic acid, acetic acid, ethanol, mannitol and CO_2 from hexoses (Müller *et al*., 1996).

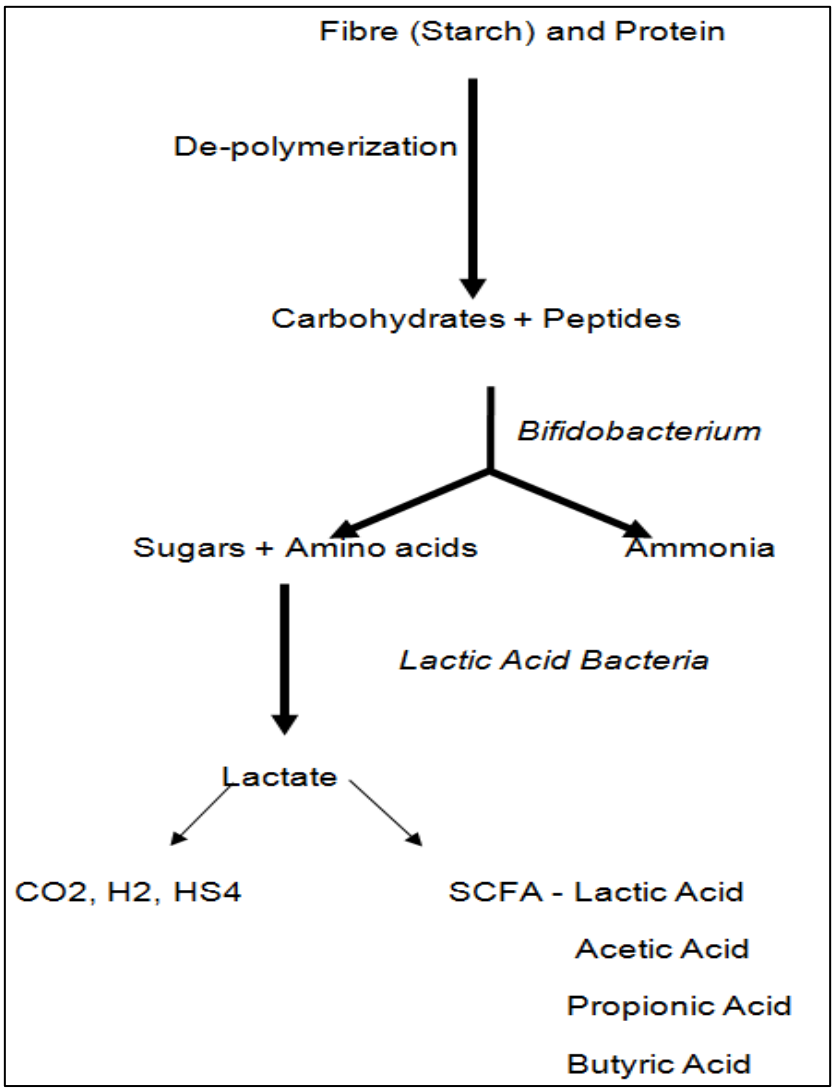

Figure 1. 2 Food fermentation by bacteria of *Bifidobacterium* and LAB and their products

Probiotics are a field of science and business that is growing fast (Olnood, Choct & Iji, 2007). The name is derived from the Greek meaning ‘for life’. This is the opposite of ‘antibiotic’ which means ‘against life’ (Ghadban, 2002). Probiotics along with prebiotics, organic acids and enzymes have been seen as a potential alternative to antibiotics (Choct, 2002).

Eli Metchnikoff was the first scientist to refer to the benefit of some bacteria and he suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907). He suggested using lactic acid bacteria in fermented milk products for longevity and maintenance of youthful energy. That was the spark for more research about LAB. At this time Henry Tissier observed low numbers of *Bifidobacteria* in children with diarrhoea and high numbers in healthy children, he suggested using these bacteria to treat diarrhoea in children (FAO, 2001)

In 1960s the name probiotic was used by Lilly & Stillwell in (1965) to name substances produced by microorganisms which promoted the growth of other microorganisms. Fuller at (1989) redefined the probiotic as a live bacterial feed supplement which beneficial effects on the host by improving its intestinal balance.

Table1. 1. Registered probiotic products for use as feed additives in poultry production

Name	LAB Types	Collection number	Chicken type
Bactocell	*Pedicoccus acidolactici*	CNC MA 18/5M	Broiler
Bioplus	*Bacillus licheniformis* *Bacillus subtilis*	DSM 5749 DSM 5750	Broiler & Turkey
Cylactin LBC	*Enterococcus faecium* *Lactobacillus acidophilus*	NCIMB 10415 CECT 4529	Broiler Laying hens
Microferm	*Enterococcus faecium*	DSM 5464	Broiler
Oralin	*Enterococcus faecium*	NCIMB 10415	Broiler
Probio, Granular	*Enterococcus faecium* *Enterococcus faecium*	DSM 4788/ ATCC 53519	Broiler

There are many criteria for selection of probiotics *in vitro* to be approved before applying *in vivo.*

- They should be generally recognized as safe (GRAS). Non- toxic and non-pathogenic (Lina *et al*., 2007)
- They should be tolerant to acid and bile which ensures their viability and capability of being biological activated within the chicken GI tract (Gibson & Fuller, 2000; Lina *et al.*, 2007)
- They should be able to adhere to the mucus and intestinal epithelium of the hosts (Jin *et al*. 1996) which ensures the bacterial maintenance in the GIT and thereby prevents their rapid removal by contraction of the gut (Jacob-sen *et al*. 1999).
- They are able to demonstrate antagonistic activity against pathogenic bac-teria by itself or via bacterial by-products (Jin *et al.*, 1998).

- They should be able to keep their viability during processing and storage
- Antimicrobial activity against potential pathogens (Tsai *et al*. 2005; Olnood *et al*. 2007; (Jin *et al.*, 1998)
- Auto-aggregation and co-aggregations with the pathogens (Kos *et al*. 2003)
- A normal resident of the host or host adapted (favorable).

The Desirable Probiotic Functions

As described by Eden in (2003):

1. Exclude (prevent colonisation) or kill pathogenic bacteria
2. Stimulate the immune system
3. Reduce inflammatory reactions
4. Enhance animal performance
5. Decrease carcass contamination
6. Increase production of volatile fatty acids
7. Increase vitamin B synthesis
8. Improve nutrient absorption
9. Decrease diarrhoea
10. Creates a restrictive physiological environment
11. Stimulates peristalsis

1.5 Action of Probiotic

1.5.1 Antagonistic activity

Generally, 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). Probiotic bacteria such as LAB are able to produce antibacterial substances, which has been shown to be inhibitory to poultry pathogens both Gram positive or negative (Jin *et al.*, 1998). The mechanisms are:

1. *Lactobacilli* are known to produce bacteriocins (peptide or protein) which have an inhibitory action against Gram positive or/and Gram negative bacteria.
2. Hydrogen peroxide inhibits the growth of pathogens through its strong oxidizing effect on the bacterial cells, or through the destruction of basic molecular structures of nucleic acid and cell proteins (Jin *et al*., 1996).
3. The bactericidal effect of organic acids (lactic, acetic, butyric and propionic) produced by *Lactobacillus*, *Enterococcus* and *Bifidobacterium*, has the ability to reduce intestinal pH (Jin *et al.*, 1997).
4. Production of volatile fatty acids by obligate anaerobes in the caeca has been suggested to have beneficial properties (Ghadban, 2002; Jin *et al.*, 1997; Nava *et al.*, 2009; Revolledo, Ferreira & Mead, 2006) due to their ability to change the expression of invasion genes in *Salmonella*. In particular, the short-chain fatty acid butyrate down-regulates expression of invasion genes in *Salmonella* species at low doses (Van Immerseel *et al*., 2006).

1.5.2 Aggregation

This is an aggregation or clumping of one bacterial cell to another bacterial cell from the same type. Co-aggregation is clumping with different type of bacteria. Auto-aggregation of LAB may be necessary for adhering to intestinal epithelial cells and with the addition of their potential co-aggregation ability they may form a barrier that prevents colonization of pathogenic microorganisms (Kos *et al*., 2003). Spencer & Chesson in (1994) suggested that co-aggregation between LAB and enteropathogens have a direct effect of excluding the pathogenic bacteria from the gastrointestinal tract.

The aggregation ability of the *Lactobacilli* may be contributed by a protein known as APF (Aggregation-Promoting Factor), which, when secreted, acts as an aggregation mediator between two bacterial cells in many *Lactobacilli* of different origin .

1.5.3 Competition for essential nutrients

Prebiotics like Fructo-oligosaccharides increase bacterial populations by promoting the growth of *Lactobacillus* and *Bifidobacterium* species. At the same time, the pH levels decrease because of the production of specific acids. As a result, the growth of Gram-negative bacteria, such as *Salmonella* and *E. coli,* are restrained as they are not able to use fructo-oligosaccharides and they are sensitive to low pH.

1.5.4 Adhesion to epithelial cells

Adhesion may be a requirement for competitive exclusion; some LAB have the ability to attach to the surface adhesion receptors of the gut, thereby excluding the harmful bacteria that could colonise using the same adhesion receptors. Since LAB adhere to epithelial cells and thus have a prolonged residence in the GIT, they could act as antibacterial agents by excluding the pathogens found on the mucosal surface.

Microorganisms adhere to the gut epithelium through polysaccharide-containing components attached to the cell wall. *Lactobacilli* require mucin for their attachment, and if the mucin content decreases, the beneficial *Lactobacilli* numbers also decrease (Mikelsaar *et al*., 1987). Additionally, the beneficial *Lactobacilli* also metabolize both the protein and sugar content of mucin and use it for energy and growth.

Competition for available binding sites on the intestinal mucosa is also affected by the pH of the luminal contents. Many of the beneficial microbiota can stimulate lower gut motility via production of short chain fatty acids and decreasing pH (Ohashi & Ushida, 2009). Larger numbers of the *Lactobacilli* will bind to the intestinal mucosal epithelial cells and exclude pathogens such as *Salmonella* and *E. coli*.

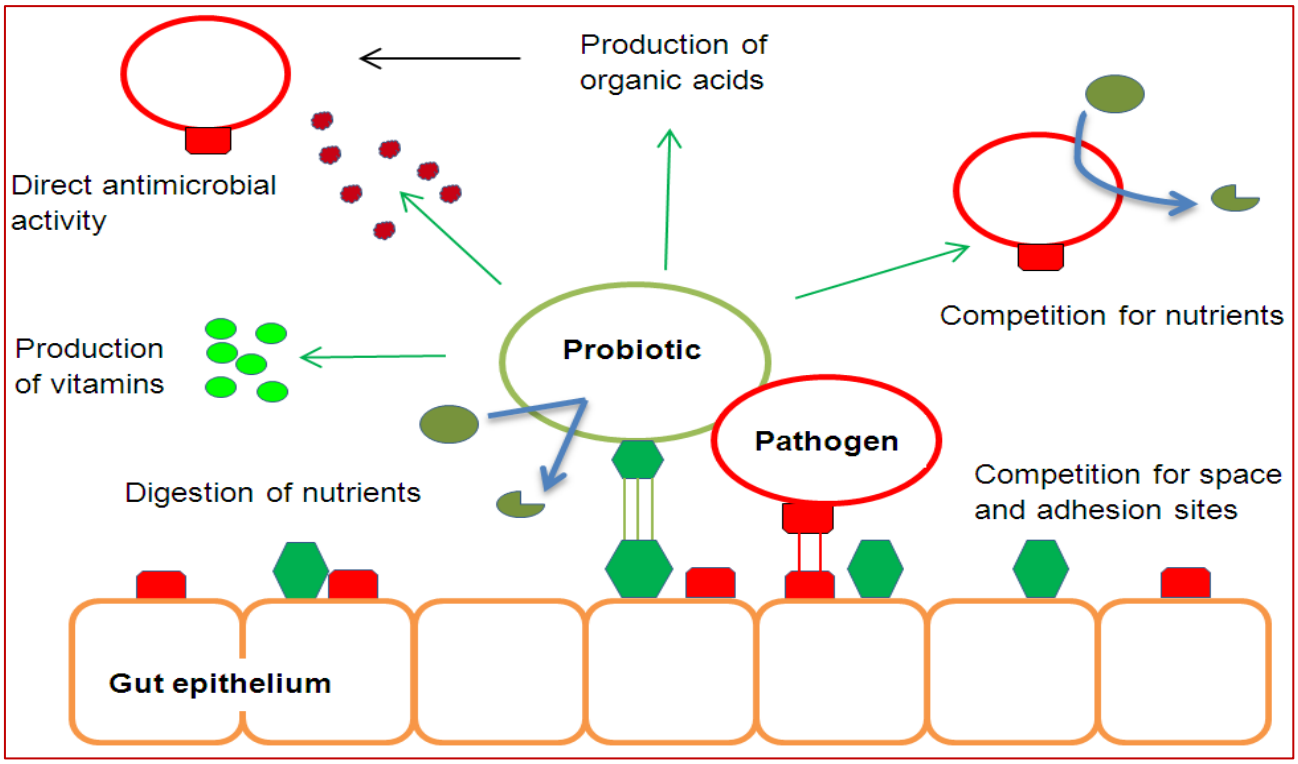

Figure1. 3. The activities of a probiotic bacterial cell in the intestinal tract. Bacteria express activities towards other bacteria such as the production of antimicrobial components, competition for space and nutrients and strong interaction with epithelial cells resulting in an influence on the host physiology. Adapted from (Snel *et al*. 2002).

1.5.5 Histological alterations of the gastrointestinal tract

The gastrointestinal tract of the newly hatched chick is in a process of development and maturation. The effects of probiotics on histological changes to intestinal villi are still unclear. The permeability of the intestinal epithelium may be altered by pathogens such as *Salmonella*, resulting in decrease of number, height and length of the villi and decrease in the digestive and absorptive activity. These changes have consequences for the health and performance of the chicken. However, there is some evidence that probiotics improve the intestinal mucosa development. Birds

fed *Bacillus subtilis* showed greater villus height in jejunum and ileum compared with control (Pelicano *et al*., 2005). Chicks and turkeys treated *with Lactobacillus reuteri* had longer villi, than birds supplied a basal diet (Dunham *et al*., 1993) .

1.6 Probiotic use in Poultry

For more than 50 years antibiotics have been used in poultry production as prophylactics and growth promoters that increase performance and decrease diseases (Edens, 2003). Many of these antibiotics are used for humans as well. Bacterial resistance to antibiotics used in humans has caused concern about the use of antibiotics in poultry and livestock production. For that reason, many countries have banned the use of antibiotic growth promoters (AGP's) in poultry and livestock (Edens, 2003). One of the most accepted natural alternatives to the use of antibiotics are probiotics.

1.6.1 Effect of probiotics on poultry performance

There are several reviews discussing the effect of probiotics on poultry performance. The general level recommended for commercial probiotics in feed additives is around 10^8 CFU/g feed continuously not a single dose (Olnood, Choct & Iji, 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.

Several researchers have reported improved feed intake, weight gain and feed conversion of chickens provided probiotics as listed in Table 1.2.

Table1. 2. Summary of publications use of the probiotics in poultry with dosages recommended and the results on growth performance.

Researcher	Type of probiotics used	Dose & route of administration	Effect on chicken performance
Jin *et al*. (1997)	*Lb. acidophilus or LAB* Mixture	Feed 1g/kg	Increase body weight Significantly($P<0.05$)
Kabir *et al*. (2004)	Protexin®Boost	Water 2 g/10 L	15% Significant higher live weight gain ($p<0.01$)
Gil de Los Santos *et al*. (2005)	*Bacillus cereus*	Feed	Increase significantly HLWG
Khaksefidi and Ghoorchi (2006)	*Bacillus subtilis*	Feed 50mg/kg	4 & 7% Significantly ($p<0.01$) HLWG
Timmerman *et al*. (2006)	Human and chicken *Lactobacillus*	Water 10^7-10^{10} /ml	1.84% slight HLWG
O'Dea *et al*. (2006)	*Lb. acidophilus* *Lb. bifidus*	Water	No significant effect on body weight
O'Dea *et al*. (2006) 2	*Lb. acidophilus* *E. faecalis*	Feed 0.5g/kg	No significant effect on body weight
Apata (2008)	*Lb. bulgaricus*	Feed 20, 40, 60 or 80 mg/kg	Significantly increase HLWG
Opalinski *et al*. (2007)	*Bacillus subtilis*	Feed 4 x10^5 /g	No difference
Faria Filho *et al*. (2006)	27 Brazilian studies	Feed	Increase weight gain
Olnood team (2007)	4 LAB	Feed 10^6/g	Not significantly improve growth
Vicente, j. (2007)	*LAB* species (FM-B11TM)	Water: (10^6 CFU/ml)	Increase body weight (2.06%)
Niba, A. (2008)	*Lb. plantarum*	FMF 10^9 CFU/g from day 1	Increase significantly weight gain
Al-Zenki team, (2009)	*P. acidilactici* (Aviguard) ™	Feed 100mg/kg, Spray 0.5ml/ chick 1day old.	No significant effect on weight gain
Savvidou (2009)	*Lb. salivarius*	FMF	Increase weight gain

1.6.2 Effectiveness of probiotics against *Salmonella*

Many researchers using probiotics to reduce *Salmonella* have met with varying degrees of success. Higgins *et al*. (2008) demonstrated that there was a significant reduction of *S.* Typhimurium (90-95%) and *S.* Enteritidis (60-70%) recovered from the caeca of day old chicks following treatment as compared with control ($P<0.05$). The Probiotic was administrated by oral gavage at a concentration of $7x10^5$ - $5x10^5$ CFU/chick while the *Salmonella* was administered orally with a concentration of about $7x10^3$ – $2x10^3$ CFU/chick. Higgins *et al*. (2008) also showed a significant reduction of *S.* Enteritidis in chicks after challenging with 10^4 CFU of *S*. Enteritidis. One hour later chicks were treated with a commercial probiotic (FM-B11) by oral administration of concentrations of 10^4, 10^6 and 10^8 CFU/Chick. Caeca contents were examined after one day and there was no significant reduction in SE with 10^4 CFU while there was a significant reduction with 10^6 and 10^8 CFU of the probiotic.

Vicente *et al*., (2008) used a commercial probiotic (FM-B11) liquid in water for one day old chicks which were challenged with 10^4 CFU *S*. Enteritidis. The probiotic was used 1 hr after SE challenge and for three consecutive days at a dose rate of 10^6 CFU/ml. The caecal contents were examined aseptically after three days of challenge and the result was a significant reduction of SE ($P<0.05$). At the same time, the team used a lyophilized culture of the same probiotic in the water for another group of chicks using the same conditions and concentrations and the result was the same as the liquid probiotic (Vicente *et al*., 2008).

Al-Zenki *et al*. (2009), used Bactocell (*Pedicoccus acidilactici*) at a concentration of 100mg/kg in dry food and Aviguard a commercial probiotic spray at 0.50 ml/chick

/day old chicks. The result was a significant decrease in *Salmonella* ($P< 0.05$) in the litter as well as in the caeca and whole body (post chill) of chicken.

Chen *et al*. (2012) used a probiotic consisting of multiple strains of LAB which reduced the incidence of *Salmonella* Typhimurium significantly in treated broiler chicks because it was more effective than mono strain probiotics due to the additive and synergistic effects.

Niba (2008) demonstrated a significant reduction of *Salmonella* Typhimurium SAL 1344 in broiler chickens by application of *Lactobacillus plantarum* NCIMB 41607 in fermented moist feed (FMF) with a concentration of 10^9 CFU/g feed provided on day 7 of age compared with a single dose by gavage of 10^7 CFU/chick at day 1 of age. The chicks were challenged with a single dose of 10^6 CFU/chick *Salmonella* Typhimurium by oral gavage on day 15 of age. *Salmonella* shedding was measured two days later until the end of the trial. Niba also showed that using LAB in FMF gave a greater reduction in *Salmonella* shedding than in if the same LAB were given in water (10^7 CFU/ml). This worker also showed that administration of LAB from day 7 of age gave a better result in reduction of *Salmonella* than if it was used from day one of age and that may be because the immune system of the chick does not fully develop until 7 days of age. The mean percentage of Birds not shedding *Salmonella* Typhimurium were 84% for FMF when *Lactobacillus plantarum* was provided from day 7 compared with 74% for same feed provided from day one of age. Only 40% and 41% birds did not shed *Salmonella* when the same LAB was supplied with water from day 1 and 7 respectively. However, the water treatment was significantly better ($P<0.001$) than the single dose of LAB on the first day (23%) and control (20%). He recommended that application of *Lb. plantarum* NCIMB 41607 in FMF could be a better way to reduce *Salmonella* in chicken than through water.

Savvidou (2009) shows the effect of *Lactobacillus salivarius* NCIMB 41606 on *Salmonella* Typhimurium in broiler chickens delivered in water and in FMF from the one day old till the end of the trial (39 days) with a concentration of 10^7 CFU/ml in water and 10^9 in FMF. *Salmonella* Typhimurium administered by oral gavage on day 14 with a concentration of 10^4 CFU/chick for both treatments. From day 17 until the end of the trial cloaca swabs were taken for *Salmonella* shedding. The results showed a significant reduction of *Salmonella* shedding ($P<0.05$) with FMF of 52% while the water treatment was not significant, only 19% compared with the control at 7% negative *Salmonella* shed. Acidified moist food (AMF) used with the same concentration of Lactic acid as FMF did not a significantly reduce shedding of *Salmonella* compared with the control. The study recommended the use of *Lactobacillus salivarius* NCIMB 41606 in FMF to reduce *Salmonella* Typhimurium in broiler Chicken (Savvidou, 2009). Heres and colleagues in (2003a) reported that Chicken using Fermented liquid food appeared less susceptible to colonization with *Salmonella.*

1.7 Probiotic Administration Methods

Many methods are available to administer probiotics to poultry in commercial production. Doyle & Erickson (2006) tried applying the probiotics *in ovo* prior to hatching, in drinking water, in dry feed and in moist feed slurries.

1.7.1 Administration of LAB through water

Administration of vaccines via drinking water is a typical practice in poultry production. However, osmosis, chlorine and calcium salts in the water could affect the survival of the LAB delivered in water. Chlorine is used as a disinfectant in piped distribution systems; a free chlorine residual of 0.2-0.5mg/L is required to reduce the risk of microbial growth (WHO, 1997). Previous studies at the University of Plymouth done by Azhar (2005) showed that LAB (*Lb*. *plantarum*) survived in hard water after 24 hours. Niba in (2008) showed that *Lactobacillus plantarum* can survive in water and can give a probiotic effect on the chicken. Savvidou (2009) demonstrated that *Lactobacillus plantarum* can survive in drinking water and can confer benefits to the chicken. Vicente team in (2008) also used probiotics (liquid and lyophilized) via water and were effective in reducing *Salmonella* as previously mentioned

1.7.2 Administration of LAB through Dry feed

When probiotics are added to dry diets, only the bacteria are present and the products of their metabolism appear in the GI tract after being consumed. Recently, Vandeplas and colleagues (2009a) used a wheat based diet for chickens with a combination of *L*actobacillus plantarum and a xylanase as a feed additive. They found the combination to be effective for reducing *Salmonella* Typhimurium infection in broilers. Jin et al. (1997) used a basal diet of maize with *Lactobacillus acidophilus* or with 12 strains of LAB and the result was an increased body weight and decrease in pathogens in the caeca. Al-Zenki et al. (2009) used the commercial product Bactocell in dry feed at a concentration of 100mg/kg and found a significant reduction in

Salmonella in chicken. However, O'Dea et al. (2006) demonstrated that there were no effects on weight gain or mortality between the control and chickens administered a commercial probiotic containing *Lactobacillus acidophilus*, *Lactobacillus bifidus* and *Enterococcus faecalis* as feed additive 0.5 g/2kg throughout the production period. Many other researchers used feed administration to test the effect of probiotic on chicken such as (Apata, 2008; Vicente et al., 2007b)). Yasar & Forbes (1999) used wet food for 1day old chicks until the end of the trial (42 days) by adding 1.3 kg water to 1 food kg and demonstrated that there was a significant increase in body weight gain ($P<0.05$) compared with dry food.

1.7.3 Administration of LAB through fermented feed

The use of fermented liquid feeding is a practice that is being adopted more widely by the pig industry. Fermented diets are made by mixing dry compound feed and water and stored for at least 8h (Russell *et al*., 1996). In fermented diets, both bacteria and their fermentation products are present. The numbers of bacteria also differ and moreover in the fermented diets bacteria grow quickly, whereas in dry diets they have to be accelerated (Scholten *et al*., 1999). Production of fermented liquid feed (FLF) depends on 3 factors:

1. Microorganism (Lactic acid bacteria, Yeast and Fungi)
2. Substrates (Carbohydrates, fibres and proteins)
3. Environnements (time, temperature, moisture content and air composition)

According to Beal *et al*. (2002b) Lactic acid bacteria fermentation of feeds provides a feed that has a pH of 3.8-4.0 and contains 150-250 mmol/L lactic acid.

Feeding FLF has also been correlated with a lower prevalence of *Salmonella* in chickens. A reduced probability of *Salmonella* colonisation in chickens fed FLF was

reported by Heres *et al*. (2003a). He also showed that broiler chickens fed FLF are less susceptible to a single oral inoculation with *Salmonella* and *Campylobacter* than chickens fed a normal dry feed. In another experiment, chickens fed FLF required a longer time after inoculation of *S.* Enteritidis, or a higher inoculation dose, for the same infection rate compared with chickens fed dry feed. Heres *et al*. (2003a) suggests that a 1000–10,000 times higher inoculation dose of *S.* Enteritidis for FLF chickens was needed to reach comparable numbers of *Salmonella* shedding as chickens fed with dry feed and one way to control the presence of *Salmonella* in broiler chickens is to make the chicken less susceptible to colonization (Heres *et al.*, 2004) .

Recently, Niba (2008) demonstrated the application of *Lactobacillus plantarum* in a moist feed for 24 hours and the effect on reduction of *Salmonella* and increasing of body weight. Niba suggested using FMF fermented by *Lactobacillus plantarum* NCIMB 41607 as an effective means of controlling *Salmonella* Typhimurium infection in poultry.

In conclusion, successful application of fermented moist feeds in poultry feeding depends on the ability to select the right strain(s) of LAB and feed substrates that able to produce repeatable fermentation results. Therefore, the resistance of such feeds to enteropathogen contamination during short storage, and their ability to reduce pathogen colonization in the gut of chickens, could have far-reaching implications for improved food and environmental safety.

In summary, salmonellosis in chicken is a worldwide disease and increasing importance of the poultry and poultry products around the world for human consumption leads to increase the possibilities of spread this disease in globally. Eradication of the disease is unrealistic but it may be controlled and reduced by using probiotics

through the drinking water, dry feed and via a fermented moist feed. The FMF approach appears to be the most effective but in practice there is a need for some engineering modification to feeding systems and this may be solved in the future. Generally, reducing the disease in poultry would lead to a consequent decrease in the incidence for humans, thereby promoting general health.

1.8 Aim and objectives:

The aim of this study was to investigate the use of *Lb. plantarum* as a potential probiotic to reduce *Salmonella* infection in chicken and its effects on the gut microflora. This aim will achieved by specific objectives:

- To investigate the beneficial effects of *Lactobacillus plantarum* NCIMB 41607 *in vitro* and *in vivo* against *Salmonella* Typhimurium infection.
- To determine the best application methods of administering L*actobacillus plantarum* to chicks
- To use the rifampicin resistance as a biomarker for detection of *Lactobacillus plantarum* for the research objectives
- To examine whether the potential positive effect of fermenting moist feed with *Lactobacillus plantarum* is due to the strain itself or due to the lactic acid produced during fermentation.
- To determine *in vitro* the effect of the *Lactobacillus plantarum* NCIMB 41607 on the microbial population of the gut by classical and molecular microbiology in commercial chicks and specific pathogen free chicks.
- To investigate the histological changes of the chicks gut using light and electron microscopy because of using *Lb. plantarum*.

- To investigate the haematological parameters of Heterophil/ lymphocyte (H/L) ratio in the chicken.
- To investigate the ability of *Lactobacillus plantarum* to attach to the chicken intestine using scanning electron microscope (SEM) and fluorescent *in situ* hybridisation (FISH) technique.

CHAPTER 2

CHAPTER 2. MATERIALS AND METHODS

2.1 Overview

This chapter will include the methods for the *in vitro* and *in vivo* experiments conducted. The following general protocols were used unless otherwise indicated. Chemicals, reagents and culture media were sourced from Sigma –Aldrich (Poole, Dorset, UK), Bioline (UK) or Oxoid Ltd (Basingstoke, UK) unless otherwise indicated.

The all *in vivo* studies were carried out at the University of Plymouth, Animal laboratory 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 30/9065.

2.2 Bacterial strains

All bacteria isolates were obtained from the University of Plymouth laboratories (UK collection). A nalidixic acid resistant *Salmonella* enterica serovar Typhimurium *Salmonella* Typhimurium SAL1344 nal^r was used. *Lactobacillus plantarum* (NCIMB 41607) was isolated from a healthy chicken gut in Plymouth in 2005. The bacteria were stored in liquid nitrogen and were activated by sub culturing three consecutive times onto on nutrient agar.

2.2.1 Selection of rifampicin resistant *Lactobacillus plantarum*

The gradient plate technique was used as a method for isolating antibiotic-resistant bacterial mutants by inoculating them onto an agar plate containing a concentration gradient of the Rifampicin (Weinberg, 1959). MRS agar (Oxoid, UK) was prepared in 100 ml aliquots, autoclaved and tempered to at 45°C. One ml of rifampicin (15mg /ml in ethanol) was added to the liquid agar and mixed well.

Briefly, MRS agar was poured into a Petri dish and allowed to set in a sloping position. A second layer of MRS agar supplemented with 15 mg/ml rifampicin was poured on top of the slope and allowed to set in a level position producing a concentration gradient of the rifampicin from 0 mg/ml at one end to the maximal concentration 15mg/ml at the opposite end.

A loop of an overnight MRS broth culture was aseptically transferred to a standard MRS plate and the MRS-rifampicin gradient plate and the culture spread over the plate. Plates were incubated at 37°C in 5% CO_2 for 48 hrs.

2.2.2 Freeze drying *Lactobacillus plantarum*

The easiest way to introduce probiotic into water is in a lyophilized freeze dried form. One litre of an overnight culture of *Lb. plantarum* MRS broth incubated at 37ºC was centrifuged at 10000rpm for 10 min. The sediment was mixed with 10 ml PBS and frozen at -20 ºC for 24 hrs. The samples were transferred to a freeze dryer (Edward, Modulyo, Italy) at -60 ºC. The viability of the resulting freeze-dried culture was assessed by mixing 10 mg with 9.9 ml PBS, followed by serial dilution and plating onto MRS agar incubated overnight at 37ºC.

2.3 Antagonistic activity to pathogens

2.3.1 Agar well diffusion assay of *Salmonella* with *Lb. plantarum*

For the agar-well diffusion assay, an overnight culture of *Salmonella* Typhimurium and *Salmonella* Enteritidis in nutrient broth (Oxoid, UK) and *Lactobacillus plantarum* in MRS broth (Oxoid, UK) at 37 ºC were used. Salmonellas were diluted to1x10^6 CFU / ml in PBS and plated onto nutrient agar. In each agar plate five wells were prepared using a sterilized cork borer of 5 mm diameter (Palaksha, Ahmed & Das, 2010). In the wells of each plate, 100 µl of the following were added:

1. Overnight broth culture of *Lactobacillus plantarum*;
2. Cell free supernatant that was obtained by centrifuging and filter sterilizing (0.2 µm pore size) the supernatant of an 18 hr culture of *Lactobacillus plantarum*.
3. Neutralized cell free supernatant, cell free supernatant was adjusted to pH 7.0 using of 1M NaOH.
4. *Lactobacillus plantarum* in fresh MRS broth.
5. *Lactobacillus plantarum* washed with saline 3 times.

The contents of the wells were allowed to diffuse into the agar for 2 h at room temperature prior to incubation at 37°C. The plates were examined after 24 h for inhibition zones.

2.3.2 Antibiotic sensitivity test

Antibiotic sensitivity of *Lactobacillus plantarum* was determined by disk diffusion using MASTERING11T antibiotic discs. *Lactobacillus plantarum* on MRS was incubated for 24 hr at 37ºC with 5% CO2. The Salmonellas on nutrient agar were incubated for 24h at 37ºC aerobically. The inhibition zones around the discs have measured by grade Vernier calliper.

2.3.3 Aggregation

2.3.4.1. Auto-aggregation

Auto-aggregation assays were applied according to the methods of Del Re *et al*. (2000) and the modified method by Kos *et al*. (2003). Some modification was applied to these methods. The *Lactobacillus plantarum* bacteria were grown for 18 hr at 37 ºC in MRS broth (Oxoid) and *Salmonella* Typhimurium *and Salmonella* Enteritidis were incubated for 18 hours at 37ºC in nutrient broth (Oxoid, UK). The bacteria were harvested by centrifugation at 4000rpm for 15 minutes, and then washed twice with PBS, re-suspended in PBS and diluted to give an Optical Density (OD) of 0.5. Five ml of each bacterial suspension in PBS were centrifuged at 4000g for 15 minutes and the bacteria were re-suspended in 4 ml of their own filtered sterilized culture supernatant fluid, mixed for 10 seconds and incubated for 4 hr at room temperature. Auto aggregation was determined by taking 1 ml of the upper suspension into a cuvette and the absorbance, (A) was measured at 600

nm at time zero and 4 hr. The equation used to calculate the percentage auto-aggregation was: (1- A4 /A0) X100.

Where A4 represents the absorbance at time t=4 hr of incubation and A0 the absorbance at t=0 hr.

2.3.4.2. Co-aggregation

For the co-aggregation method, *Lactobacillus plantarum* were grown for 18 hr at 37ºC in MRS broth (Oxoid, UK), *Salmonella* was cultured in the same conditions in nutrient broth (Oxoid, UK). The bacteria were harvested by centrifugation at 4000rpm for 15 minutes, washed twice with PBS, and re-suspended in PBS to give an OD of 0.5. Two ml of each *Salmonella* bacteria was added to two ml of $1x10^9$ *Lactobacillus plantarum* and mixed for 10 seconds. Control tubes were set up at the same time containing 4 ml of each bacterial suspension at alone. The absorbance (A) at 600 nm of the suspensions was measured after mixing 0.1 ml of each suspension an transferred to a cuvette containing 0.4 ml of PBS and the viability of the samples were examined by placing into nutrient agar for *Salmonella* and MRS agar for *Lactobacillus plantarum* after 5 hr of incubation at room temperature. The following equation was applied: The percentage of co-aggregation was calculated using the equation of (Handley *et al*., 1987):

$$\text{Co-aggregation (\%)} = ((\mathrm{Ax} + \mathrm{Ay})/2 - \mathrm{A}(\mathrm{X} + \mathrm{Y}))/((\mathrm{Ax} + \mathrm{Ay})/2)\,\mathrm{X100})$$

Where x represents *Lactobacillus plantarum* and y represents *Salmonella* and (X+Y) the mixture.

2.3.4.3. Preparing aggregation samples for SEM examination

Scanning Electron Microscope was used to see the interaction between the bacteria itself (auto-aggregation) and interaction with other types of bacteria (co-aggregation). Thermal cover slips are inserted into the suspension of both bacteria as well as in suspension of individual bacteria. After 10 min the cover slips (coated with the bacteria) were aseptically removed from the suspension. Cover slips were fixed with 2.5% Glutaraldehyde for 30 min and washed with increasing alcohol concentrations for 10 min in each 30%, 50%, 70%, 90% and twice in 100% alcohol. The samples were placed in a critical point dryer to dehydrate the sample. Dried samples were placed on brass stubs and were coated with gold in a sputter coating unit FINE COAT/JFC 1100 (JEOL, Japan). The samples were examined under scanning electron microscope (JEOL, 5600, Japan).

2.3.4 Survival of *Lactobacillus plantarum* in water

Lyophilized *Lactobacillus plantarum* prepared in laboratories of the University of Plymouth was used in this experiment. 120 mg of Lyophilized *Lb. plantarum* was added to 120 ml tap water to make suspension of $1x10^9$ CFU/ml. The suspension divided to 6 sterile tubes and placed at 4°C, 15°C, 20°C, 30°C, 37°C and 60°C for 24 hr. One ml of each sample was serially diluted in 9 ml of PBS (OXOID, UK) be-

fore and after incubation for 24 hrs. Appropriate dilutions were plated on MRS Agar (OXOID, UK) for enumeration of *Lb. plantarum*. Plates were incubated at 37 °C for 24h. Colonies were counted using a Colony Counter (Gallenkamp, UK). This process was replicated three times. The treatments were designed to conform to the environmental conditions in the room temperature where the birds are held is usually 20-23 ºC; also, the temperature under the heaters that are used for the first 3 weeks of chicks age which is around 34ºC. None of the samples were sterilized. In another experiment, viability was checked after keeping freeze dry bacteria in fridge for 22 days.

2.4 Simulated Digestive System of Chicken Model

Ten grams of the following feed treatments were added to 70ml distilled water plus 10^9 CFU/ml *Salmonella* Typhimurium or *S.* Enteritidis in a flask and subjected to an *in vitro* model of the poultry digestive tract Figure 2.1:

- Fermented moist feed (FMF).
- *Lactobacillus plantarum* suspended in feed (DW or Feed).
- Control (CON) feed.

The *in vitro* model was divided into 3 stages:

Stage 1 crop: pH 4.6 (80µl 37% HCl) for 45min at 41.4ºC.

Stage 2 gizzard/ proventiculus: pH 2.5 with 0.320g pepsin for 90min at 41.4ºC.

Stage 3 small intestine: pH 6.2 (with NaHCO3) plus 0.320g pancreatic and 0.210g

bile salts for 150 min at 41.4ºC.

Lactobacilli and *Salmonellas* colonies were enumerated in 0, 45, 135 and 285 min by serial dilution, and plating onto MRS and nutrient agar respectively. All treatments were applied in triplicate.

Figure 2. 1. The simulated digestive system of chicken that used for the *in vitro* experiment

***In vivo* materials and methods**

2.5 Housing and feeding protocol

All feeding experiments were conducted at the University of Plymouth, Animal facility. Birds were housed in pens, measuring 100 X 82 cm. The chicks were given *ad libitum* access to fresh feed and water. The amount of feed and water consumption was measured daily. The feed was provided in plastic troughs, water was provided in graduated plastic conical drinkers (1.3 L). All drinkers and feeders were washed daily and disinfected weekly. The temperature was controlled by ceramic brooder lamps, it was 34ºC in the first week, and reduced by 5ºC every week until 20-22ºC and measured by temperature logger (Tiny tag, tv-4050, UK). Ventilation was programmed. Lighting with white light (700 Lux/m2/s Lx-101 lux meter, Lurton, Taiwan) for 14 hr a day. The chicks were observed twice a day and weighed on a weekly basis. All birds were tagged using coloured plastic leg rings for identification purposes.

2.6 Growth performance protocol

All chicks were weighed every week. The average daily feed intake (ADFi) was calculated by subtracting daily feed refusals and dividing by the number of chickens in each pen to get the mean. Growth performance was assessed using the feed conversion rate (FCR) of feed intake divided by weight gain.

2.7 Experimental chicks

Three experimental trials are conducted with two types of chicks. Specific pathogen free (SPF) white Leghorn chicks (VALO, Germany) were obtained as fertilized eggs and incubated for 21 days in an egg incubator (Cuvatutto, Italy). Ross day old chicks were obtained from P D Hooks Hatcheries (Kentisbere, Devon, UK) and transported to the animal facility of Plymouth University. On arrival or hatch, chicks were randomly divided into groups.

2.8 Treatment groups

2.8.1 Control feed

A commercial chick crumb feed obtained from BOCM Pauls Ltd, (Wherstead, IP9 2AX, UK) with the composition shown in Table 2.1 was used. The feed contained AVATEC 150 mg/kg premix with a concentration of Lasalocid sodium 90 mg/kg for prevention from coccidiosis in broiler chicks.

Table 2. 1 The chick feed composition which was used for the all trials.

Quantity of feed	composition%	Nutritional	
Wheat	100-40	Methionine	0.35
Soya bean extract	25-50	Vitamin A IU/kg	12000
Barley, Oats	10- 0	Vitamin D3 IU/kg	5000
Salt		Vitamin E IU/kg	40
Vitamins		Selenium mg/kg	0.3
Minerals		Copper sulphate	20

2.8.2 Fermented moist feed (FMF)

FMF was prepared by mixing the feed with water (1: 1.2 feed: water) and inoculating with 10^9 CFU/ml rifampicin resistant *Lactobacillus plantarum*. The broth culture was prepared by inoculating 10 ml MRS broth with *Lactobacillus plantarum* and incubating at 37ºC for 24 h. The resulting feed was incubated at 30ºC in polythene bags for 24 hr prior to feeding. The pH of FMF was recorded using a pH electrode (pH213 microprocessor pH meter, Hanna instrument, Lisbone, Portugal). The mean pH of the fermented feed before inoculation was 5.85 and when delivered to chicken was 4.44. The mean lactic acid concentration was 310 mmol/L (2.7%).

2.8.3 Water treatment

Freeze dry *Lactobacillus plantarum* 10^{12} CFU/g was added to drinking water at the rate of one pram per litre of to give a final concentration of 10^9 CFU/ml.

2.8.4 Dry Feed with *Lb. plantarum*

One gram of freeze dry *Lb. plantarum* was added to 1 kg of feed in a sterile bag and mixed well to give a final concentration of 10^9 CFU/ml.

2.8.5 Acidified Moist Feed (AMF)

The acid moist feed (AMF) was prepared by adding Lactic acid (PURBAC 88) to wet feed at a rate of 30 ml/kg to give a concentration 310 mmol/L in wet feet.

2.8.6 Acidified Moist Feed and drinking water (AW)

Feed with lactic acid as AMF and drinking water with *Lb. plantarum* as WAT group.

2.9 *Salmonella* Typhimurium SAL 1344 nal^r infection study

Salmonella Typhimurium of 10^6 CFU/ml was gavage orally by 1ml syringe. Before the challenge, all birds were dosed with 0.2 ml sodium bicarbonate to neutralize the acidic environment in the upper GI tract.

2.9.1 Cloacal swab

The swabs were diluted in 1 ml of PBS and serial dilution was followed by PBS. The samples plated for 24 h at 37ºC on MRS which used for LAB and Hicrome for detecting and differentiated by *E. coli* and other *Enterobacteriacae*.

The swabs plated on XLD supplemented with nalidixic acid (20µl/ml) and incubated at 37ºC for 24 hrs. To verify there are negative for *Salmonella*, 1 ml suspension was added to 9 ml of peptone buffer water broth (enrichment) and incubated overnight. One ml of the suspension was added to Rappaport- Vassiliadis *Salmonella* media (RVS) as a selective medium for *Salmonella* and incubated for 3 days at 37 ºC. The resulting suspension was plated on XLD agar and incubated at 37ºC for 24 hrs. Following table showed the laboratory tests to compare between *Salmonella* Typhimurium and *E. coli*.

Table 2.2. The laboratory tests to compare *S*. Typhimurium with *E. coli*

Laboratory test	S. Typhimurium	*E. coli*
Catalase	+ve	-ve
Oxidase	-ve	-ve
Indole	-ve	+ve
Motile	+ve	-ve
Lactose fermentation	-ve	+ve
H_2S production	+ve	-ve
Gram stain	-ve	-ve
Colony in MacConcky agar	Pink	Yellow & +ve bile salt precipitations
Colony on XLD agar	Black	Yellow
Colony on chromogenic medium	Colourless	Dark blue -to- violet

2.10 Chick GIT dissections

The chicks were killed by cervical dislocation. Sterile scissors and scalpel blade were used to aseptically open the peritoneal cavity. The entire gastrointestinal tract was removed from the peritoneal cavity (Fig. 3.1). The digesta of the duodenum, jejunum, ileum and caeca was removed separately later and divided into two samples for standard and molecular microbiology. Liver and spleen were aseptically removed. The organs were weighed in stomacher bags, tenfold PBS added and homogenised a stomacher (Bag mixer® Interscience 788860, France). One ml of each dilution added to 9 ml of PBS for 3 subsequent serial dilutions to investigate the bacterial numbers.

2.11 Standard (culture based) microbiology

XLD agar was used for detecting *Salmonella*, MRS agar for LAB, Hicrome^{TM} coliform agar (Sigma-Aldrich, Dorset, UK) was used for detecting and differentiated of *E. coli* (violet to dark blue) and other Enterobacteriacae (pink or yellow). For identification of bacteria all the colonies were Gram stained and sub-cultured on TSA (Tryptone Soya Agar) at 37ºC overnight and biochemical test of Catalase, Oxidase, Indol and Glucose fermentation were applied.

2.12 Molecular microbial investigation

All molecular work and protocols were carried out in a Labcaire PCR workstation (Labcaire System Ltd, Clevedon, UK).

2.12.1 Bacterial DNA extraction

Typically, digesta from ileum and caeca of two chicks from the same pen was pooled into one sample. Three methods for DNA extraction were used, a Kit method, modified kit method and Phenol-chloroform method to select the one with the highest purity yield and most cost effective method.

2.12.2 Kit method

A QIAamp stool mini kit (QUIAGEN, West Sussex, UK) was used with slight modification to the manufacturer's instruction. Two hundred mg of sample was prepared in a sterilized Eppendorff tube, and DNA extracted by the following four

stages.

1- Lysis stage: The lysis of bacterial cells of the sample was incubated at 37ºC for 30 min in a fresh lysozyme solution (50mg/ml TE buffer). TE was prepared by adding 500µl Tris with 100µl EDTA in 49.8 ml grade water, then 700 µl of ASL buffer was added and mixed for 1 min. The mixture was placed on a hot plate at 90ºC for 5 min and mixed for 15 seconds with centrifugation for 1 min at 13000rpm.
2- Inhibitor removal stage: An inhibitor tablet was added to 800 µl of the supernatant mixed for 1 min, and centrifuge for 3 min at 13000rpm. The supernatant was centrifuged for 3 min.
3- Protein removal: 200µl of the supernatant was mixed with 20 µl of proteinase K and 200µl of AL buffer was mixed for 15 seconds and incubated at 70ºC for 10 min then 200 µl of 100% ethanol was added.
4- Clean-up: All samples were applied to a QIAamp column and centrifuged for 1 min. The column was placed into to a new collection tube centrifuged for 1 min 500ul of AW1 in to collection tube and 500µl (AW2) with 1 min centrifugation. Eluted DNA was transferred to a new Eppendorf tube and the DNA quantity and purity were determined using a Nanodrop-100 Spectrophotometer.

2.12.3 Modified kit method

This was the same as the kit method up to the clean-up stage (step 4). DNA was precipated with 50ml 100% ethanol centrifuged for 5 min at 13000 rpm. Ethanol when added to water containing DNA will force the DNA to aggregate with other DNA molecules and with the help of centrifugation all DNA will be expelled outside

and precipated on the internal surface of the tube. Cleaned from acids with 70% ethanol 50µl and centrifuged for 5 min at 13000rpm. After drying for 10 min DNA was resuspended with 50µl DNA grade water. The DNA was purified by adding 20 µl phenol mixing then centrifuged for 5 min at 13000 rpm. The phenol is a less polar charge than water molecules, so the protein will dissolve in phenol and the centrifugation will increase the separation of water containing DNA from phenol containing protein. The supernatant containing DNA was taken and 40 µl of chloroform added followed by mixing and centrifugation for 5 min at 13000 rpm. The chloroform was used to clean all remains of phenol. The DNA concentration and protein with humic acids determined by Nanodrop® 100 Spectrophotometer.

2.12.4 Phenol-chloroform method

Three solutions were prepared:

1. Lysis solution: 50mM Tris/ Cl pH 8, 25mM EDTA, 3% SDS, 1.2% PVP
2. Extraction solution: 10 mM/L Tris/Cl pH 8, 1mM/L EDTA, 0.3M Na acetate, 1.2% PVP.
3. TE solution: Tris – EDTA (TE): 10mM Tris/Cl (1.57 g/l), 2mM EDTA (0.37g/l) adjusted to pH8.0

Two hundred mg of sample, 0.5ml of fresh lysozyme (50 mg/ml in TE) was added, mixed, and incubated at 37 °C for 30 min. 35µl of lysis solution was added and mixed gently. Subsequently, 400µl of warm extraction solution (60 °C) was added to the sample and mixed gently. An equal volume of ice cold phenol was added and left 10min. Then, 1ml of phenol / chloroform was added and mixed gently. The samples were centrifuged at 13000 rpm, for 10 min, in a bench top centrifuge (Rotina 46, Tuttlingen, Germany). The clear upper layer was

carefully removed to a new tube and 0.54 of the total volume of the ice-cold iso-propanol was added to precipitate the DNA. The samples were left for 10 min and spun at 4000 rpm for 10 min. The supernatant was removed leaving the pellet on the bottom of the tube. The pellet was washed by adding 1ml 70% ethanol and centrifuged. The supernatant was removed. The samples were dried, with the top off for 10mins. Then, the pellet re-dissolved in molecular grade water and stored at 4 °C.

2.12.5 Spectrophotometric assay

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 has about 50-220 ng/ µl. Protein purity (A260/A280) and Humic acid purity (A260/ A230) >1.7 are good.

2.13 PCR (polymerase chain reaction)

2.13.1 PCR for pure bacterial colony culture

The PCR was performed with the forward primer 27F (specific for Bacteria) (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse oligonucleotide primer 1492R (5'-GGTTACCTTGTTACGACTT-3'), at 94°C for 12 min (for initial denaturation) followed by 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1.5 min; followed by a final extension period of 12 min at 72°C (Merrifield, 2009).

2.13.2 PCR for DGGE (denaturing gradient gel electrophoresis)

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') (Merrifield, 2009) These primers correspond to position 341 – 534 in the 16S rRNA of *E. coli* which produces a fragment of 193 bp. Each PCR tube contained 1µl of primer P2 and P3 (50 pmol/µl, MWG-Biotech AG, Germany), 1 µl DNA template, 12.5 µl of Ready Mix Taq DNA polymerase and were made up 25 µl with 9.5 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.

2.13.3 Agarose gel electrophoresis

Seven µl of the PCR products were then separated by electrophoresis on a 1% Agarose gel (Lonza, Rockland ME, USA). A mixture of 0.70 g of agarose powder and 70ml of TEA buffer (Tris/ EDTA/Acid) was dissolved in microwave for 1 min with mixing. Seven µl of PCR product was loaded in the wells of the gel. Seven µl of the 100bp DNA ladder (Bioline, 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).

2.14 Denaturing gradient gel electrophoresis (DGGE)

The DGGE was made using a DGGE-2001 system (CBS scientific, USA). Fifteen µl of PCR products were run on acrylamide gels (16 cmX16cmX1mm) 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 (20 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 60ºC in 1 x 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. Briefly, the gel was soaked in fixation buffer (200ml dH2O containing 20 µl gold CYBER safe DNA stain) and scanned in a Bio-Rad Gel-Doc and optimized for analysis of UV light. All the samples were triplicates per treatment. The bands were cut aseptically under the UV light and kept overnight in Eppendorf tubes with 50 µl DNA grade water.

2.15 PCR for Ribosomal inter-space area (PCR-RISA)

The DNA of the bacteria was amplified using PCR specified for RISA that involves a region of the rRNA gene between small 16S and large 23S subunits. The 2 primers were used were: 1406F (TGAACACACCGCCCGT) and 23R (GGGTTBCCCCATTCRG) bacterial 23S rRNA (Merrifield, 2009) obtained from MWG op-

eron, Eurofins, Germany. The PCR kit reagents were used (QIAGEN Ltd., West Sussex, UK). A mixture of 1µl of each bacterial DNA extraction, 12.5 Taq polymerase, 1µl of each primer (100 pmol), and water completed to 25µl, the mixture was prepared in an ultraviolet cabinet (PCR workstation Labcaire). Samples were amplified in a PCR thermacycler (TECHNE, Model TC-312-UK). The PCR program was as follows: at 94ºC for 5 min as denaturing then one loop with 35 cycles at 94ºC for 30 s, 52ºC for 30 s, 72ºC for 1 min plus an extension of 7 min at 72ºC (Borneman & Triplett, 1997).

2.16 Poly Acrylamide gel electrophoresis RISA (PAGE- RISA)

This method was used with 3.4 ml of 5% Acrylamide, 16 ml distilled water, 400 µl X50 TAE, 100 µl Ammonium per-sulphate (APS), 20 µl dyes and 20 µl of TEMED. The mixture was poured slowly into vertical Acrylamide gel electrophoresis apparatus (Small Mighty II, USA). The comb was put on the top of the gel. The gel was left for 30 min to set. 15 µl of the PCR-RISA samples and a DNA marker ladder were added to the wells and left for 45 min at 80 mAmp. Subsequently, the gel was stained by 6 µl cyber gold stain (Invitrogen, UK) with 60 ml of distilled water in a dark place with shaking. The image was visualized by Doc-Gel device (Bio Rad, Japan) and analysed using Quantity One software and PRIMER v.6 software.

2.17 Preparing samples of DGGE bands for sequencing

Three µl of diffusion DNA in molecular grade water overnight added to 12.5 µl of Ready Mix Taq polymerase with 7.5 µl of molecular water, 1 µl of primer 2, 1 µl of primer 1 which have no GC clamp and completed with 3 µl of re-PCR products. The mixture runs using the same program of PCR-DGGE. The PCR products were cleaned using Bioline Biotech company sure clean purification kit according to manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260 nm (Thermo scientific Nano Drop 100, DE, USA), Only 30 µl of diluted to 20-30 ng/µl was sent for sequencing centre of GATC biotechnology in London and the sequencing results send via their website: http://www.gatc-biotech.com/en/index.html. Sequences were 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 their approximate phylogenetic relationships.

2.18 Histology

2.18.1 Scanning Electron Microscope (SEM)

The intestine samples (5mm) length were fixed in 2.5% Glutaraldehyde. The samples were washed with buffer (0.1 M sodium cacodylate) two times for 15 minutes each. Dehydration was achieved by placing the samples in graded ethanol solutions (30%, 50%, 70%, 90% and 2 times in 100%) were used for 15 min each. Samples were transferred to a critical point temperature (CPT) drier (EMITECH

K850, Ashford, Kent, UK) to remove all ethanol. Dried samples were mounted on aluminium stubs and coated with gold using a Gold sputter coater machine (EMITECH K850, KENT, UK). Samples were then screened using an electron microscope (JEOUL 5600LV, Tokyo, Japan) and all the pictures were documented. SEM images were taken at several magnifications for general structural investigation of the intestine and the height of the villi and investigations of microbial communities.

2.18.2 Light microscopy

Intestine samples (1 cm) length was cleaned with PBS and then fixed in 4% formaldehyde until used. The dissected samples were dehydrated by immersion in a tissue processor (Leica, Germany) and treated with methanol 50%, 70%, 90%, 100% and 100% then embedded in paraffin wax. The samples placed in wax small blocks (5 X 3 X 3 cm) to prepare for cutting using a microtome (Leica, Germany). Sections five µm thick were cut and placed in water at 50 ºC for two minutes. The samples were placed on slides. Slides containing paraffin sections were placed in a slide holder and put it in an autostainer (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 stained slides were mounted and examined under light microscope. The aims of histology and SEM were to measure the villus height of all treatment groups and the composition layers of the intestine as well as ability to find the bacterial colonization in the intestine as developed method of bacterial detection in its host.

2.19 Haematology

Blood samples were taken from three chicks per treatments from wing vein or directly from the heart after killing the chicks. One ml syringe and 23 gauge needles was used and the blood collected in anticoagulant tubes with ethylene diamine tetra acetic acid (EDTA). Differential leukocyte counts of lymphocytes, heterophils, basophils, eosinophil and monocytes were counted by smearing 5 µl on a microscopic slide. The slides were allowed to air dry and then fixed in 95% methanol, stained with 5% Giemsa stain (BDH, UK) for 10 min and mounted in DPX. Counts were calculated directly and digital images were taken for documentation using a DCM 130 digital camera (Brunel microscopes Ltd, UK).

2.20 Fluorescent *in situ* hybridisation (FISH) technique

2.20.1 Fluorescent *in situ* hybridisation (FISH) of tissue sections

Intestine samples (10 mm) length were fixed in freshly prepared 4% paraformaldehyde for 24 h and was replaced by PBS and stored until used. One ml of hybridisation solution containing 180 µl of 0.9 mM NaCl, 20 µl of 1mM Tris-HCl pH 8.0, 1 µl of 10% SDS, and 799 µl of ddH2O) and formamide if required. Fifty ml of washing buffer (1 ml 1MTris pH 8.0, 9 ml 5 M NaCl to 50 ml ddH2O). The specific fluorescent probe for LAB and *Salmonella* Typhimurium were designed with aid of the Primer3primer tool website http://biotools.umassmed.edu/bioapps/primer3_www.cgi and ordered from Eurofin MWG, Germany http://www.eurofinsdna.com/home.html as required. The probe

designed for LAB contained 20 nucleotides (5’ GGA ACT CCATGT GTA GCG GT 3’) marked with FITC fluorescent dye and for *Salmonella* Typhimurium was 18 nucleotides (5’ AAT CAC TTC ACC TAC GTG 3’) marked with HEX fluorescent dye. The samples were dehydrated and embedded in paraffin wax and sliced to 5 µm thickness using standard procedures as mentioned in histology section 2.11. The Leebers *et al*, (2011) protocol with major modifications was applied in this study for FISH. The samples were placed on positively charged slides (SuperFrost plus, Braunschweig, Germany). De-paraffination was achieved by immersing all slides in histolin or Xylene in Coplin jars (2 x 10 min). Subsequently, all the samples were rehydrated by transfer to a series of aqueous ethanol solutions of 90%, 70%, 50% and 30% then washed in PBS for 5 min. The samples were hybridized by adding 20 µl of hybridisation buffer spotted onto samples and 2 µl of the specific FISH probes added. The hybridisation was carried out overnight at 50 ºC in a humid chamber (50 ml falcon tube with wet tissue paper). After hybridisation each slide was washed with washing buffer containing 5 µg/l DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) for 15 min at 50 ºC to stains the nucleic acids of the tissues. All the slides were rinsed with distilled water and air dried. One drop of fluroshield (sigma) was added. The slides were covered by coverslip. The slides were examined with many filters (Carl Zeiss, Germany) for visualisation of DAPI, FITC and HEX (TRITC), with an Axioplan II epifluorescence microscope with a 100-W mercury lamp. The images were taken using equipped digital camera Nikon DS-QiMc.

2.20.2 Fluorescent *in situ* hybridisation (FISH) of bacterial pure culture

The method for pure culture was different but with the same fluorescent probe, hybridisation buffer and washing buffer. The modified procedure from Waines (2011) which composed of four stages of fixation, dehydration, hybridisation and drying. The broth sample centrifuged and resuspend with PBS then fixed with 4% paraformaldehyde (3:1 fixative: sample) for Gram negative bacteria and with ethanol for Gram positive bacteria. The suspension incubated in the fridge for 3-12 hr then centrifuged for 5 min twice to remove all residual paraformaldehyde. The resulting pellets were resuspended in 1 ml PBS and 1 ml ethanol. About 10-30 µl of suspension was dropped on positive slide and left to dry at room temperature. Dehydration was by dipping slides into ethanol 50%, 70%, 100% for three min each and the samples were dried. Ten µl of hybridisation buffer was mixed with 1 µl of specific probe and applied to sample on the slide. The prepared slide was incubated at 50 ºC for 3 hr in a humid chamber. The hybridisation buffer was washed out using washing buffer. The slides were dipped in double distilled water then dried and mounted by fluroshield (sigma) followed by examination with a Nikon Eclipse 80i epifluorescence microscope with Nikon digital camera DS-Qi1Mc.

2.21 Statistical analysis

All data statistics were carried out using Minitab v.16 statistical software (Minitab, Coventry, UK). Significance was accepted at level of $P< 0.05$. Results are presented as mean ± standard error (SE) unless otherwise indicated. Typically a two

way ANOVA and Fisher LSD were used for normally distributed data. DGGE and RISA results, PRIMER v.6 (Plymouth Routine In Multivariate Ecological Research) software was used and for each gel, non-metric multidimensional scaling (nMDS) analysis was used to represent the relative similarities between the different conditions represented on each gel. 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 groups. The species richness and the microbial diversity in different were determined by using Shannon's diversity index.

CHAPTER 3

CHAPTER 3: LABORATORY WORK FOR SELECTION OF PROBIOTIC & *IN VITRO* EFFECT OF *LACTOBACILLUS PLANTARUM* NCIMB 41607 RIFAMPICIN RESISTANT ON *SALMONELLA* TYPHIMURIUM AND *S.* ENTERITIDIS IN DIGESTIVE SYSTEM OF CHICKEN

3.1 Introduction

In order for a probiotic treatment to be successfully applied *in vivo*, the organism must be able to survive in the gastrointestinal tract (GIT). It is also useful to be able to demonstrate that the organism survives passage through the GIT. The objectives of these initial studies were to confirm and whether a novel naturally occurring rifampicin resistant strain of *Lactobacillus plantarum* can survive in the conditions of the chicken digestive system, inhibit *Salmonella* Typhimurium and *Salmonella* Enteritidis, survive in water, have good viability after lyophilisation and produce good quality FMF.

The aim of this study was to assess the effect of *Lactobacillus plantarum* NCIMB 41607 on Salmonellae in a simulated chicken digestive system. The objectives were to isolate a naturally occurring rifampicin resistant *Lactobacillus plantarum* to use as a marker for subsequent studies. To determine whether the rifampicin resistant isolate can survive in conditions of the GIT, has the potential for reducing pathogens (*Salmonella* Typhimurium and Enteritidis), can ferment feed at 30°C for 24hr to produce 10^9 CFU/g and more than 175 mmol of lactic acid and has the ability to survive in water at different temperatures.

3.2 Materials and Methods

The *in vitro* methods were described in Chapter Two. The selection of rifampicin resistant *Lactobacillus plantarum* was described in Section 2.2, the freeze-drying Section 2.5. Its ability to ferment and survive in water were described in Sections 2.9.2 and 2.6, respectively. The antagonistic activity of *Lactobacillus plantarum* against *Salmonella* was described in Section 2.3. Auto-aggregation and co-aggregation were described in Sections 2.5. Antibiotic sensitivity of *Lactobacillus plantarum* described in Section 2.4. The simulated digestive system of chicken as described in Section 2.4.

3.3 Results

3.3.1 Selection of rifampicin resistant *Lactobacillus plantarum*

The colonies that grew at high the concentration of rifampicin were rifampicin resistant. To confirm the resistance phenotype, the mutant colonies were re-streaked on to MRS-rifampicin plate and incubated for 24h at 37ºC. Single colonies were plated on MRS agar containing no rifampicin. Also from time to time, the bacteria streaked on rifampicin-MRS agar to ensure this phenomenon was still persistent.

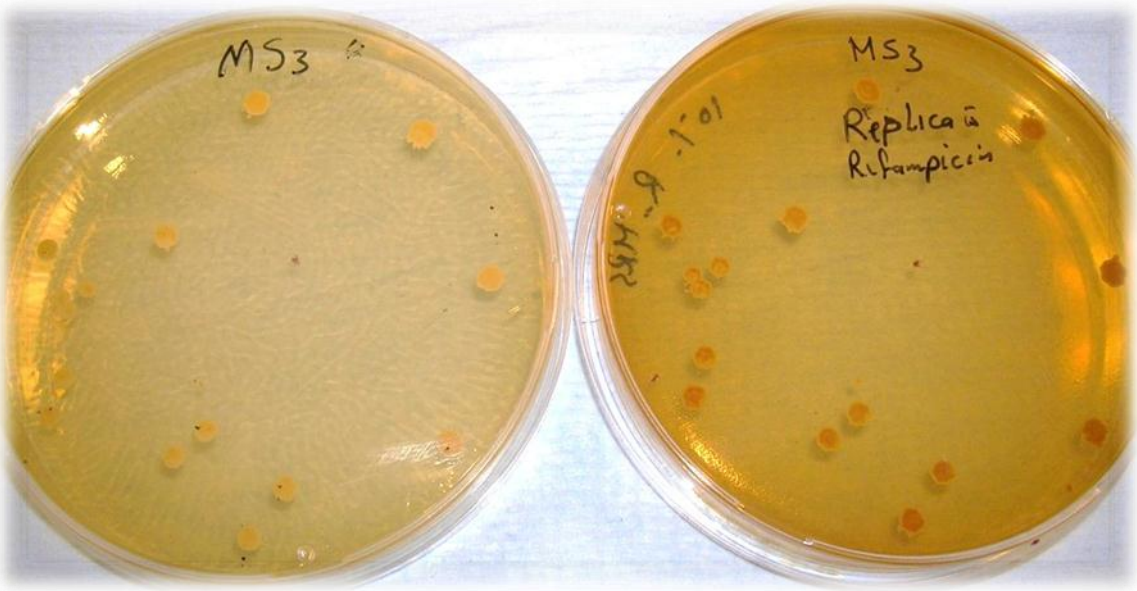

Figure 3. 1. Replica plating of *Lactobacillus plantarum* from MRS agar (left) to rifampicin-MRS agar (right).

3.3.2 Agar well diffusion assay

The inhibition zones of Salmonella produced by *Lb. plantarum* were significantly different compared with the control and the results are presented in Table 3.1 and Figure 3.4.

Table 3. 1. Inhibition zones of both *Salmonella* ($1x10^6$ CFU/ml) by *Lb. plantarum* in an agar well diffusion assay. Mean± SE (mm).

Lactobacillus plantarum **$1x10^8$ CFU/ml, 100µl**	***S.* Typhimurium**	***S.* Enteritidis**
Broth (24hr), pH 3.8	6.3 ±0.03	5.7 ±0. 0
Filtered supernatant broth (24hr), pH 4.3.	5.0 ± 0.03	5.03 ±0.09
Washed *Lb. plantarum* (0 hr.), pH 6.8,	1.8 ±0.06	2 ± 0.09
Neutralised supernatant (24 hr.), pH 6.8.	0.0 ±0.00	0 ± 0.00
Fresh broth (0 hr.), pH6.8.	0.0 ±0.00	0 ± 0. 00

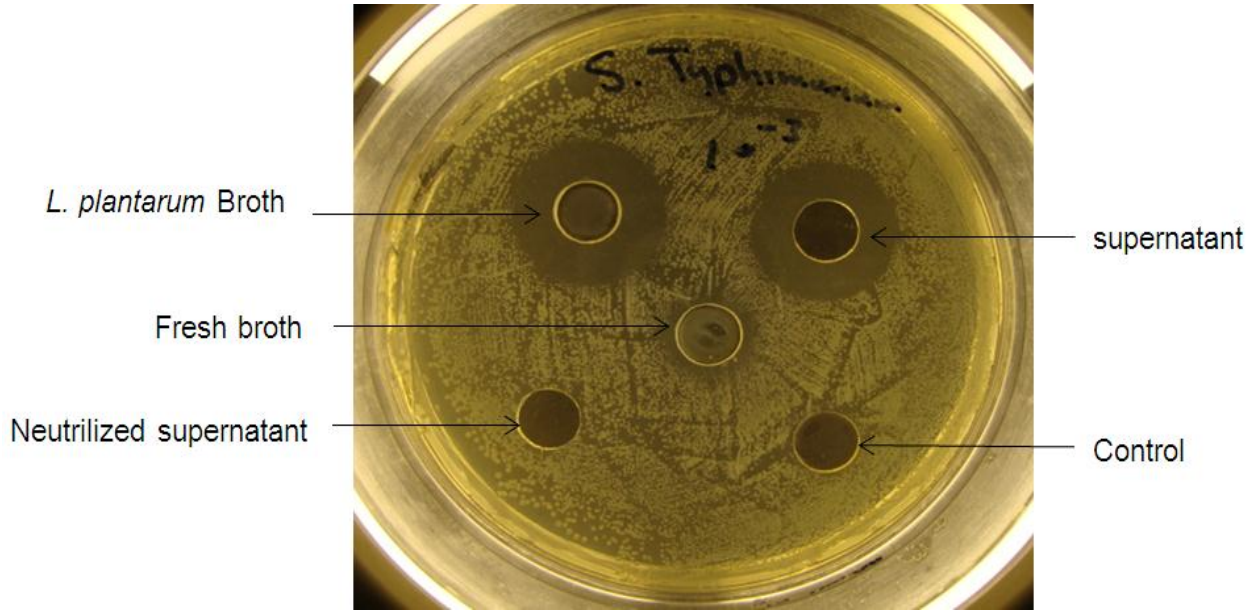

Figure 3. 2. Inhibition zone of *Lactobacillus plantarum* with *Salmonella* Typhimurium.

3.3.3 Auto-aggregation

The results show that *Lb. plantarum* has a greater aggregation than both *Salmonella*. The both *Salmonella* were almost same aggregation ability.

Table 3. 2. Auto-aggregation percentage of *Lactobacillus plantarum* and both *Salmonella* Typhimurium and *Salmonella* Enteritidis after Del Re *et al* method.

Bacteria	**OD in A0 (nm)**	**OD in A4 (nm)**	**Auto-aggregation%**
Lactobacillus plantarum	0.130	0.110	16
Salmonella Typhimurium	0.110	0.080	9.27
Salmonella Enteritidis	0.110	0.084	9.24

3.3.4 Co-aggregation

The result of OD for control tubes *Lactobacillus plantarum* at absorbance 600 nm was 0.130 *Salmonella* Typhimurium 0.113 and *Salmonella* Enteritidis 0.100. At the same time the mixture with *Salmonella* Typhimurium 5 hours later was 0.085 and 0.077 with *S.* Enteritidis. The percentage co-aggregation was calculated according to equation 2 and the results are presented in Table 3.3.

Table 3. 3. Co-aggregation percentage using Handley *et al*. equation (1987).

Auto-aggregation	*Lactobacillus plantarum*	**12%**
	Salmonella Typhimurium	7%
	Salmonella Enteritidis	6%
Co-aggregation	*Lb. plantarum* + *S.* Typhimurium	30%
	Lb. plantarum + *S.* Enteritidis	27%

3.3.5 Aggregation visualised by scanning electron microscope

Scanning electron micrographs of the auto-aggregation and co-aggregation of the mixture of *Lactobacillus plantarum* with *Salmonella* Typhimurium are shown in Figures 3.5 and 3.6.

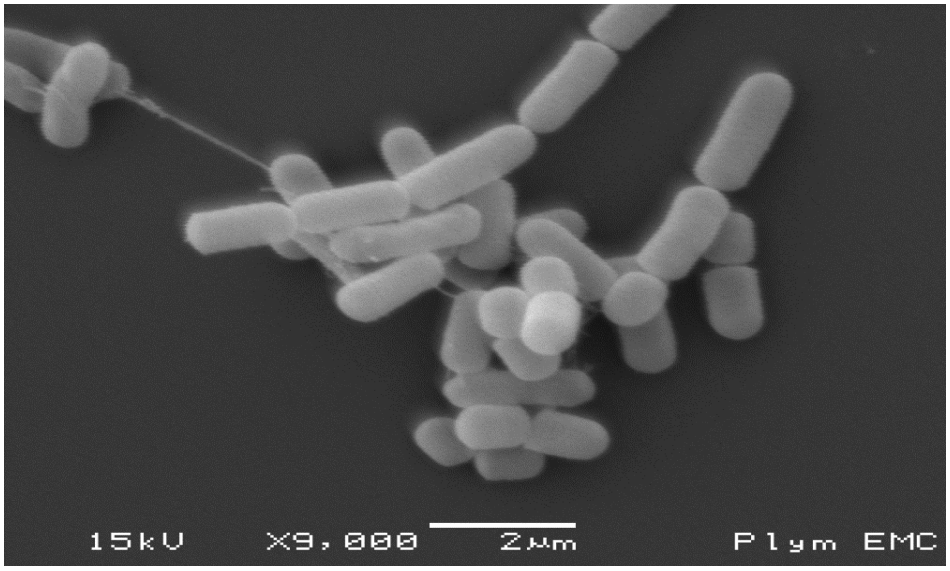

Figure 3. 4. Auto-aggregation of *Lb. plantarum* showing the aggregation using SEM.

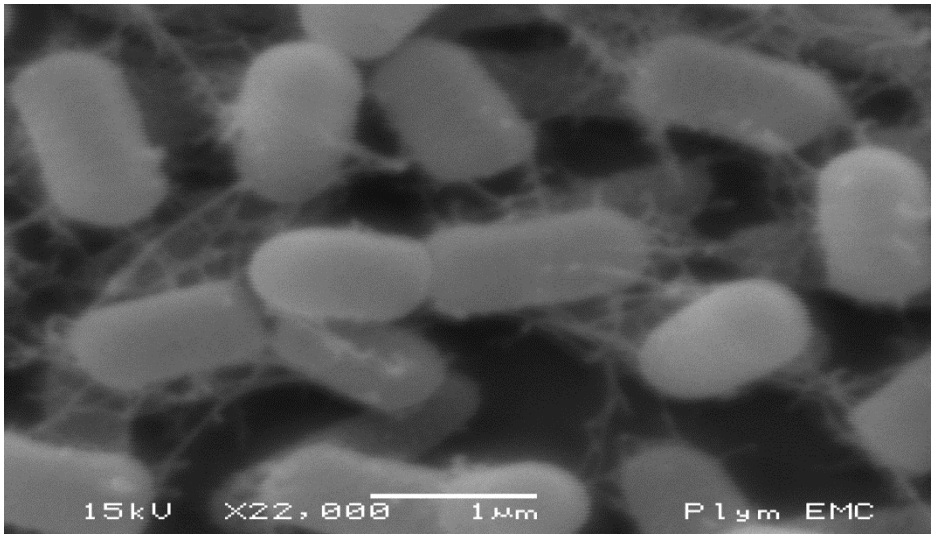

Figure 3. 5. Auto-aggregation of *Lb. plantarum* showing the protein strand of aggregation.

3.3.6 Freeze dried *Lactobacillus plantarum*

In this study the viability of the freeze dried culture was very good, with a yield of 10^{12} CFU/g of freeze dried material. Three grams of freeze dried bacteria were produced from one litre of overnight broth of *Lb. plantarum* culture. The viability results of freeze-dried bacteria kept in water in the fridge were very high surviving percentage.

3.3.7 Antibiotic sensitivity test

The results showed many types of antibiotic can effects on growth of *Lactobacillus plantarum* and *Salmonellas*. The higher dose of antibiotic will effect on both normal flora bacteria and pathogenic bacteria. May be that one of the reasons for limitation using of antibiotics as feed additive

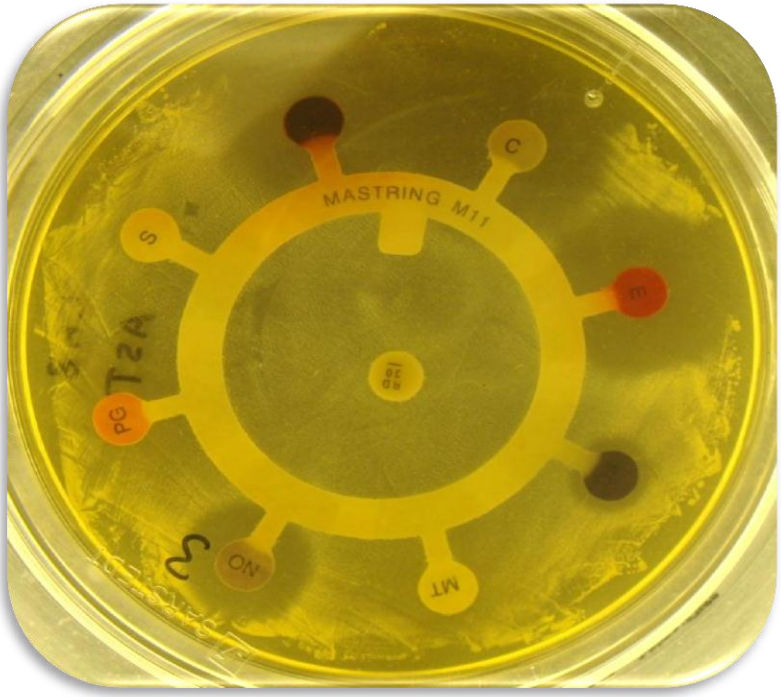

Figure 3. 6**.** Effect of antibiotics on *the Lactobacillus plantarum using MASTRING M11.*

Table3. 4. Mean inhibition zones (mm) of *Lactobacillus plantarum using many types of antibiotics. R= resistance, S= sensitive*

Antibiotics		*Lactobacillus plantarum*
Chloramphenicol	25 µg	S
Gentamycin	10 µg	S
Tetracycline	10µg	S
Ampicillin	10 µg	S
Erythromycin	5µg	S
Clindamycin	2 µg	R
Novobiocine	5 µg	S
Rifampicin	30 µg	R
Penicillin G	1 unit	R
Trimethoprim	1.25	R
Streptomycin	10 µg	R

3.3.8 Survival of *Lactobacillus plantarum* in water

The initial count of *Lactobacillus plantarum* added to drinking water was $1x10^8$ CFU/ml. This study showed that the tap water has no significant effect on the number of *Lb. plantarum* 24 later at temperature 4, 15, 20, 30, 37°C but not 60°C, when no *Lb. plantarum* survived Table 2.7. The mineral content of water did not affect the survival of LAB (Azhar, 2005). Suggesting that water type does not significantly affect the number of live lactic acid bacteria. LAB preferably grow under moderate temperatures (Adams & Moss, 2003).

Table 3. 5. *Lactobacillus plantarum* survival numbers after 24 hr in water of different temperatures (ºC). (CFU / ml), mean± SE.

Time (hr)	Temperature (ºC)					
	4	15	20	30	37	60
0	8.0	8.0	8.0	8.0	8.0	8.0
24	7.79 ±0.06	7.85 ±0.03	7.7 ±0.03	7.42 ±0.11	7.36 ±0.18	0

The results showed no significant difference between all the groups but extremely significant difference ($P<0.001$) between water temperature 60ºC & other treatments.

3.3.9 Fermentation ability of *Lactobacillus plantarum*

Lactobacillus plantarum produced FMF contained 10^9 CFU/g of *Lb. plantarum* in chicken feed. The pH of the FMF after 24 hr of fermentation at 30ºC was 4.5. Lactic and acetic acid production were 175 mmol/L and 5 mmol/L respectively High numbers of *Lactobacilli* (>10^9 CFU/ml), a high concentration of lactic acid (>150 mM) and a low pH (<4.5) in the fermented feed could make chickens less susceptible to *Salmonella* infections (Heres *et al.*, 2003a).

3.3.10 FMF and *Lactobacillus plantarum* in drinking water effect on reduction of *Salmonella* Typhimurium and *Salmonella* Enteritidis in simulated digestive system of bird

In FMF, *Salmonella* Typhimurium and *Salmonella* Enteritidis, (45min), were reduced from 10^7 CFU/ml to 10^6 CFU/ml at the end of stage 1. At the end of stage 2, no more *Salmonella* detected (less than 10^2 CFU/ml).

Lactobacillus plantarum survived in stage1 and even increased in number after 45 min. At the end of stage 2, (135 min) *Lactobacillus plantarum* was reduced from 10^9 CFU/ml to 10^7 CFU/ml and further reduced to by the end of the experiment (Tables 3.6 and 3.7).

In DW at the end of stage 1, (45 min), there was little reduction for *Salmonella* Typhimurium from log 7.4 CFU/ml to log10 7.2 CFU/ml but at the end of stage 2, 135min, the *Salmonella* Typhimurium was reduced to log10 4.0 CFU/ml.

For *Salmonella* Enteritidis in drinking water (DW) there was a greater reduction in min 45, end of stage 1, than *Salmonella* Typhimurium, from Log 7.0 CFU/ml to Log 5.9 CFU/ml. At the end of stage 2, 135 min, no more *Salmonella* was detected.

Table 3.6. Numbers (Log_{10} CFU/ml) of *Lactobacillus plantarum* and *Salmonella* Enteritidis in FMF and drinking water in chicken simulated digestive system process. Time 1, 45, 135 and 285 min correspond to ingestion time resident in crop, proventiculus and small intestine respectively.

Time	**CON1**	**CON2**	**FMF**		**Drinking water**	
min	*Salmonella* Enteritidis	*Lb. plantarum*	*Salmonella* Enteritidis	*Lb. plantarum*	*Salmonella* Enteritidis	*Lb. plantarum*
1	7.5±0.15 ^{a1}	8.0±0.16 a1	7.3±0.26 a1	8.2±0.32 a1	7.0±0.00 ^{a2}	8.02±0.07 a1
45	7.5±0.03 ab1	8.1±0.10 ab1	6.0±0.13 a2	8.6±0.06 ab2	5.9±0.00 ^{a2}	8.1±0.10 ab1
135	5.2±0.20 ^{b}	6.9±0.42 b1	N.D	7±0.14 ^{b1}	N.D	6.9±0.12 b1
285	6.5±0.09 ^{b}	6.7±0.15 b1	N.D	6.7±0.00 b1	N.D	6.7±0.07 b1

(N.D. Not detected level less than Log_{10} 2.0); $^{a, b}$ data with the same superscripts with the same column are not significantly different (P>0.05); $^{1, 2}$ data with the same superscript for the same organism in the same row are not significantly different (P>0.05).

Salmonella Typhimurium survived in higher numbers than *Salmonella* Enteritidis.

Table 3. 7. Numbers (Log_{10} CFU/ml) of *Lactobacillus plantarum* and *Salmonella* Typhimurium in FMF and drinking water in chicken simulated digestive system process. Time 1, 45, 135 and 285 min correspond to ingestion time resident in crop, proventiculus and small intestine respectively.

Time	**CON1**	**CON2**	**FMF**		**Drinking water**	
min	*S.* Typhimurium	*Lb. plantarum*	*S.* Typhimurium	*Lb. plantarum*	*S.* Typhimurium	*Lb. plantarum*
1	7.4 ±0.13 ^{a1}	7.8 ±0.06^{a}	7.0 ±0.02	9.0 ±0.0 $_{1a}$	7.4 ± 0.17 ^{a1}	8.1 ±0.17 $_{a}$
45	7.3 ±0. 02 ^{a1}	8.1 ±0.10^{2}	5.7 ±0.09	8.9 ±0.24 $_{a}$	7.2 ±0.17 ^{a1}	8.0 ±0.27 $_{ab2}$
135	4.4 ±0.48^{1}	7.6 ±0.52 $_{a2}$	ND	7.4 ±0.45 $_{b2}$	4.1 ±0.27^{1}	7.6 ±0.29 $_{b2}$
285	6.1 ±0.10^{1}	7.7 ±0.01 $_{a2}$	ND	7.5 ±0.26 $_{b2}$	6.0 ±0.36^{1}	7.9 ±0.24 $_{ab}$

(N.D. Not detected level less than Log_{10} 2.0); $^{a, b}$ data with the same superscripts with the same column are not significantly different (P>0.05); $^{1, 2}$ data with the same superscript for the same organism in the same row are not significantly different (P>0.05).

The control treatment showed the proventiculus and ventriculus (stage2) had a greater effect on both salmonellas than the small intestine part where both Salmonellas increased in number.

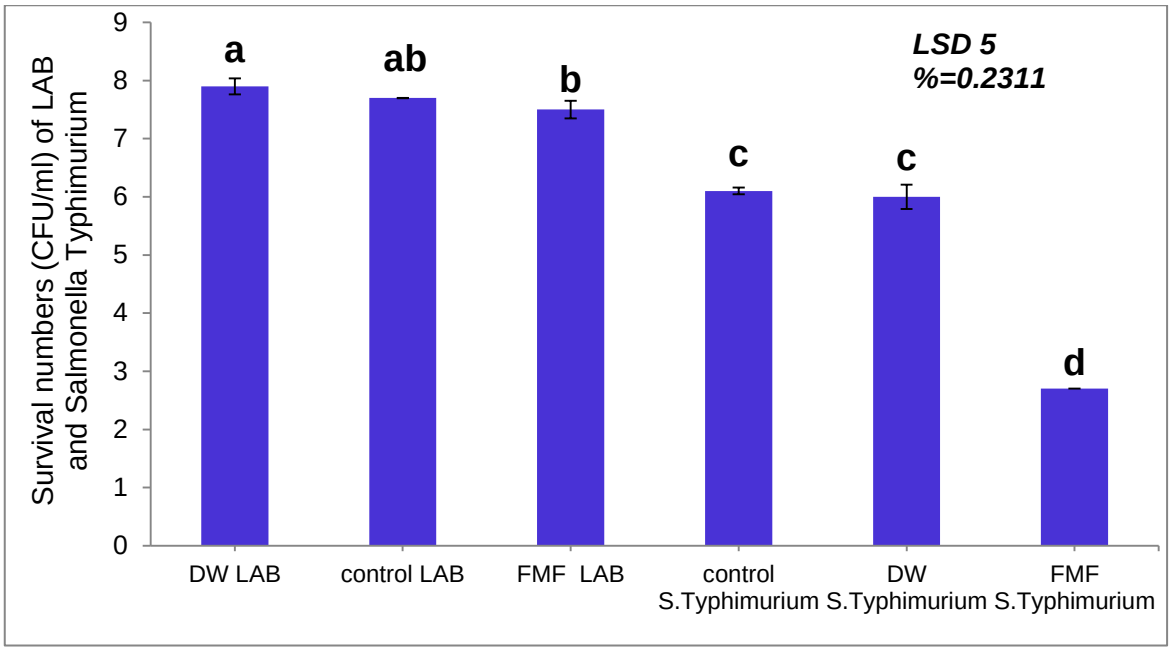

Figure 3. 8. Total survival numbers (Log CFU/ml) of LAB and *Salmonella* Typhimurium in in the simulated digestive system of chicken after 285 minutes.

On the other hand, the survival of LAB was very high significant (P<0.001) while the *S.* Enteritidis were highly significant reduced in FMF and DW groups compared to control group.

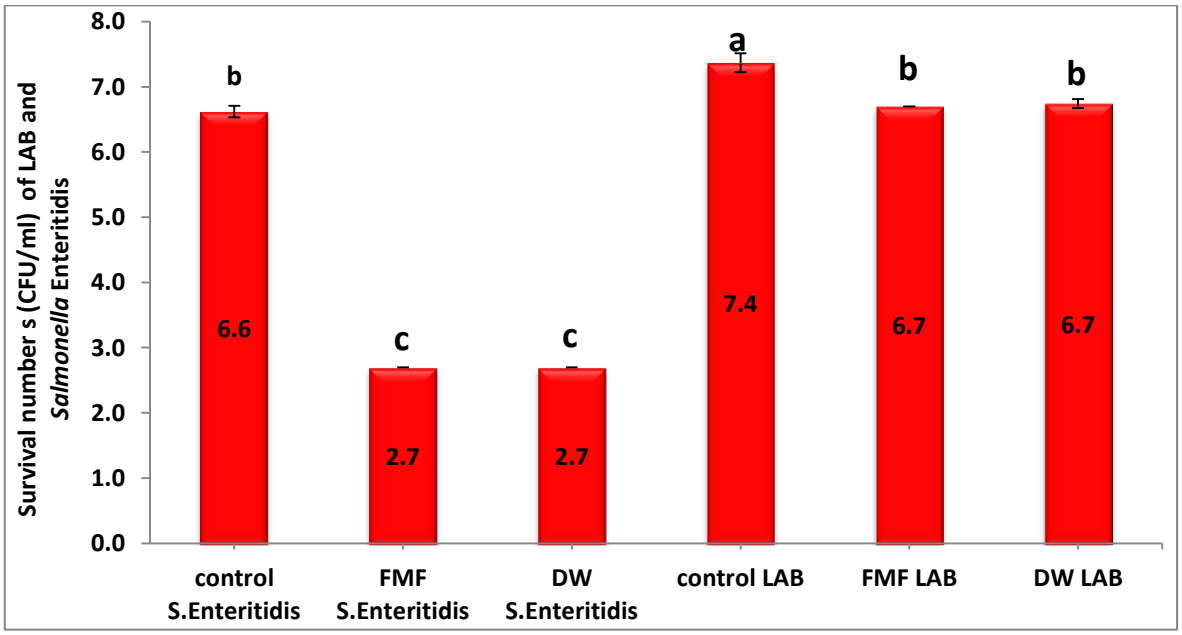

Figure 3. 9. Survival numbers (Log CFU/ml) of LAB and *S.* Enteritidis in the simulated digestive system of chicken after 285 minutes.

3.3.11 Replica plating

The survival rifampicin resistant phenotype was confirmed by plating on rifampicin MRS agar.

3.4 Discussion

These studies demonstrate that *Lactobacillus plantarum* (NCIMB 41607) has probiotic properties, interacts with both Salmonellas and survives the conditions in the gastrointestinal tract of the chicken. Many types of antibiotics have effects on normal gut microflora including *Lactobacillus plantarum* and this is one of the reasons to limit the use of antibiotics in the broiler industry. *Lactobacillus plantarum* showed resistance to the many types of antibiotics that used widely in veterinary medicine such as penicillin, streptomycin, trimethoprim, Sulphathiaxazole and methicillin. However, antibiotics such as chloramphenicol, tetracycline, ampicillin and erythromycin, which used widely in animal treatments, inhibit *Lactobacillus plantarum* and this is may be one of the reasons to use alternatives such as probiotics instead of antibiotics. Aggregation is an important phenomenon to select any bacterial organism as a probiotic because aggregation ability means the bacteria may be able to colonize the gut and it is related to cell adherence properties (Del Re *et al.*, 2000; Vandevoorde, Christiaens & Verstraete, 1992). Therefore, *Lb. plantarum* was examined for its ability to auto-aggregate and co-aggregate with both *Salmonella* which is suggested as a defence mechanism against pathogens (Spencer & Chesson, 1994). Broth-grown *Lb. plantarum* were additionally examined for auto- aggregation ability resuspended in their culture supernatant fluid. *Lactobacillus plantarum* showed a strong aggregating phenotype that was not lost

after washing and suspending of the cells in PBS.

Agar diffusion assay and agar spot method results showed that the LAB products and cell-free culture supernatants from LAB inhibits *Salmonella* Typhimurium while after neutralization the culture supernatants has no effect on *Salmonella*. This suggests that the production of organic acids from glucose fermentation lower the culture pH and inhibit *Salmonella*. This observation was confirmed by analysis of the LAB products by HLPC and the principal products were lactic and acetic acid.

Administration of probiotics in the drinking water is the most useful and easily accepted practice for producers. *Lactobacillus plantarum* found to survive in water after 24h over a range of temperatures. When providing poultry with water in commercial poultry units, the water stays in the delivery system for several hours. During this time the Lactobacilli are exposed to a variety of environmental conditions, temperature, the chemistry of the water, the nature of the pipework and the resident microflora present in biofilms on the surface of the delivery systems.

Lactobacillus plantarum produced fermented feed with low pH (<4.5) that contains about 175 mmol/L lactic acid and $1x10^9$ CFU/g during a 24h fermentation at 30°C. In pigs FMF proved to be a good alternative to AGPs (Beal, 2010; Brooks, 2008). Pigs will readily accept liquid feed with dry matter concentrations as low as 149 g/kg (Brooks *et al*., 2001). However, for chickens the dry matter content needs to be raised to around 450 g/kg for the feed to be acceptable (Heres *et al*., 2003b). Beal and her team in 2005 allowed wheat and barley to ferment spontaneously for 24 hr at 30ºC with no starter culture. They found the mean concentration of lactic acid was 60 mmol/L, acetic acid 23 mmol/L, butyric acid 17 mmol/L and ethanol 15 mmol/L. After fermentation for 24 h only 9 of 300 fermentations produced more than 75 mM lactic acid, which is the level considered to be bactericidal against

Salmonella in liquid pig feed (Beal *et al.*, 2002b). This suggested that spontaneous fermentation cannot be depended upon to produce sufficient SCFAs to prevent the proliferation of enteropathogens (Beal *et al*., 2002b).

In freeze-drying the frozen water is removed by sublimation, therefore reducing damage to biological structures. However, the level of cell viability after freeze drying varies according to numerous factors including the strain of microorganisms and also the efficacy of the protective agents used (Jagannath, Raju & Bawa, 2010). During the processing and storage of freeze dried food, oxygen content, high temperature, low pH, water activity and elevated solute concentration may all affect the viability of probiotic organisms (Carvalho *et al*., 2004). Rapid freezing is the best methods to increase the percentage of survival bacteria in Lyophilisation because reduce the mobility of water then decrease the effect of osmotic shock (Mooe, Jeewon & Young, 1999). Water content is an important parameter for the stability of dried cultures. In general, microorganisms survive better at low water activity. But, over drying may reduce the viability and stability of micro-organisms (Z. Manel *et al*., 2009). Lastly, the proof of efficacy of the probiotic bacteria in broilers is required *in vitro* simulated digestive system to prove the efficacy and later *in vivo* studies.

The application of *Lactobacillus plantarum* in DW group in the simulated digestive system of the chicken showed the numbers of both serotypes *Salmonella* were reduced in the acidic parts of the simulated digestive tract but then recovered when pH buffered above pH 6. Addition of *Lb. plantarum* prevented recovery of *Salmonella* Enteritidis but not *Salmonella* Typhimurium, suggesting an inhibitory effect on *Salmonella* Enteritidis. When FMF added *Salmonella* Typhimurium failed to recover numbers during stage 3 and no *Salmonella* Enteritidis was detectable at the end

of stage 2. It is likely that lactic acid present in FMF increased inhibitory effect of *Lactobacillus plantarum*. FMF was the best method to reduce *Salmonella* in a simulated digestive system of poultry. One of the objectives of this study was the detection of *Lactobacillus plantarum* by using replica plating technique on MRS rifampicin agar and the benefit of that is to use rifampicin as a marker for the *Lactobacillus plantarum* in *in vivo* research.

The effect of FMF may be attributed to the lactic acid produced by fermentation (Beal *et al*., 2002a; Brooks, 2008; Makras *et al*., 2006; Niba, 2008; Savvidou, 2009).

CHAPTER 4

Pilot studies

CHAPTER 4: Pilot studies

Pilot study 1

4.1 Introduction

This chapter divided into two pilot studies. The first pilot study dealt with the effects of moist feed fermented by *Lactobacillus plantarum* (FMF) on *Salmonella* Typhimurium and the microbial population of the chicks gut. The objectives were to assess the acceptability of FMF, examine any changes in the microflora of the SPF chicks gut due to feeding FMF and assess the ability of the rifampicin resistant *Lb. plantarum* isolate to survive in the gut conditions and the ability of FMF to reduce *Salmonella* infection.

The second pilot study assessed the effect of *Lactobacillus plantarum* in drinking water or in dry feed on the microbial population of the chicken gut without infection by *Salmonella* Typhimurium. The objectives were to examine the effect of *Lb. plantarum* in drinking water or feed on the chicken gut microbial population. Growth performance was monitored for any adverse effects.

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 methods. 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 total.

The basis of molecular approaches lies in the development of techniques con-

cerned DNA extraction from the samples and the subsequent application of PCR techniques to amplify gene sequences. Thus, facilitating diversity studies by fingerprinting and sequencing by denaturing gradient gel electrophoresis (DGGE), a technique first described by (Muyzer, De Waal & Uitterlinden, 1993) following by cutting the DGGE gel bands and re-PCR without GC clamp and cleaning. The last stage is sequencing followed by taxonomy to determine the bacterial species.

The purpose of the molecular microbial ecology investigations was to identify changes in the bacterial community as influenced by factors of feed type and age.

4.2 Experimental design and treatments

Fourteen SPF chicks were randomly allocated to two treatments of 7 chicks per treatment and each group allocated into 2 pens. The control (CON) group was fed a chick's feed (BOCM Pauls Ltd, Wherstead, UK). The FMF group was fed the same feed fermented by *Lactobacillus plantarum* NCIMB 41607 at 30ºC for 24 hrs, from day one of age. On day 3 of the trial 0.2 ml of 10^5 CFU of *Salmonella* Typhimurium were gavage orally by syringe as described in section 2.10.4. Cloacae swabs were taken from chicks twice on day 5 and 8 of trials to determine the presence of *Salmonella* as described in Section 2.10.5.

On day 14, the ileum and the caeca were aseptically removed and used to detect *Salmonella* and microflora population by classical and molecular microbiology as described in Section 2.10 - 2.12.

4.3 Results

4.3.1 Cloacal swabs

All chicken in both groups were positive for *Salmonella* Typhimurium nal^r on day 5 and 8 of the trial.

4.3.2 Classic microbiology

The numbers of *Salmonella* Typhimurium in the caeca were higher than the Ileum in the control group (7.3 and 4.05 CFU/g, respectively). LAB (detected by MRS agar) but not *Lb. plantarum* (detected by rifampicin MRS agar) were also higher in the caeca than ileum (5.2 and 3.4 CFU/g, respectively). One control chick died on day 6 of the experiment. In the FMF group, no *Salmonella* Typhimurium was detected except for one chick with a low level (10^3 CFU/g). *Lactobacillus plantarum* had survived in the digestive system of chicken. Caeca had higher numbers of rifampicin resistant *Lactobacillus plantarum* than the ileum (9.46 and 8.73 CFU/g, respectively). Feeding FMF fermented with *Lb. plantarum* significantly reduced *Salmonella* Typhimurium in the caeca (P< 0.5).

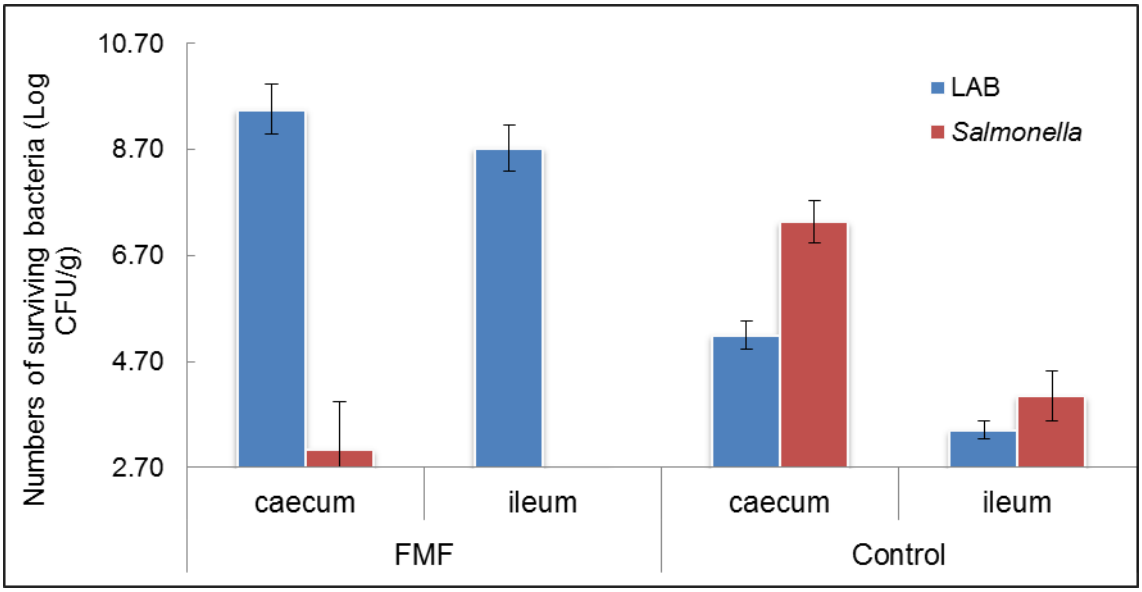

Figure 4. 1. Survival numbers of LAB and *Salmonella* Typhimurium (Log CFU/g) in caeca and ileum of control and treated groups. (1/6; one chick of 6 was positive for *Salmonella*).

4.3.3 Molecular microbiology

4.3.3.1. PCR-RISA

The PCR-RISA (Figure 4.2) results in the presence of gel bands each of which represents an operational taxonomic unit (OTU) in the agarose gel. The numbers of OTU in the gel fingerprints of caeca were higher than ileum in the treated group. The result indicates the effects of FMF on the increase the microbial population in the digestive system of the chicken.

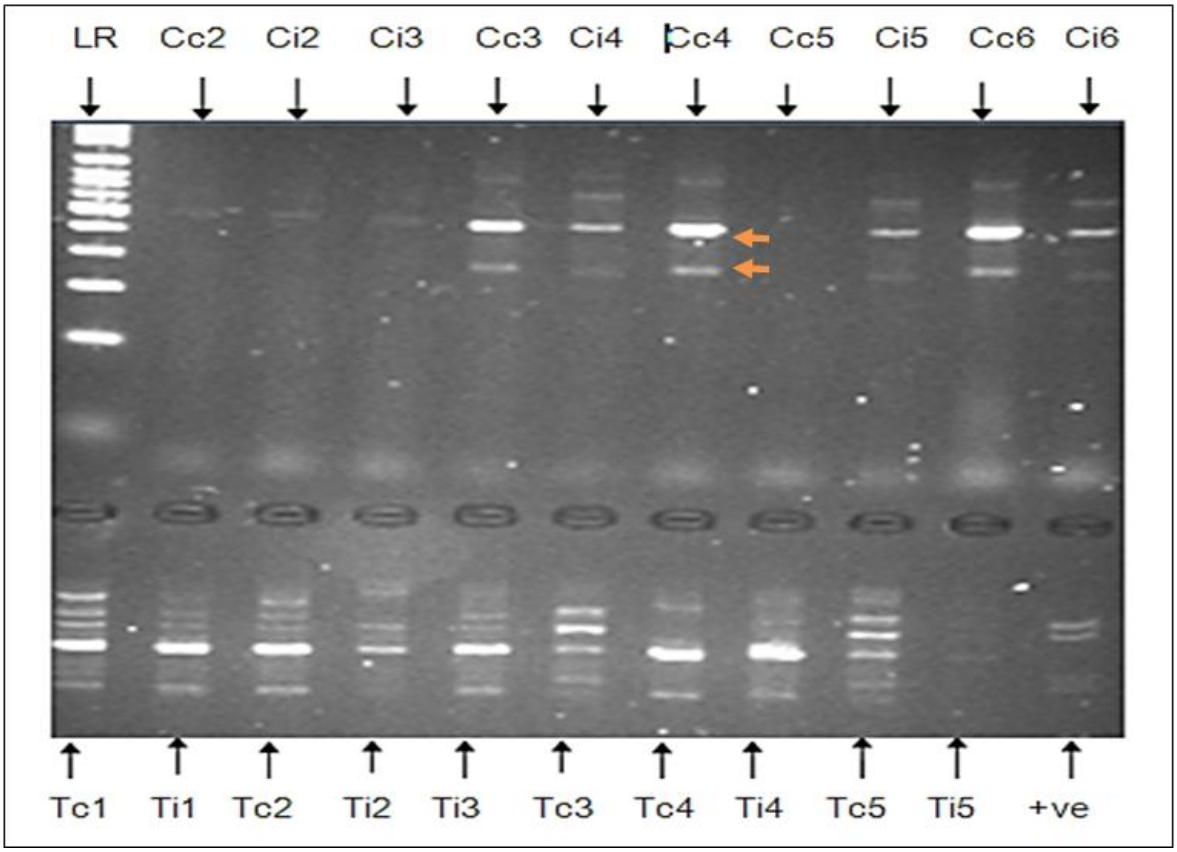

Figure 4. 2. The PCR-RISA negative image on a 2% agarose gel. The gel bands represent bacterial communities' presents in chicks gut samples. (LR; 100 pb DNA ladder, C= control, T= treatment, i= ileum, c= caeca, arrows= gel bands).

4.3.1.2 DGGE of ingesta in ileum and caeca

Many different bands are shown in both caeca and ileum of treated and control chicks in the DGGE image. DGGE image shows the gel bands which are called operative taxonomy units (OTU) in each sample Figure 4.3.

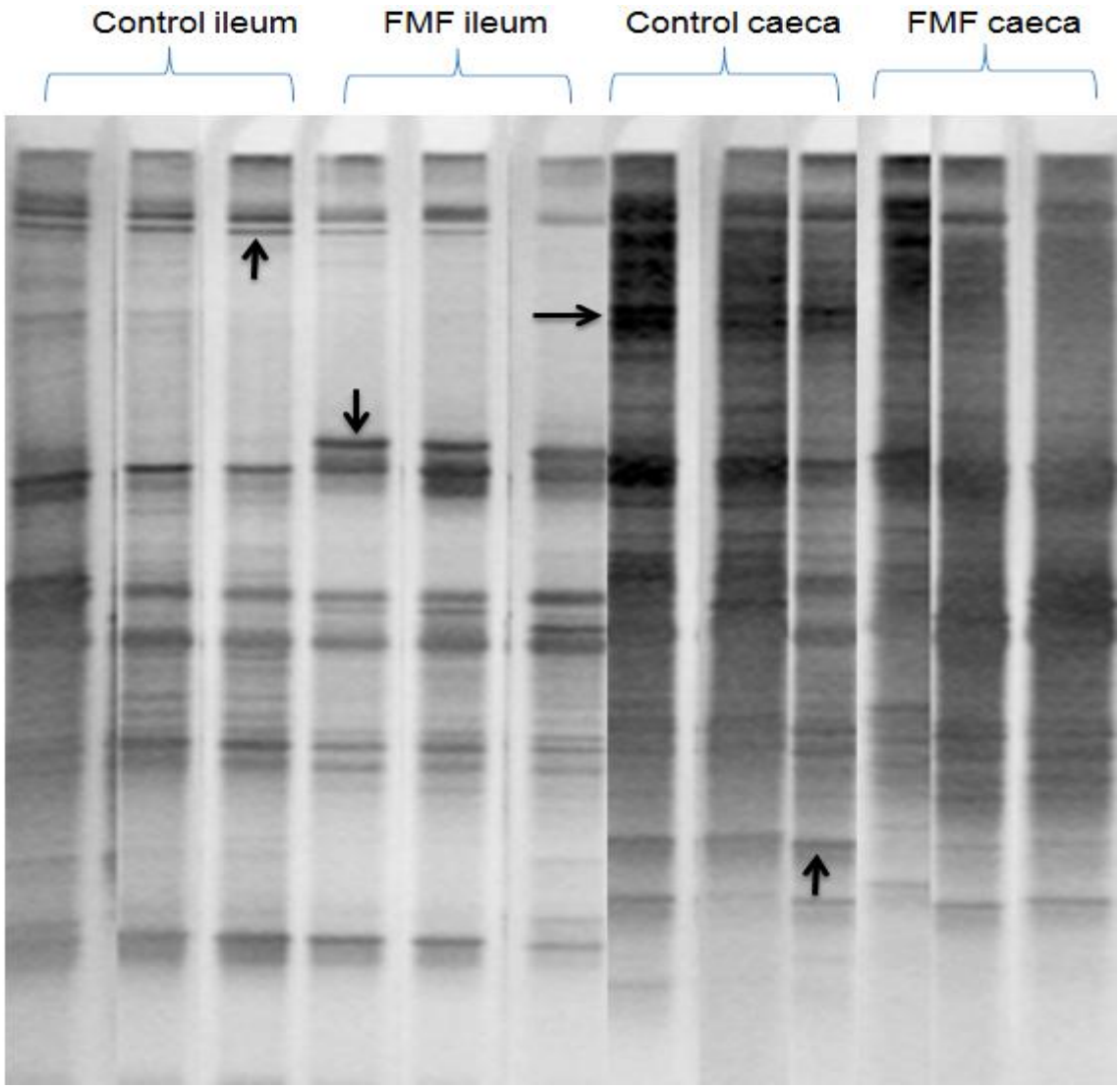

Figure 4. 3. DGGE fingerprints of ingesta in caeca and ileum of treated and control groups chicks 14 days old. Arrows are represents band numbers or operative taxonomy unite (OTU) in each sample which refers to richness in the samples.

The similarity of bacterial population within and between the groups were measured by non-metric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 4.4. The half matrix similarity showed in Table 4.1.

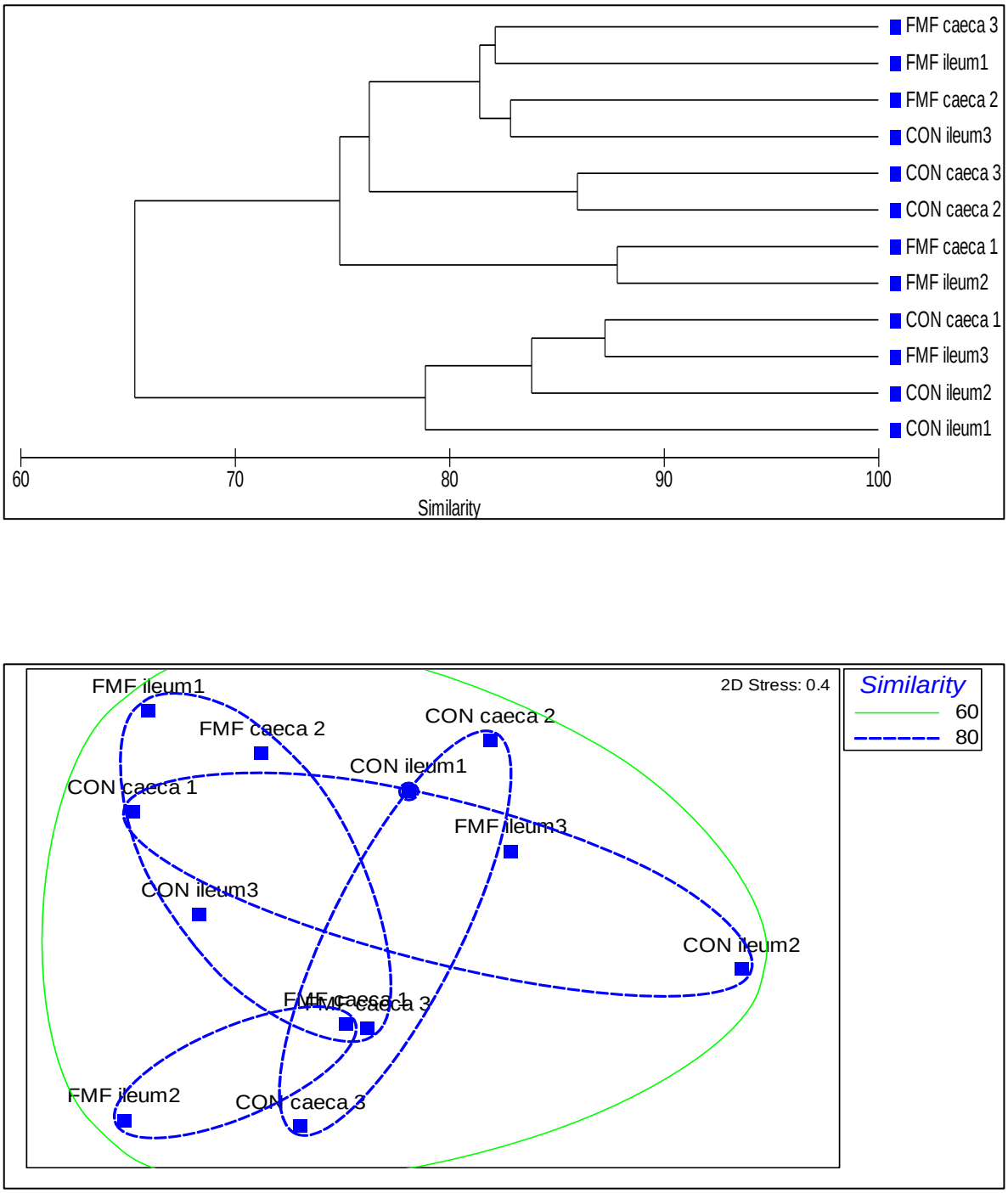

Figure 4. 4. (A) Cluster analysis and (B) non-metric multidimensional scaling (MDS) analysis plot of DGGE fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in poultry caeca and ileum. (CON) = control, (FMF) = fermented moist feed groups. 1-3 denotes replicate number in each sample. (n=12).

Table 4. 1. The half matrix similarity of bacterial population of DGGE fingerprints of caeca (c) and ileum (il) showing the similarities between the replicates group.

GROUP	CON il1	CON il2	CON il3	Trt il1	Trt il2	Trt il3	CON c1	CON c2	CON c3	Trt c1	Trt c2	Trt c3
CON il1	**100**											
CON il2	**73**	**100**										
CON il3	**80**	**63**	**100**									
Trt il1	**79**	**66**	**81**	**100**								
Trt il2	**66**	**48**	**81**	**71**	**100**							
Trt il3	**83**	**86**	**71**	**73**	**56**	**100**						
CON c1	**81**	**82**	**69**	**78**	**55**	**87**	**100**					
CON c2	**64**	**59**	**65**	**73**	**72**	**61**	**64**	**100**				
CON c3	**74**	**65**	**76**	**81**	**76**	**73**	**71**	**86**	**100**			
Trt c1	**64**	**46**	**77**	**69**	**88**	**54**	**53**	**70**	**74**	**100**		
Trt c2	**77**	**61**	**83**	**83**	**76**	**67**	**65**	**75**	**80**	**72**	**100**	
Trt c3	**71**	**60**	**80**	**82**	**83**	**66**	**68**	**77**	**84**	**79**	**82**	**100**

Note: CON = control, Trt = treatment groups. 1-3 denotes replicate number in each sample. (n=12).

This table indicates the analyses of control caeca and ilea bacterial populations' similarity within the sample replicates were 74 and 72% respectively. While the treated group caeca and ilea were 78 and 67%, respectively. The bacterial population similarity between caeca and ilea in the control group was 71% and the treatment group was 74%. These results show no difference in the bacterial species of the same group but the numbers of bacteria settled in the caeca and the ileum is different. Shannon's diversity index was used to display the microbial population richness in the ceca and ilea samples without significantly different between all groups Figure 4.5.

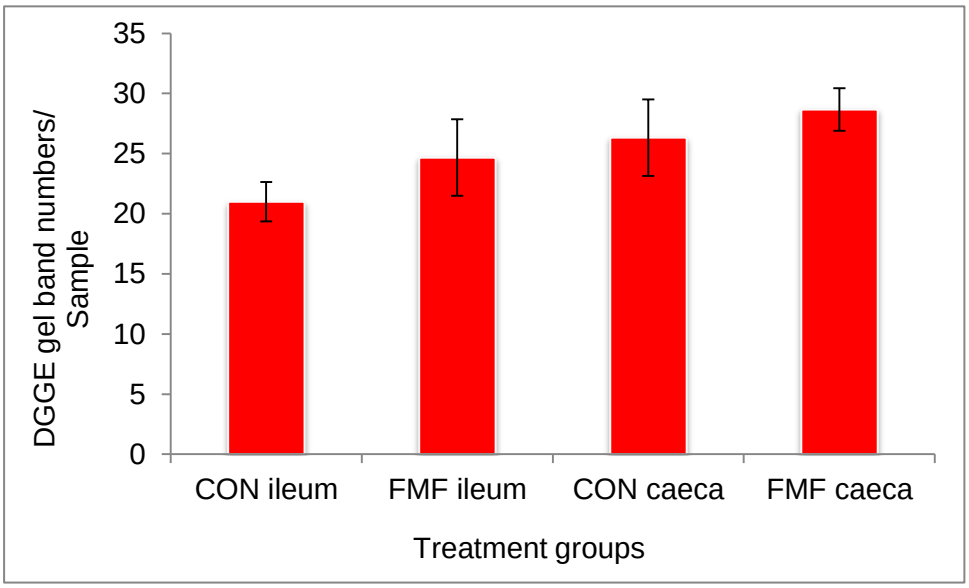

Figure 4. 5. Average band numbers in chicken caeca and ilea in control and treated group samples detected in DGGE fingerprints. (n=11).

4.4 Discussion

Two methods of traditional and molecular technique were used to detect the bacterial populations in the chicks gut. The classical microbiology results of this study suggest that FMF produced by *Lb. plantarum* was effective in reducing of *Salmonella* Typhimurium. The cloacal swabs at day 8 of the trial showed all birds were positive for *Salmonella* but at the end of the trial there were none detected. These results agree with the results of many researchers (Heres *et al.*, 2004; Niba, 2008; Patterson & Burkholder, 2003; Savvidou, 2009) . In this trial FMF fermented by *Lb. plantarum* was used which produced lactic acid and this may decrease the pH value in the intestinal chyme which may inhibit *Salmonella* colonisation by creating a biological barrier (Canibe *et al*., 2005). FMF has been successfully introduced in pig nutrition and now recognized as a powerful tool in the control of *Salmonella* in swine herds as well as preventing weaning diarrhea (Brooks *et al.*, 2001). Heres *et al.* in (2004) claims FMF can protect broiler against *Salmonella* and *Campylobacter*. FMF improves gut health due to acidification of the digestive system and this forms a hostile environment for acid sensitive pathogenic bacteria like *Salmonella* (Engberg *et al*., 2010). Niba in 2008 and Savvidou in (2009) claims that FMF is an effective method to control *Salmonella* Typhimurium infection in poultry.

A molecular technique of RISA revealed that the numbers of the gel bands or operative taxonomy unite (OTU) were higher in the caeca of the treated group than others. From the DGGE image Fig. 4.3, the different bands appear in control and treated groups may be refer to change of the microorganism population of the intestine because may be due to adding probiotic to the treated group. The DGGE results indicate the band numbers in the caeca and ileum of FMF were more than the control group. These results of molecular microbiology support the results of

classical microbiology that shows that the numbers of bacteria in caeca were higher than other organs of chicks gut. In poultry, the caeca are the favoured sites for colonization of bacteria and that may be because the slow movement of food in the caeca compared with other parts of the gut. Adding probiotics to feed had a highly significantly effects (P< 0.5) on caecal bacteria population species richness and this is indicated by increased numbers of bands in DGGE fingerprints. These results indicate that adding *Lactobacillus plantarum* has increased the diversity of microbial population in the gut and thereby decrease the possibility of colonisation by pathogens (Dillon et al., 2005).

4.5 Conclusion

The results indicate the survival of *Lactobacillus plantarum* NCIMB 41607 in the gastrointestinal tract of chickens. This is a very important criterion for a micro-organism to be selected as probiotic. The second conclusions are the success of using a rifampicin resistant isolate of *Lb. plantarum, which* can be detected by the simple replica plating method to demonstrate the survival of the organism. The third conclusions are a significant reduction of *Salmonella* Typhimurium nal^r NCIMB1344 in the digestive system of chicken by feeding moist feed fermented by *Lactobacillus plantarum.* The high acceptability of FMF for the chicken and the lastly FMF treatments has changed the microbial population diversity in the gastro-intestinal tract of chickens. Increasing the bacterial population diversity may lead to a decrease in the ability of pathogens to colonise.

Pilot study 2

The effect of Probiotic (*Lactobacillus plantarum* NCIMB 41607) in feed and drinking water on changing of microbial populations in specific pathogen free (SPF) chicks

4.6 Experimental design and treatments

Twenty one day-one old SPF White Leghorn chicks were randomly allocated to three treatments of 7 per treatment. Each treatment was divided into two randomized pens with six pens. The three treatments comprised a control, water and a feed group (*Lb. plantarum* 10^9 CFU/ml added to the drinking water and the same concentration to the feed). The feed and water were supplied *ad libitum*. Feed and water intake was measured on a daily basis and live weight weekly. Cloacal swabs were taken from the birds on day 7. At day 10, three chicks /pen were killed and the rest are killed at the end of the trial (day 20). Post-mortem 400 mg of chicks gut contents (duodenum, jejunum, Ileum and caeca) were aseptically removed and used for the assessment of gut microflora population changes using standard microbiology (culture methods) and molecular microbiology as described in Section 2.11 and 2.12. Including DNA extractions, polymerase chain reaction (PCR) followed by Agarose gel electrophoresis (AGE), Agarose-Ribosomal inter space analysis (Agarose-RISA) and Poly acrylamide gel electrophoresis-RISA (PAGR-RISA) and denaturant grade gel electrophoresis (DGGE) analysis and lastly gene sequences. Selected bands (OTU) of DGGE gel were aseptically separated and sequenced according to the band represent many groups or a unique band for particular groups and match BLAST at NCBI to confirm the name of the bacteria species.

4.7 Results

4.7.1 Growth performance

Growth performance and FCR are demonstrated in Table (4.2). Chicken weight increased by over 73, 76 and 77% in control, feed and water groups, respectively. The Feed conversion rate (FCR) was calculated by dividing total feed intake on the total weight gain. The FCR of all groups were very high 4.8 - 5.2 may be because this type of chicks is not particularly for meat production. The survival rate for all the group was 100%. The average daily feed intake (ADFi) and water intake were not significantly different between groups. The weight gain and daily weight gain (DWG, g/chick/day) were same for all groups.

Table 4. 2. The growth performance of SPF Leghorn chick's age 20 days.

Growth performance	CONTROL	FMF	WATER	P. value
Initial weight (g)	39 ±1.3 [a]	37 ±1.1 [a]	39 ±2.3 [a]	0.123
Final weight (g)	144 ±6 [a]	161 ±3.2 [a]	162 ±3.1 [a]	0.114
Weight gain (g)	105 ±2.5 [a]	123 ±4.4 [a]	123 ±4.6 [a]	0.067
ADFi (g)	31 ±7.01 [a]	31 ±7.01 [a]	29 ±5.79 [a]	0.991
ADG (g)	6 ±0.37 [b]	6 ±0.27 [a]	6 ±0.2 [a]	0.067
Water intake	44 ±5.05 [a]	42 ±4.90 [a]	43 ±4.78 [a]	0.985
FCR	5.2	5.2	4.8	

(ADFi =average daily feed intake, ADG =average daily gain, FCR =feed conversion rate). [(a, b)] data with the same superscript in the same row are not significantly different (P<0.05).

4.7.2 Cloacal swabbing

On day 7 all the chicks were swabbed and the results are shown in Figure 4.4. The chicks of feed and water groups showed a higher population of LAB than the control group. The results showed an increase in the number of bacteria in the treated groups compared to control group.

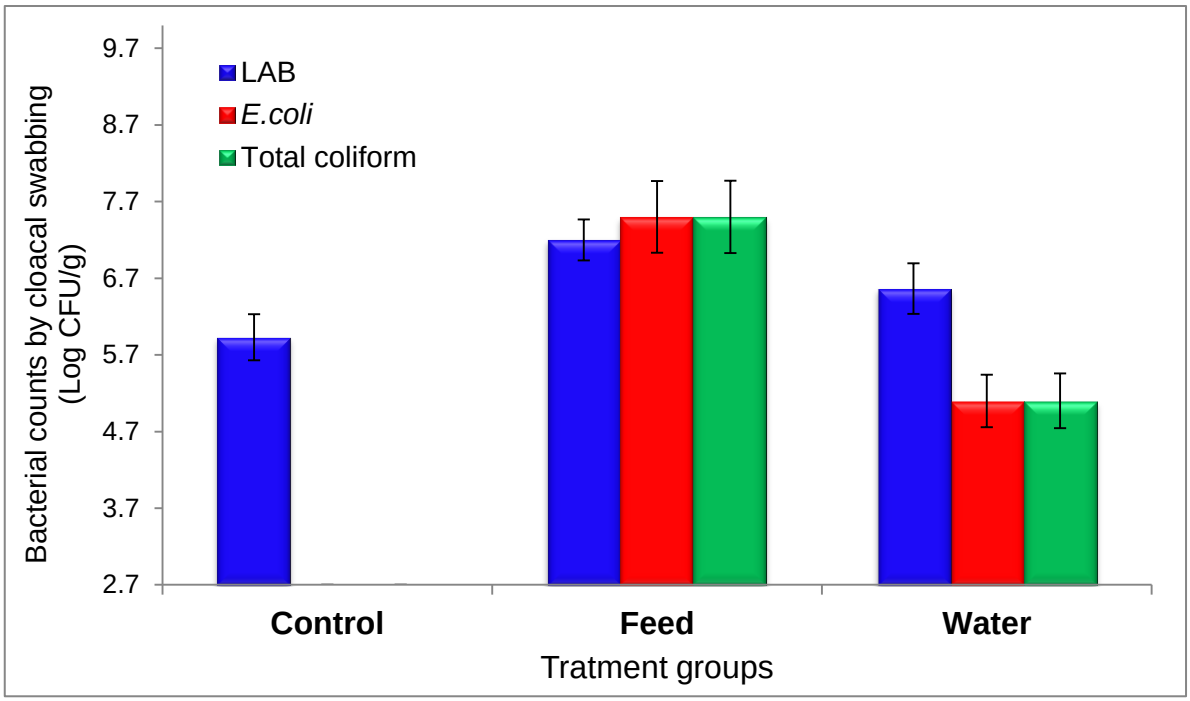

Figure 4. 5. Chicks' bacterial counts of LAB and total coliform in day 7 of age by cloaca swabs.

The result suggests that adding *Lactobacillus plantarum* could be contributing to stabilize gut microflora, which is an important factor of improvement of productive parameter.

4.7.3 Chicks at age 10 days

The results of microbial population on selective media show that the number of LAB in the caeca of control group was significantly higher (P< 0.5) than other organs (ileum, duodenum and jejunum) in the same group as well as higher than total coliform. *E. coli* was not detected in any organs in day 10 of the trial.

The LAB in Ileum of the feed group were significantly (P < 0.5) higher than other organs in the same group. LAB was higher than total Coliforms in the Ileum, jejunum and duodenum but equal in the caeca. LAB in water group was significantly higher (P< 0.5) in the caeca than other organs and significantly higher than total coliform but not in the caeca itself. The LAB were counted in MRS agar and then plated into rifampicin MRS agar to confirm the presence of *Lactobacillus plantarum* that was used in this trial.

The bacteria counted in chicks gut were demonstrated in Table 4.3. In general the numbers of bacteria in the caeca were higher than ileum on day 10 of the trial. The caeca of water group has a higher bacterial count than the others.

Table 4. 3. Bacterial composition of chicken gut isolates from caeca, ileum, jejunum and duodenum at age 10 days. (n=30).

Bacteria counts (Log CFU/g) & ratio	CONTROL				FEED				WATER				P. value between groups				P. value within groups		
	Caeca	Ileum	Jeju	Duod	Caeca	Ileum	Jeju	Duod	Caeca	Ileum	Jeju	Duod	Caeca p. value	Ileum p. value	Jeju P. value	Duod P. value	CON	FEED	WATER
LAB	7.94 ±1.0 a1	4.8 ±2.5 b2	4.53 ±2.3 a2	5.23 ±0.8 a2	7.76 ±0.6 a2	8.59 ±1.1 a1	7.36 ±0.3 a3	7.17 ±0.5 a3	8.91 ± 0.9 a1	5.34 ±0.7 b2	4.77 ±0.4 a3	5.20 ±0.5 a2	0.59	0.27	0.35	0.09	0.56	0.47	0.01
E. coli	ND	ND	ND	ND	8.09 ±1.2 b1	6.48 ±0.5 a1	5.79 ±0.5 a1	5.27 ±0.4 b1	10.53 ±0.1 a1	6.80 ±0.6 b2	6.00 ±0.6 a3	6.04 ±0.5 a2	0.00	0.00	0.00	0.00	NA	0.12	0.01
Enterobacteriacae other than *E. coli*	6.04 ±1.0 a1	6.26 ±0.6 a1	6.68 ±o.5 a1	6.40 ±0.4 a1	7.00 ±0.5 a1	7.19 ±0.4 a1	7.70 ±0.1 a1	7.46 ±1.2 a1	6.53 ±0.8 a1	7.29 ±1.1 a1	7.52 ±0.4 a1	7.51 ±0.8 a1	0.71	0.62	0.23	0.62	0.93	0.92	0.80
Total coliform	6.04 ±1.0 c1	6.26 ±0.6 a1	6.68 ±0.5 a1	6.40 ±0.4 a1	8.59 ±1.0 b1	7.38 ±0.4 a1	7.71 ±0.1 a1	7.58 ±1.1 a1	10.54 ±0.1 a1	8.00 ±0.6 a2	7.70 ±0.2 a3	7.60 ±0.7 a3	0.03	0.15	0.13	0.53	0.93	0.71	0.01
LAB : *E. coli*	1.3	0.8	0.7	0.8	0.9	1.2	1.0	0.9	0.9	0.7	0.6	0.7							
LAB: total coli-form	2.9	1.8	1.7	1.9	1.0	1.3	1.3	0.9	0.9	0.8	0.8	0.9							

(a, b, c, d) data with the same superscript in the same row and same organ are not significantly different (P>0.05). (1, 2, 3) data with same numbers superscript in the same feed group are not significant different. Jeju= jejunum, Duod= duodenum, CON= control, ND= not detected.

4.7.4. Chicks at day 20

The *E. coli* and coliform were not detected in all organs in day 20 of the trial in the control group.

In the Feed group the LAB and coliform in caeca were significantly (P< 0.5) higher than other organs in the same group. LAB were higher than total coliforms in the ileum but not significant. LAB were higher than total Coliforms in all organs of Feed group.

Water group was different, the total coliform were higher than LAB in the caeca and ileum but not in jejunum and duodenum. .

The bacteria counted in all organs were demonstrated in Table 4.4. The numbers of bacteria in caeca were higher than the ileum in all treatments of SPF chicks in day 20 of the trial. The caeca of feed group has higher bacterial counts than others. The LAB on MRS agar was plated onto rifampicin MRS agar to confirm the bacteria of *Lactobacillus plantarum*, which used in this trial.

Table 4.4. Bacterial counts (CFU/ml) and ratio in chicks gut at age 20 days (n=30).

Bacteria counts (Log CFU/g) & ratio	**CONTROL**				**FEED**				**WATER**				**P. value between groups**				**P. value with-in groups**		
	Caeca	**Ileum**	**Jeju**	**Duod**	**Caeca**	**Ileum**	**Jeju**	**Duod**	**Caeca**	**Ileum**	**Jeju**	**Duod**	Caeca p. value	Ileum p. value	Jeju P. value	Duod P. value	CON	FEED	WATER
LAB	5.52 ±0.8 ^{a1}	4.95 ±1.0 ^{b1}	6.22 ±0.4 ^{a1}	5.63 ±0.7 ^{a1}	7.87 ±0.6 ^{a1}	7.14 ±0.4 ^{a1}	5.95 ±0.8 ^{a1}	6.61 ±0.6 ^{a1}	6.56 ±0.2 ^{a1}	6.45 ±0.4 ^{b1}	6.22 ±0.7 ^{a1}	5.39 ±0.7 ^{a2}	.08	.13	.97	.44	.70	.24	.42
E. coli	ND	ND	ND	ND	9.69 ±0.8 ^{a1}	4.93 ±0.4 ^{b2}	4.10 ±0.4 ^{b3}	4.93 ±0.6 ^{a2}	8.76 ±0.7 ^{b1}	6.82 ±1.3 ^{a2}	5.52 ±0.8 ^{a3}	4.51 ±0.4 ^{a4}	.00	.00	.00	.00	NA	.00	.04
LAB : *E. coli*	2.0	1.8	2.3	2.1	0.8	1.4	1.4	1.3	0.7	0.9	1.1	1.2							

$^{(a, b, c, d)}$ data with the same superscript in the same raw and same organ are not significantly different (P>0.05). $^{(1, 2, 3, 4)}$ data with same superscript numbers in same treated group are not significant different. ND= not detected level Log 2.7 CFU/g. Jeju= jejunum, Duod= duodenum, CON= control.

4.7.5. Molecular microbiology (DGGE and RISA)

4.7.5.1. Spectrophotometric assay

All the results of DNA concentrations in caeca, ileum, jejunum and duodenum samples were more than 50 ng /µl. The protein contamination of 260/280 is higher than 1.7 as well as the humic acid of 260/230.

4.7.5.2. DGGE analysis of bacterial community before infection (day 10)

The gut caeca of the chicks was investigated at the age of 10 days (A) and 20 days (B) The DGGE image showed the bands are called operative taxonomy unite (OTU) in each samples 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 caeca DGGE fingerprints is shown in Table 4.5 indicates the average similarity within the same group is 65% at day 10 and 60% in day 20, water 58% at day 10 and 64% at day 20, feed group 55% at day 10 and 41% at day 20. The average bacterial population similarity between control groups at day 10 and 20 was 64%, while the water group was 48% and a feed group was 48%.

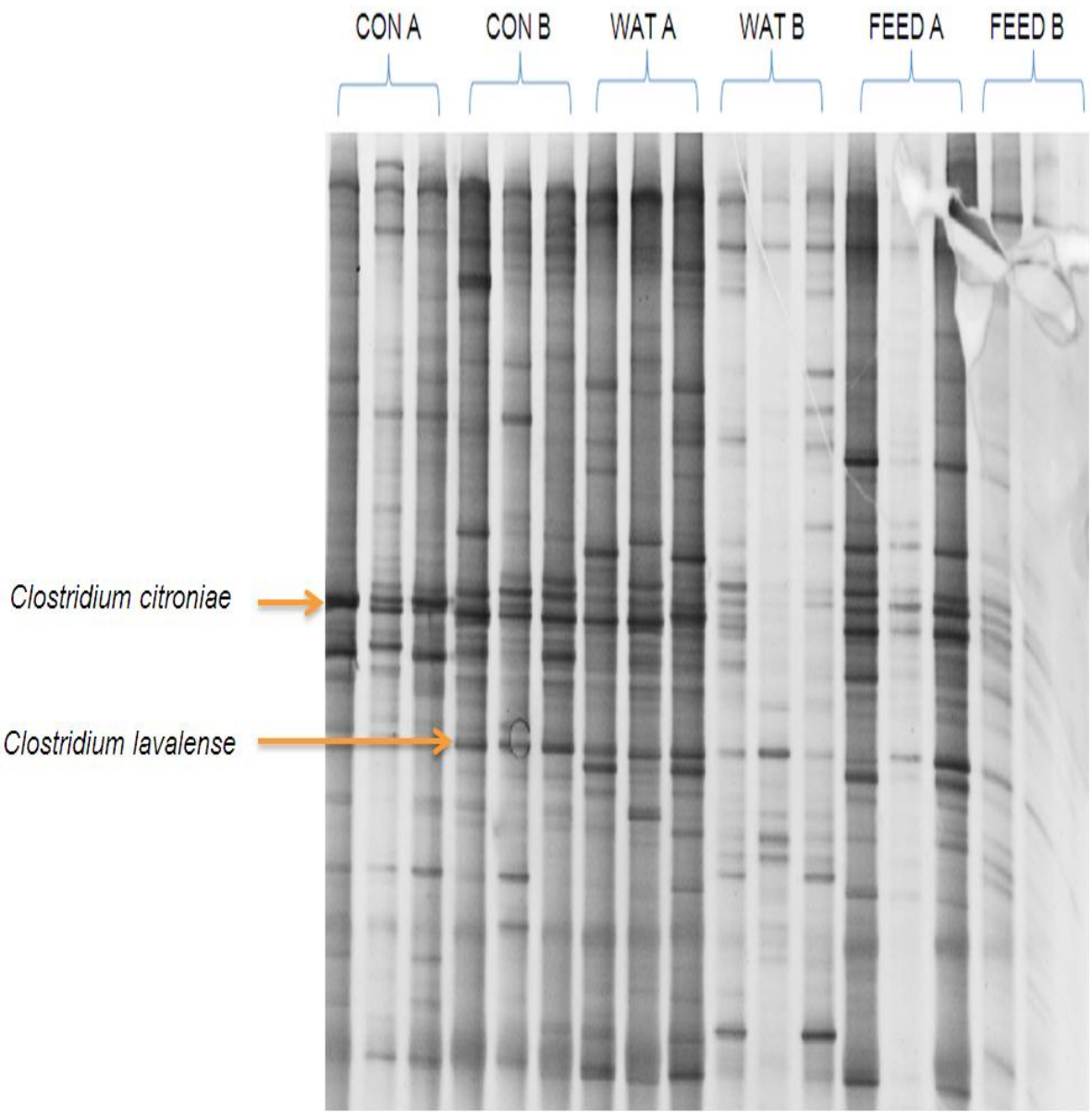

Figure 4. 6. The chicken caeca DGGE image of cyber gold stained 40-60% DGGE gel, analysis of the intestinal contents of caeca using a banding pattern, showing a banding pattern of PCR- amplified bacterial 16SrRNA fragments. Each Band or operational taxonomic unit (OTU) represents one species of bacteria present in the caeca of all treatments in day 10 (A) and day 20 (B). (n=18). The bands sequence results summarized in Section 4.3.3.

Table 4. 2. The half matrix similarity of caeca DGGE fingerprints showed the similarities between the replicates and the groups.

GROUP	CON A1	CON A2	CON A3	CON B1	CON B2	CON B3	WAT A1	WAT A2	WAT A3	WAT B1	WAT B2	WAT B3	FEED A1	FEED A2	FEED A3	FEED B1	FEED B2	FEED B3
CON A1	100																	
CON A2	71	100																
CON A3	70	54	100															
CON B1	74	57	71	100														
CON B2	68	49	68	65	100													
CON B3	63	67	58	70	44	100												
WAT A1	63	57	56	66	50	63	100											
WAT A2	68	50	73	75	68	58	63	100										
WAT A3	55	53	40	51	39	58	63	47	100									
WAT B1	66	61	62	58	56	59	55	58	46	100								
WAT B2	59	65	53	45	50	45	45	43	39	63	100							
WAT B3	65	64	64	54	54	49	52	52	42	64	66	100						
FEED A1	61	64	48	55	42	58	63	49	60	55	58	51	100					
FEED A2	65	65	62	63	53	55	59	55	45	59	72	64	65	100				
FEED A3	63	50	68	70	61	60	55	73	42	57	44	51	47	54	100			
FEED B1	46	40	56	38	48	26	31	41	22	65	56	55	33	43	38	100		
FEED B2	59	54	55	62	48	61	69	61	74	50	42	46	59	51	58	32	100	
FEED B3	61	50	67	64	60	47	45	60	33	52	47	61	40	53	61	45	44	100

CON = control, WAT= water groups, A= 10 days, B= 20 days. 1-3 refers to replicate a number in each case. (n=18).

Adding probiotics to feed or water has had a positive effect on caecal bacteria population species richness indicated by numbers of bands in DGGE fingerprints present Figure 4.7. Shannon's diversity index was used to display the microbial population diversity in the ceca samples Figures 4.8.

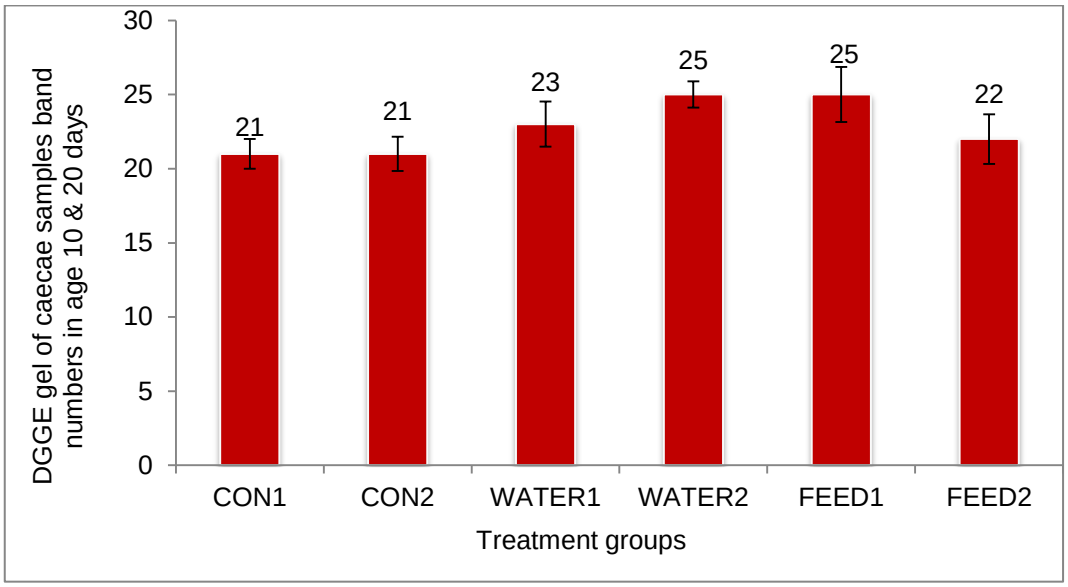

Figure 4. 7. Average DGGE gel band numbers in the chicken caeca group samples detected in DGGE fingerprints of all treatments. A= 10 days and B = 20 days, (n=18).

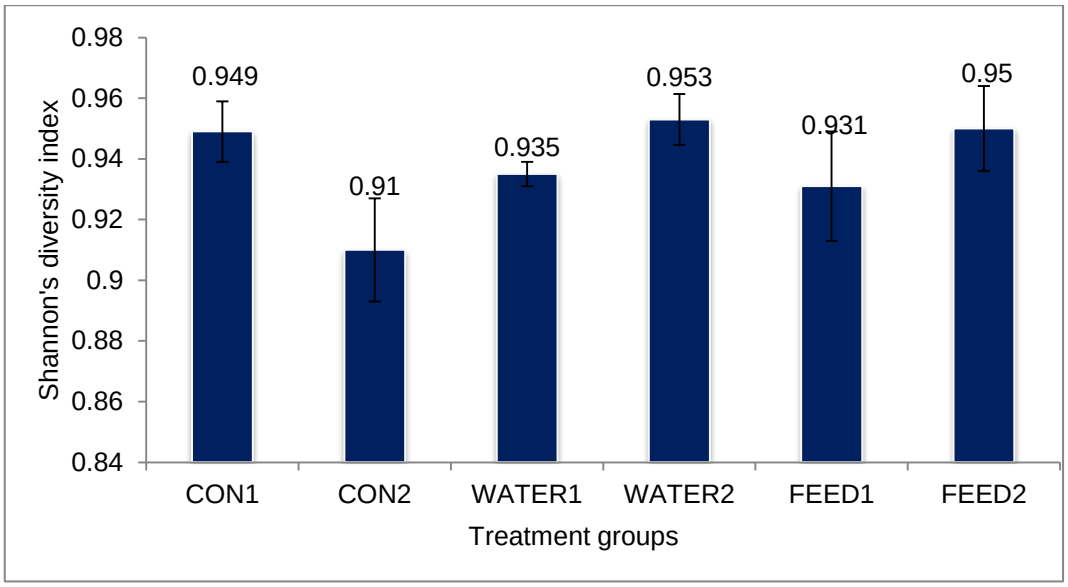

Figure 4. 8. Shannon's microbial diversity of chicks' caeca detected in DGGE fingerprints of all treatments. (n=18).

The ileum of the chicks was investigated at the age of 10 and 20 days (B). The ileum DGGE image showed the bands (OTU) in each samples Figure 4.7.

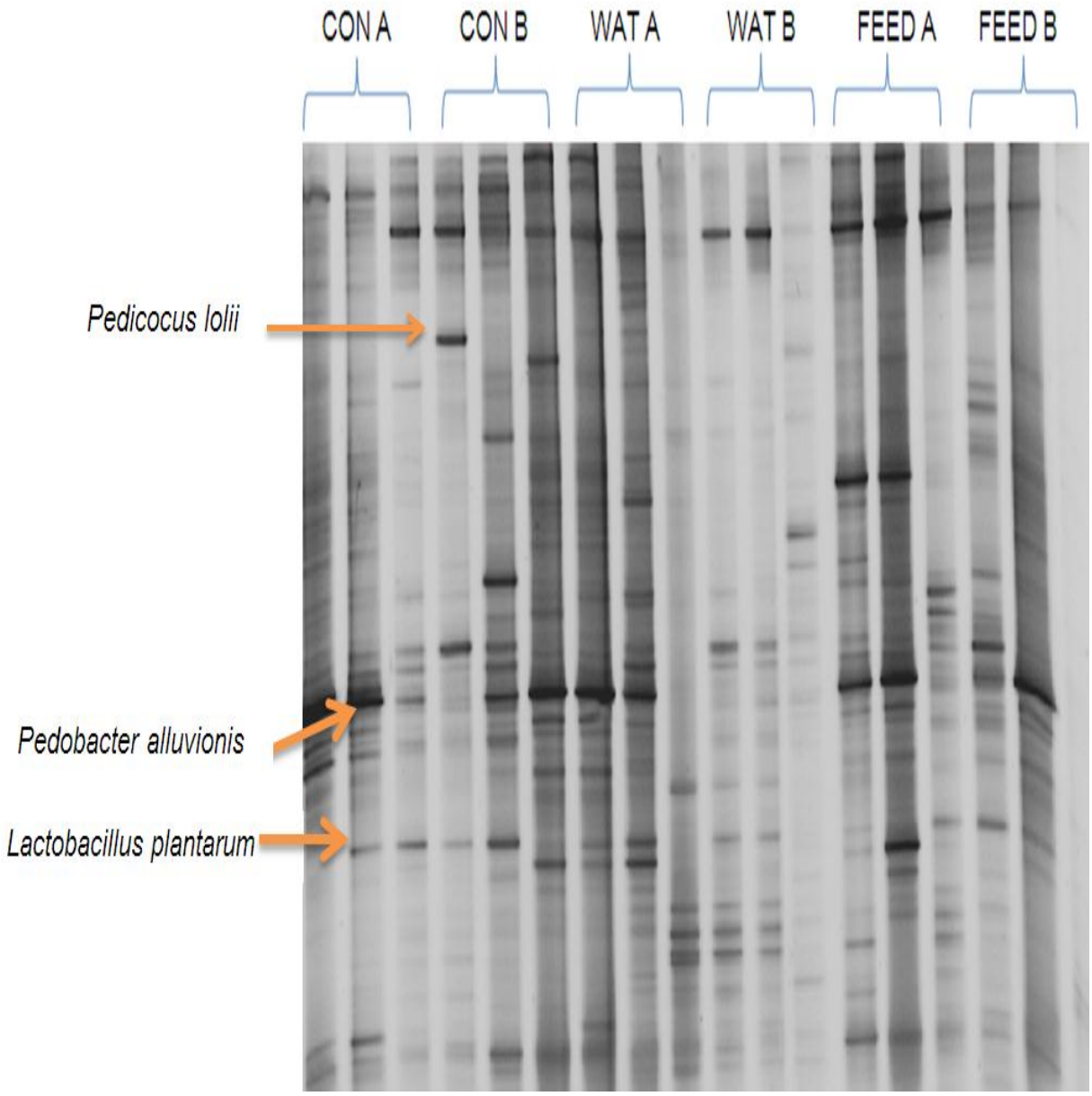

Figure 4.9. The chicken ileum DGGE image of cyber gold stained 40-60% DGGE gel, analysis of the intestinal contents of ileum using a banding pattern, showing a banding pattern of PCR- amplified bacterial 16SrRNA fragments. Each Band or operational taxonomic unit (OTU) represents one species of bacteria present on the ileum of all treatments in day 10 (A) and day 20 (B). The bands sequence results summarised in Section 4.3.3. (n=18).

The cluster and MDS analyses of the same fingerprints are shown in Figure 4.8.

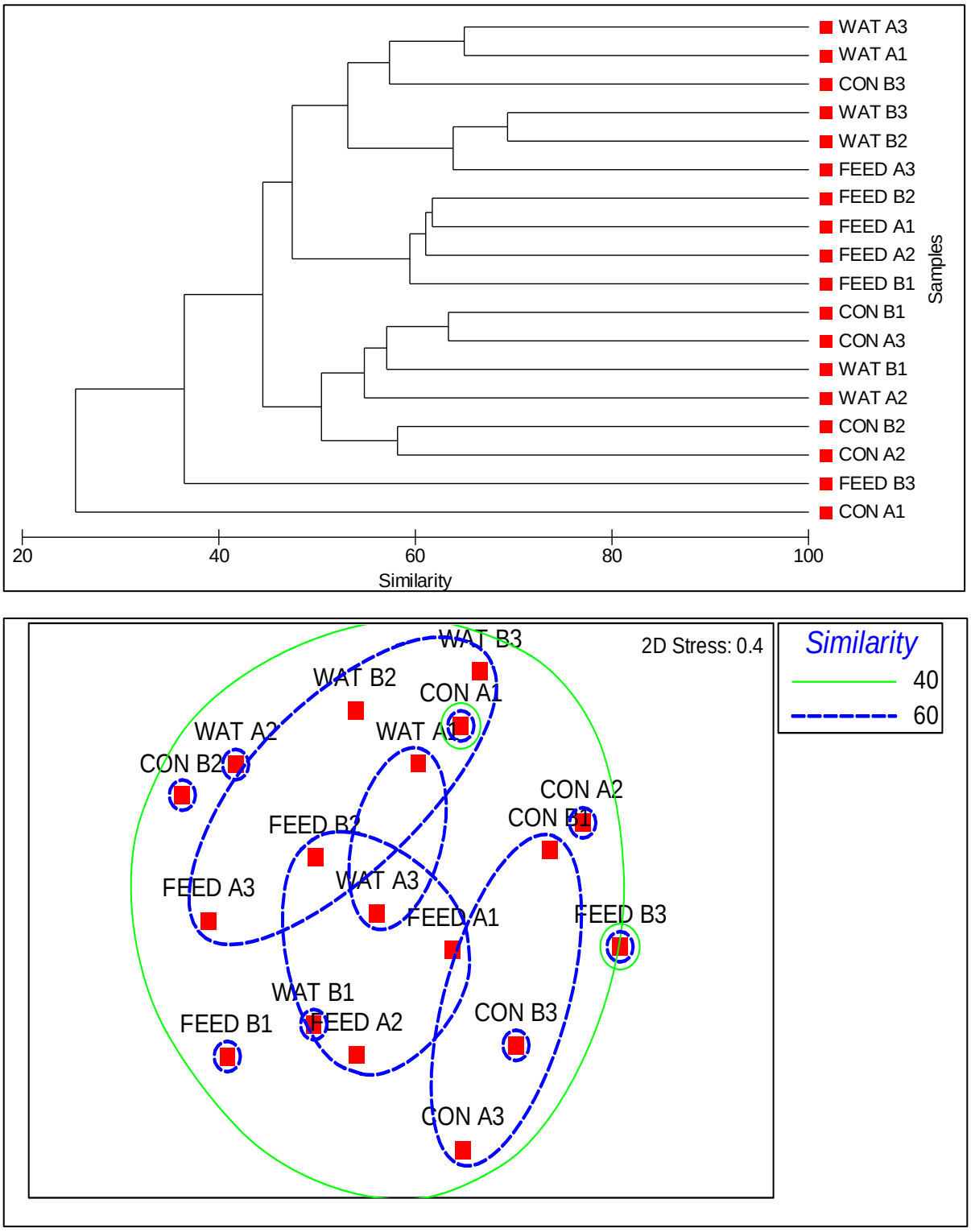

Figure 4.10. Cluster and MDS analyses of DGGE fingerprint of the ileum similarity relationship between the bacterial populations at age 10 & 20 days showed the similarity between the replicates of the groups. 1-3 denotes replicate number in each case. (n=18).

Ileal bacteria population similarity is higher within the samples from same treatments than those from other groups. The average similarity within the same group is 33% at day 10 and 49% on day 20, water 60% at day 10 and 60% at day 20, feed group 55% at day 10 and 44% at day 20.

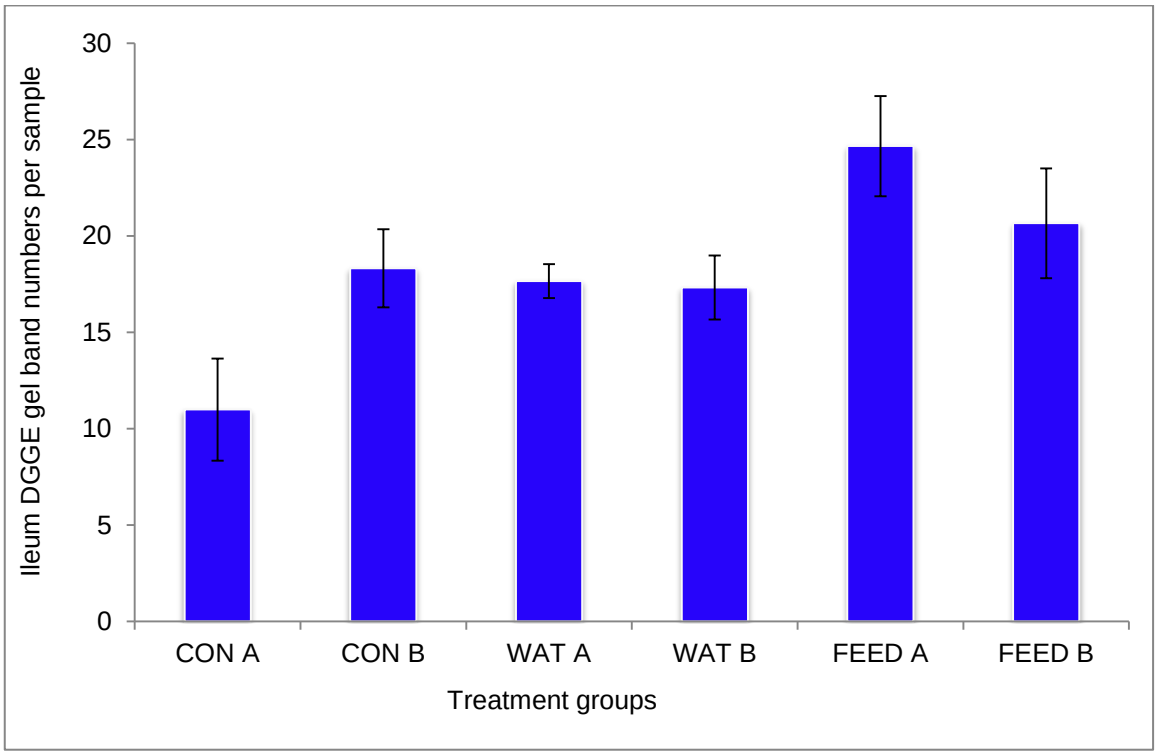

Figure 4. 11. Average DGGE gel band numbers in the chicken ileum group samples detected in DGGE fingerprints of all treatments. (n=18).

The DGGE gel image of jejunum showed many different bands

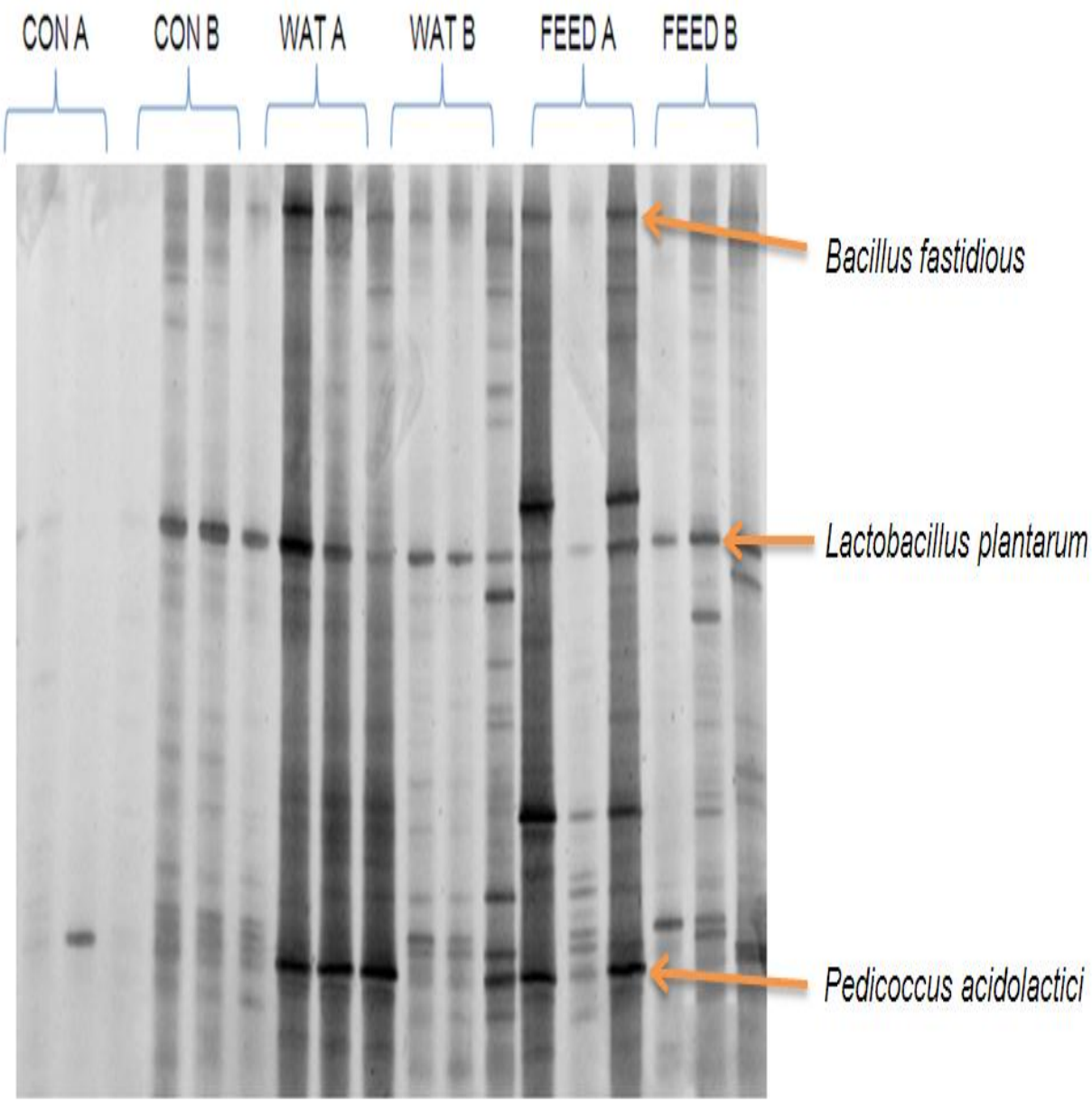

Figure 4. 12 The DGGE gel image of chicken ileum contents in day 10 (A) and day 20 (B), showing a banding pattern of PCR- amplified bacterial 16S rRNA fragments. Each gel band represents (OTU), the bands sequence results summarised in Section 4.3.3. (n=18).

The duodenums of the chicks were also investigated and five bands were sequenced but unfortunately were below the required standard and sequencing data was zero.

4.7.5.3. Sequence analysis:

A positive sequencing was done for only 7 out of the 13 PCR fragments which were analysed. The results of the trial sequence analysis shown in Table 4.6. The other samples sequencing quality were below the required standard and sequencing data was zero.

Table 4. 3. Summary results of the sequencing analysis from cutting bands of DGGE fingerprints of poultry gut content samples.

Band number	NCBI Accession number	Query coverage	Max. Identity	NCBI BLAST matches
Jejunum1	NR_042394.1	100%	95%	*Lactobacillus plantarum* strain NRRL B-14768
Jejunum2	NR_044829.1	100%	100%	*Bacillus fastidious* strain DSM 91 16S rRNA
Jejunum3	NR_042057.1	100%	100%	*Pedicoccus acidilactici* DSM 20284 strain DSM 20284 16S rRNA
Ileum 1	NR_041640.1	96%	96%	*Pedicoccus lolii* strain NGRI 0510Q 16S rRNA
Ileum 2	NR_044382.1	85%	85%	*Pedobacter alluvionis* strain NWER-II11 16S rRNA
Caeca 2	NR_044289.1	77%	97%	*Clostridium lavalense* strain CCRI-9842 16S rRNA
Caeca 3	NR_043681.1	100%	85%	*Clostridium citroniae* strain RMA 16102 16S rRNA

4.7.6. RISA analysis of bacterial community profiles

Agarose-RISA results revealed few numbers of bands per each sample ranged from 2-3 bands (OTU). The bands were not very separated even with changing the concentration of agarose gel and increasing the time of running as shown in Figures 4.10.

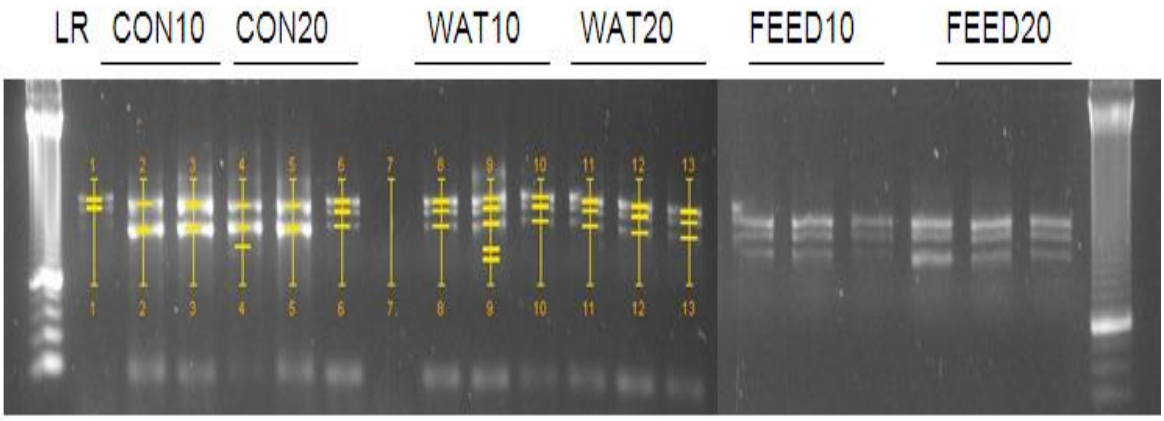

Figure 4.13. Agarose - RISA image of chicks' caeca. LR= 100 bp DNA ladder, CON= control, WAT= water group. (10, 20= 10, 20 days), yellow marks are numbering the bands using Quantity One software.

4.7.6.1. PAGE – RISA (Poly Acrylamide Gel Electrophoresis)

PAGE-RISA analysis revealed complex microbial communities present in all replicates from all intestinal samples. The total numbers of different species present in each sample ranged 4 – 7; some of them were common to all samples and all replicates.

The caeca RISA image showed the bands (OTU) in each sample Figure 4.11.

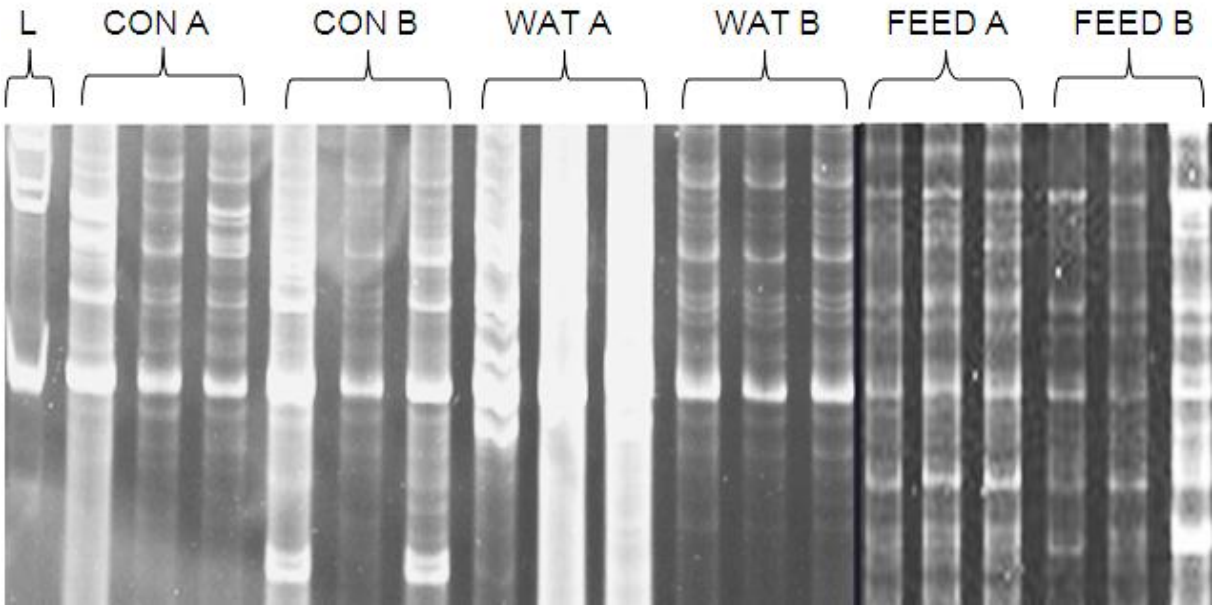

Figure 4. 14**.** PAGE-RISA image of the caeca in all groups, banding patterns represent fingerprints of the bacterial communities present in the samples. LR= 100 bp DNA ladder, (n = 12).

The half matrix similarity of the caeca of RISA fingerprints is shown in Table 4.7 indicates the average similarity within the caeca control group is 71% at day 10 and 60% on day 20. The average bacterial population similarity between the caeca before and after infection was 71%. Water group similarity is 78% at day 10 and 84% at day 20, feed group 75% at day 10 and 72% at day 20. The average bacterial population similarity between control groups at day 10 and 20 was 69%, while the water group was 59% and a feed group was 72%.

Table 4. 4. Half matrix similarity of caeca of all groups RISA-PAGE fingerprints showed the similarities between the replicates of the treatment groups in age 10 and 20 days.

Caeca	CON A1	CON A2	CON A3	CON B1	CON B2	CON B3	WAT A1	WAT A2	WAT A3	WAT B1	WAT B2	WAT B3	FEED A1	FEED A2	FEED A3	FEED B1	FEED B2	FEED B3
CON A1	100																	
CON A2	64	100																
CON A3	62	86	100															
CON B1	68	66	73	100														
CON B2	75	62	59	48	100													
CON B3	87	70	65	57	74	100												
WAT A1	78	71	69	70	67	72	100											
WAT A2	65	67	74	95	48	56	69	100										
WAT A3	64	64	71	96	46	54	69	95	100									
WAT B1	58	94	81	62	56	64	65	63	60	100								
WAT B2	55	75	68	49	56	63	69	49	47	78	100							
WAT B3	53	90	77	58	53	59	60	58	56	95	80	100						
FEED A1	85	70	68	75	66	74	94	72	73	64	67	59	100					
FEED A2	64	100	86	66	62	71	72	67	64	94	75	90	70	100				
FEED A3	62	86	100	73	59	65	69	74	71	81	68	77	68	86	100			
FEED B1	74	71	66	86	52	62	77	83	84	67	53	63	82	71	66	100		
FEED B2	90	71	68	60	85	88	81	60	58	65	63	60	80	71	68	65	100	
FEED B3	87	70	65	57	74	100	72	57	54	64	62	59	74	70	65	62	88	100

CON = control, WAT= water groups, A= 10 days, B= 20 days. 1-3 refers to replicate a number in each case. (n=18).

RISA fingerprints of ileum analysis of (MDS) and cluster are shown in Figure 4.12.

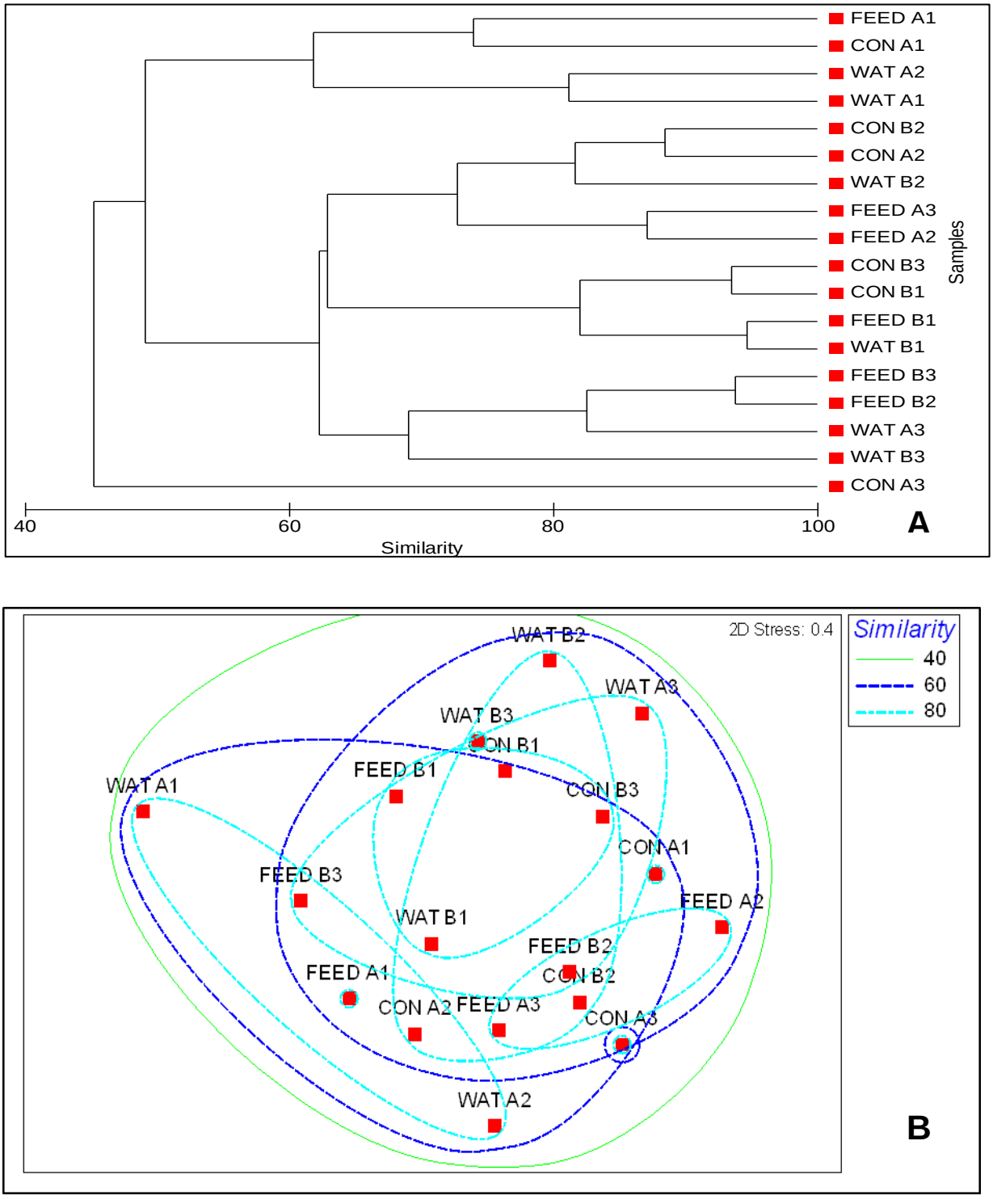

Figure 4. 15. The similarity of microbial population of RISA-PAGE method for chicks' ileum in all treatments showed the similarities between the replicates of the groups using cluster and MDS analyses of the RISA gel image.

4.8. Discussion

Two methods of probiotic delivery were used, feed and drinking water methods and both of them were acceptable by chicks but the water method was more easily applied. There are no significant differences in the health or growth of the three treated groups. Feed and water groups had high levels of LAB and total coliform compared to control group. Adding probiotics in feed and water groups lead to increased microbial populations richness and diversity but did not affect the bacterial ratio (LAB: total coliform) and on the growth performance.

The total bacteria detected in the caeca of feed group was more than other groups in all organs and that agreed with the conclusions of many researchers such as Casagrande Proietti *et al*., 2009; Gabriel & Mallet, 2006; Owens *et al*., 2008; and Salanitro *et al*., 1978 and this may be because continuing adding Lb. plantarum to the feed and water. Netherwood *et al*. (1999) added probiotics to chicken feed and reported no significant difference in the numbers of viable bacteria between the treated groups by culturing method.

A molecular microbial population analysis was used. DNA extraction was modified to get highest yields and more purified DNA. The Kit modified method gave the best results and that may be because using ethanol for precipitation of DNA and using phenol and chloroform increased the purity of the DNA yield.

PCR-DGGE analyses were undertaken in order to investigate the effect of feed types on the bacterial populations. Without exception, each investigation produced clear evidence of a large degree of variation within the bacterial populations individually and between groups. There are some problems using PCR for the micro-

bial population analyses such as variation in gene copy numbers, but this is not the major cause of bias. This problem can be avoided by normalization of the DNA. The primer sets used during these studies yields a relatively small fragment (193bp) and it is presumed that a larger fragment would have led to greater success in species-level identification. It is also possible that different primer sets and PCR conditions will affect band separation, and influencing the results. Any trialling of new primer sets, changes in PCR conditions or re-positioning of the GC clamp onto the reverse primer or even changing the DGGE gel concentration may it will effect on the results.

Another limiting factor regarding the excision of bands for sequencing was the closeness of bands. The single bands refer to a single species but this is not the case every time. Many bands may migrate to the same denaturant level resulting in an accurate indication of genotypic diversity and abundance (Hume et al., 2003). The term 'operative taxonomic unit' (OTU) has previously been proposed as a term suitable for use in recognition of the fact that this may not be the case (Gafan and Spratt 2005). Indeed, Gafan and Spratt (2005) and Sekiguchi *et al*. (2001) demonstrated that single bands may, under tighter denaturing gradient conditions, yield 'sub-bands' (representing in some cases both Gram-negative and Gram-positive bacteria) and as a consequence suggest a cautionary approach to analysis. Analysis of the gels used in this study suggested that some re-runs of gels and manipulation of the gradients subsequently utilized might have improved band isolation for downstream processing, as many bands were in close proximity to each other. However, time and cost prevent this.

The DGGE analysis separate DNA on the basis of sequence dissimilarities (Netherwood *et al.*, 1999). DGGE results revealed that the numbers of bacterial

species (DGGE gel band numbers) in the caeca of 10 and 20 days of the feed and water groups were more than all organs in all groups. Indeed, the differences of bands in caeca, ileum, jejunum and duodenum indicated the changes in predominant microflora as chicks aged and type of feed. The band numbers in the duodenum were lower than all organs. In general, the band numbers in the feed and water groups were higher but not significantly so from the control group, and this may be because adding Lb. plantarum in the feed and water. The high species richness in gut microflora is associated with decreased ability of pathogens to colonize the gut (Dillon *et al.*, 2005).

The sequence analysis of DGGE bands was helpful to know the types of the bacteria in the population. The most family BLAST results were Bacillaceae, including *Lactobacillus plantarum.* The sequencing revealed the variety of bacteria in the chicken gut. Some species were common between all groups and other is unique for subjected group.

The RISA technique was used with two methods, Agarose-RISA and PAGE-RISA. The analyses of Agarose-RISA images were not valued because the band numbers of all treatments were limited. The gel band numbers were higher using poly acrylamide gel electrophoresis (PAGE – RISA). There is a difference in band numbers even within the same group as well as with other groups. The diversity was different according to the age and diet and as assessed by species richness and evenness.

4.9. Conclusions

This study confirmed the result of last study namely the survival of *Lactobacillus plantarum* through the GIT of the chicken and the possibility of using rifampicin as a biomarker for detection *Lactobacillus plantarum*. The use of molecular techniques does not exclude traditional techniques but they can used together to get more accurate results. The difference in the bacterial population species richness between 10 and 20 days indicated the changes in predominant microflora as chicks aged and may be the influence of the type of feed and by continuing addition of LAB). Adding probiotics increased the bacterial populations in feed and water groups and as Dillon *et al*., (2005) suggests a higher species richness in the gut means less ability for pathogens to colonize the gut.

DGGE is very helpful tool to understand the very complex bacterial populations in the gut and detect the changes in the intestinal microbial populations as a response to feed additives administration.

CHAPTER 5

CHAPTER 5: The effect of *Lactobacillus plantarum* NCIMB 41607 on survival of *Salmonella enterica* serovar Typhimurium SAL1344 nal^r and changing of microbial populations in broiler chicken

5.1 Introduction

The eating of chicken meat contaminated with *Salmonella* is the biggest source of Salmonellosis in Humans (Revolledo, Ferreira & Mead, 2006). Preventing colonization of the chicken gut with *Salmonella* is the best way to reduce contamination of chicken carcasses and subsequent transmission to humans (Dunkley *et al.*, 2009). In order to reduce *Salmonella* in chicken, dietary interventions are being considered and including inclusion of probiotics, organic acids and FMF. Probiotics and moist feed fermented with probiotic (FMF) may offer a way to reduce *Salmonella* in chicken (Niba, 2008). Delivery of probiotic to chicken can be by drinking water, food or allowing the feed to ferment by adding probiotic Lactic acid bacteria to moist feed (1: 1.2 feed: water) to produce a feed containing at least 150mmol/l lactic acid and low pH<4.5 with at least 10^9 CFU/ml of LAB. This has been shown to reduce contamination of feed by *Salmonella* (Beal *et al.*, 2002b) and it is considered a biosafe method by which gut and host health can improve. In addition, these feeds resist enteropathogens contamination prior to feeding. FMF can also act as a carrier for a high concentration of Lactic acid (>150mmol/L) and high numbers of lactic acid bacteria 10^9 CFU/ml to be delivered to the bird (Heres *et al*., 2003c). Spontaneous fermentation of moist feed is unacceptable as it fails to give consistently good results, so controlled fermentation with carefully selected organisms, that rapidly produce a high concentration of lactic acid, is desirable (Brooks, 2008).

In poultry, the acidity of the crop and gizzard are the most important single factor resisting the passage of *Salmonella* through the upper part of the gut but FMF can improve the acidic barrier function (Heres *et al.*, 2003a). The chick is more susceptible to pathogens during the first 4 days post hatch (Wells *et al*., 1998), So the age at which FMF or probiotic is introduced to birds might be important because the post hatch period is of major immunological significance to the chicks.

In this experimental study, a rifampicin resistant strain of *Lb. plantarum* was selected. The study reported here was designed to: examine the effect of administration of rifampicin resistant *Lb. plantarum* in water or fermented moist feed on *Salmonella* infection in poultry, observe changes in the intestinal ecology of chicken by molecular methods and to assess histological alteration of intestinal villi and the possibility of transmission of *Salmonella* to the liver and spleen.

5.1.1 Experimental design and treatments

One hundred chicks Ross (P D Hooks Hatcheries Kentisbere, Devon, UK) were obtained and housed in 1 x 1.2 m and randomly allocated to five treatments of 20 per treatment. Each treatment was divided randomly between pens with 15 pens. The five treatments were control, FMF, AMF, water group and acid water (AW) group. The treatments detailed in Section 2.10.2. Preparing feed was described in Section 2.7. *Lactobacillus plantarum* and *Salmonella* Typhimurium strains were used as described in Section 2.2. All chicks were orally gavage with 0.2 ml of 10^6 CFU of *Salmonella* Typhimurium SAL 1344 on day 14 of age as described in Section 2.8.

Twenty five birds were randomly selected and tested for the presence of bacteria

particularly *Salmonella* species by cloacal swabbing weekly as described in Section 2.10.3. The growth performance was measured as described in Section 2.8.1. On day 14 and 36 of the trial chicks were killed as described in Section 2.10. Sections of ilea were taken for histological assessment, fluorescent *in-situ* hybridisation (FISH) using fluorescent probes specific for *Lb. plantarum* and *Salmonella* Typhimurium and electron microscope to detect the attachment of bacteria to the chicken intestine. The whole liver and spleen were taken for detection of *Salmonella*. Blood samples (2ml) were taken from the wing vein for total white blood cells and the heterophil / Lymphocyte ratio to assess physiological stress. Samples of the ileal and caecal contents were taken to assess gut microflora population changes using classical culture dependent and molecular methods including PCR followed by PAGE-RISA, DGGE analysis and finally gene sequences as detailed in Sections 2.10 - 2.12.

The analysis of bacterial community structure is summarised in Figure 5.1.

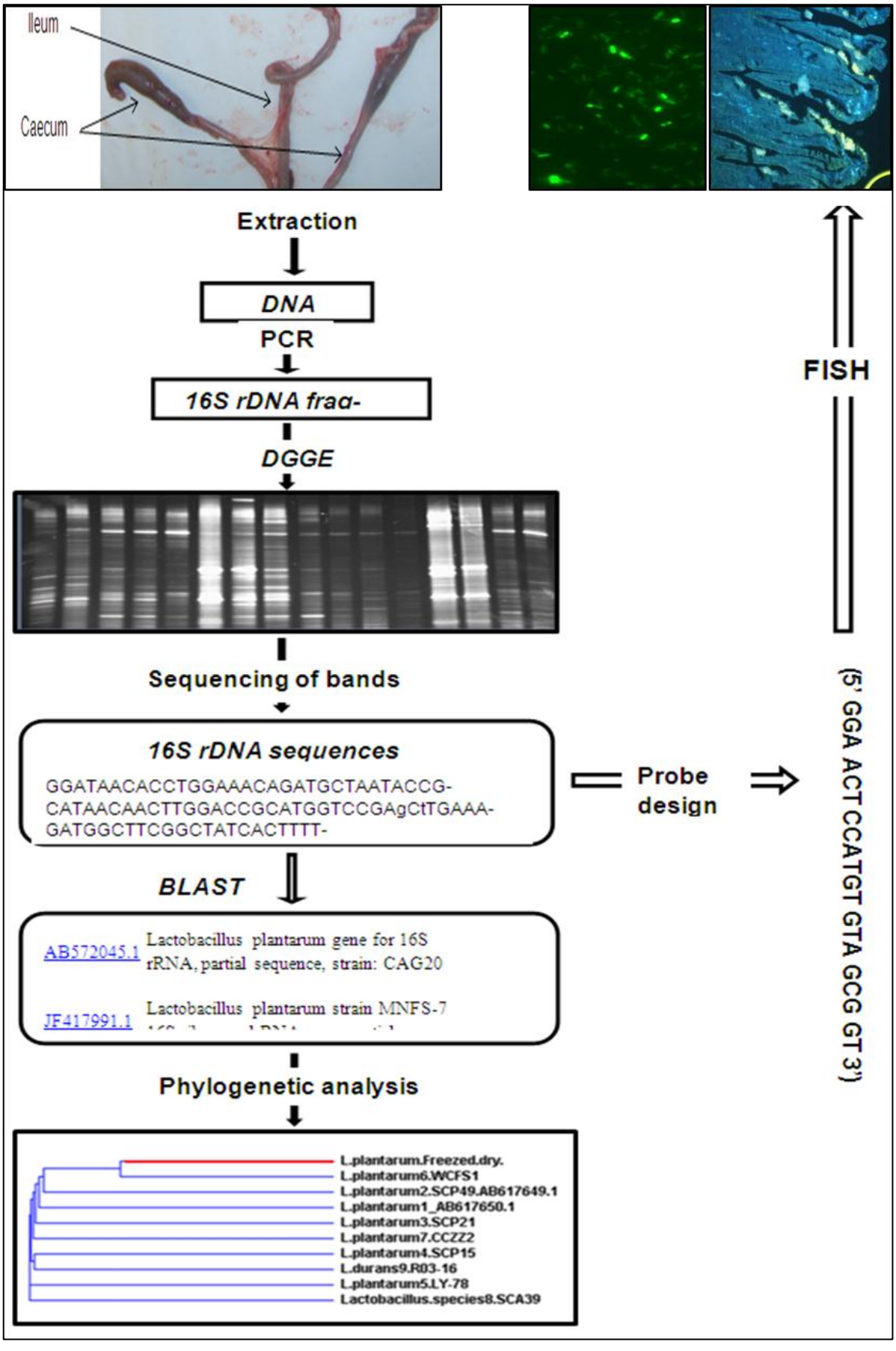
Ileum
Caecum
Extraction
DNA
PCR
16S rDNA frag-
DGGE
Sequencing of bands
16S rDNA sequences
GGATAACACCTGGAAACAGATGCTAATACCG-
CATAACAACTTGGACCGCATGGTCCGAgCtTGAAA-
GATGGCTTCGGCTATCACTTTT-
Probe design
FISH
(5' GGA ACT CCATGT GTA GCG GT 3')
BLAST
AB572045.1 Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: CAG20
JF417991.1 Lactobacillus plantarum strain MNFS-7
Phylogenetic analysis
L.plantarum.Freezed.dry.
L.plantarum6.WCFS1
L.plantarum2.SCP49.AB617649.1
L.plantarum1_AB617650.1
L.plantarum3.SCP21
L.plantarum7.CCZZ2
L.plantarum4.SCP15
L.durans9.R03-16
L.plantarum5.LY-78
Lactobacillus.species8.SCA39

Figure 5.1. Flow diagram showing the different steps in the analysis of microbial population structure by PCR-DGGE. DNA is extracted from chick's gut ingesta and amplify the 16S rRNA by PCR then the PCR products separated by DGGE. The band pattern of the predominant community members represented in the DGGE. Selected band excised and re-amplified and sequences stored in nucleotide databases. The sequence information can be used to design an oligonucleotide probe for the detection of a specific bacterial population by fluorescent *in situ* hybridisation (FISH). The left top photo represents the sample (Gut tissue or ingesta) and the left top photos are the FISH image results of pure culture and the gut tissue showing the LAB bacteria after incubation with a green fluorescent-labelled oligonucleotide probe specific for the *Lactobacillus plantarum.*

5.2 Results

5.2.1 Growth performance

In all treatments, there were no mortalities. Growth performance and FCR were showed in Table 5.1. There was no significant difference in FCR. Feed intake increased from week one to the end of the trial (day 36). The average daily feed intake (ADFi) in the control group was significantly higher ($P<0.05$) from all groups except the water group, this may be because the dry type feed for both control and water groups compared wet feed of another group as showed in Table 5.1.

The weight gain which measured as daily weight gain (DWG, g/chick/day) are presented in Table 5.1 which shows to ADG increased from week 1 to week 4 but not in week 5.

The water intake was higher and significantly higher different ($P<0.05$) in the treatments of control and water groups compared with FMF, acid and acid water group and that's simply because the feed has a high level of water compared with a control or water groups which were supplied with dry feed.

5.2.2 Cloacal swabbing

To make sure the birds were free from *Salmonella,* 25 birds were randomly selected and cloacal swabs were taken. Biochemical tests were applied for the two colonies isolated from the samples as follows: small white colonies (nutrient agar) Gram positive, cocci, Catalase +ve, Glucose fermentation +ve acid produced but no gas and black colonies on Baird parker Egg agar identified the genus *Staphylococcus*. A gram negative rod from big creamy colonies on nutrient agar, big yellow colonies on XLD agar, big colonies in MacConkey agar with biochemical tests; catalase +ve, Oxidase –ve, Indole +ve and Glucose fermentation +ve for acid and gas was identified as *Escherichia coli.* The results of cloaca swabbing in the first week were negative for *Salmonella* even with enrichment in buffered peptone water (BPW) broth. After challenge with *Salmonella* at day 14 all of the chicks were positive for *Salmonella* in the first week after challenge. While in the second week all of them were negative.

Table 5. 1. The growth performance of chicken after 5 weeks feeding on experimental diets (mean ±SE).

Growth perfor-mance	Control	FMF	Water	Acid water	Acid	P. value
Initial weight (g)	43 ±1 [a]	42 ±0. 6 [a]	45 ±0. 3 [a]	44 ±1. 4 [a]	44 ±0. 4 [a]	0.285
Final weight (g)	1033 ±31 [a]	815 ±6. 5 [b]	913 ±65 [ab]	762 ±54. 6 [c]	661 ±51. 6 [c]	0.002
Weight gain (g)	990 ±31 [a]	773 ±7 [bc]	868 ±65 [ab]	718 ±56 [cd]	617 ±52 [d]	0.002
ADFi (g)	72 ±12.7 [a]	54 ±1.4 [a]	83 ±1.0 [b]	48 ±1.6 [b]	41±5.5 [b]	0.004
ADG (g)	28 ±0.9 [a]	22 ±0.2 [b]	25 ±1.9 [ab]	20 ±1.6 [bc]	18 ±1.5 [c]	0.002
FCR	2.6 ±0.43 [ab]	2.5 ±0.06 [b]	3.3 ±0.19 [a]	2.4 ±0.21 [b]	2.2 ±0.32 [b]	0.005
Water intake (ml)	130 ±15 [a]	59 ±1 [b]	147 ±9 [a]	53 ±2 [b]	65 ±15 [b]	0.000

FMF=fermented moist feed, ADFi =average daily feed intake, ADG =average daily gain, FCR =feed conversion rate. [(a, b, c)] data with the same superscript in the same row and for the same organ are not significantly different (P>0.05).

5.2.3 Classic microbiology (Culture dependent investigation)

5.2.3.1 Bacterial counts at day 14

Numbers of LAB and Coliforms were higher in the caeca than ileum in all treatments Table 5.2, there was no significant difference ($P<0.05$) in numbers LAB and Coliforms within the same group. There was a significant difference ($P<0.05$) between FMF and other groups in the numbers of bacteria.

Table 5.2 shows that for FMF and water groups LAB counts were higher than other groups. That may be because of the high numbers of *Lactobacillus plantarum* fed with these treatments.

Table 5. 2. Bacterial numbers (Log CFU/g) of chicken gut isolates from caeca and ileum in different treatments (day 14).

Bacteria counts (Log CFU/g) & ratio	CONTROL		FMF		WATER		Acid-Water		Acid		P. value between groups		p. value within groups				
	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	CON	FMF	WATER	AW	Acid
LAB	7.1 ±0.4 ^{bc1}	6.4 ±0.2 ^{bc1}	9.1 ±0.3 ^{a1}	8.8 ±0.1 ^{a1}	7.7 ±0.8 ^{b1}	7.1 ±0.6 ^{b1}	6.9 ±0.6 ^{bc1}	6.1 ±0.7 ^{bc1}	6.2 ±0.4 ^{c1}	5.6 ±0.4 ^{c1}	.01	.00	.17	.47	.45	.43	.34
E. coli	6.8 ±0.5 ^{bc1}	5.7 ±0.4b ^{c1}	6.9 ±0.3 ^{a1}	3.9 ±0.4 ^{a1}	7.3 ±0.2 ^{b1}	5.2 ±0.3 ^{b1}	7.2 ±0.5 ^{bc1}	4.0 ±0.6 ^{bc1}	6.1 ±0.7 ^{c1}	5.4 ±0.3 ^{c1}	.40	.01	.17	.47	.45	.43	.34
Enterobacteriacae other than *E. coli*	4.0 ±0.7^{b1}	2.7 ±0.0 ^{b1}	6.5 ±0.2 ^{a1}	3.9 ±0.6 ^{a2}	6.1 ±0.3 ^{a1}	4.1 ±0.7 ^{a2}	3.3 ±0.6 ^{b1}	3.1 ±0.4 ^{b1}	3.3 ±0.6 ^{b1}	3.5 ±0.6 ^{b1}	.00	.29	.80	.00	.03	.77	.79
Total coliform	6.8 ±0.5 ^{a1}	5.7 ±0.4b ^{a1}	7.2 ±0.2 ^{a1}	4.3 ±0.5 ^{a1}	7.4 ±0.3 ^{a1}	5.7 ±0.5 ^{a1}	7.2 ±0.5 ^{a1}	4.1 ±0.5 ^{a1}	6.1 ±0.7 ^{a1}	5.5 ±0.3 ^{a1}	.34	.02	.15	.43	.39	.43	.38
LAB : T. coliform	1.04	1.12	1.26	1.05	1.04	1.25	0.96	1.49	1.02	1.02							
LAB : *E. coli*	1.04	1.12	0.32	2.26	1.05	1.37	0.96	1.53	1.02	1.04							

$^{(a, b, c, d)}$ data with the same superscript in the same row and for the same organ are not significantly different (P>0.05). $^{(1, 2, 3, 4)}$ data with same superscript numbers in same treated group are not significant different. ND= Log 2.7 CFU/g. CON= control.

5.2.3.2. Challenge chicks with *Salmonella* Typhimurium

At day 17, all chicks' swabs were positive for infection with *Salmonella*. This result shows that none of the treatments used in this trial were able to protect the chicken from a high dose infection with *Salmonella* Typhimurium. The results of liver and spleen were negative for bacterial contamination at all times before and after infection with *Salmonella*.

5.2.3.3. Bacterial count results at the end of trial

The results of traditional cultural dependent work were negative for *Salmonella* detection in all chicks including the control group. The LAB and Coliform numbers in the caeca were higher than in the ileum and these results were not significantly different to the results before infection. There was a significant difference ($P<0.05$) between the caeca and ileum within the same group in the numbers of LAB as well as Coliform (except the ACID group). There is a significant difference ($P<0.05$) between the LAB in the caeca of all the groups. The LAB in ileum was significantly higher ($P< 0.05$) in FMF and water groups compared to control group but not with other groups. The LAB colonies presented in MRS agar were transferred to rifampicin- MRS agar to confirm the presence of *Lactobacillus plantarum.*

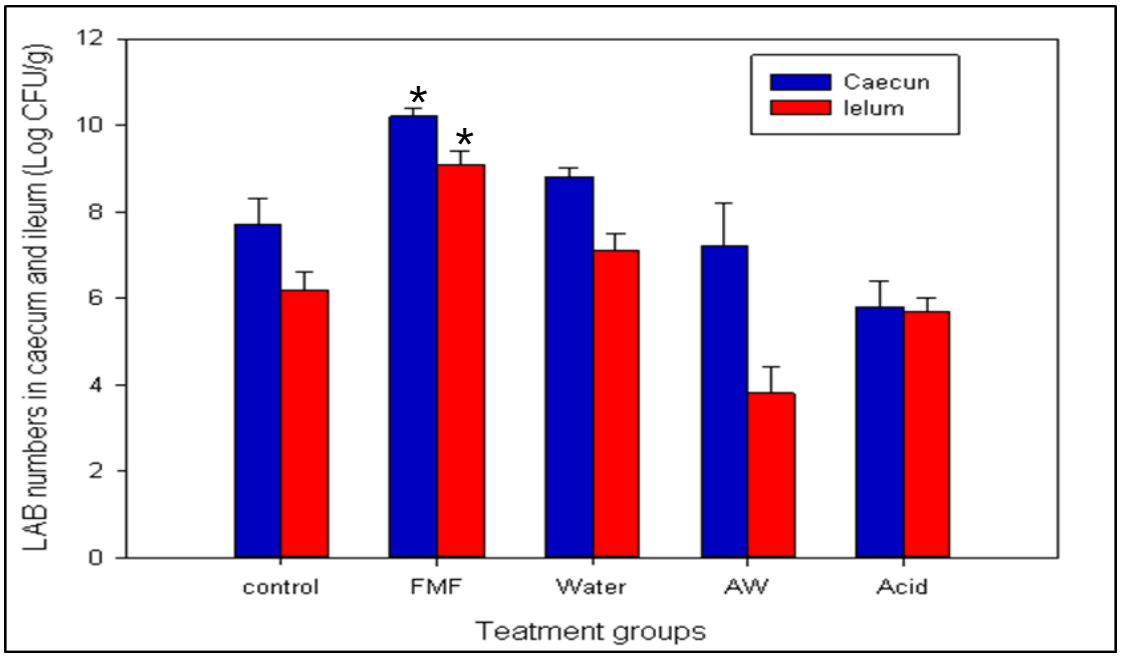

Figure 5. 2. The LAB accounts (Log CFU/ g) in caeca and ileum of chicks at age 36 days.*significantly different with the same organ in all treatments. (n= 30).

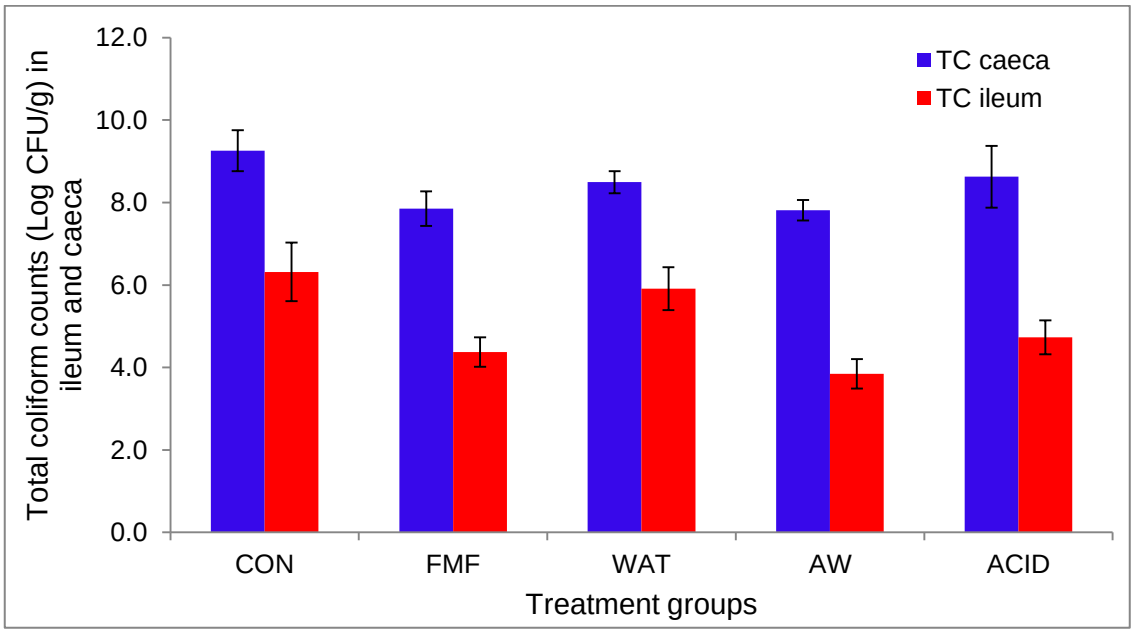

Figure 5. 3. Total Coliforms (TC) accounts (Log CFU/g) in caeca and ileum at age 36 days. (n=30).

Infection with *Salmonella* Typhimurium had no statistically significant effect on the numbers of LAB in the ileum in all groups.

In general, the numbers of LAB in caeca were higher than ileum before and after infection. The numbers of LAB in FMF caeca were significantly higher (P<0.05) compared to other treatment groups as shown in Figure 5.4.

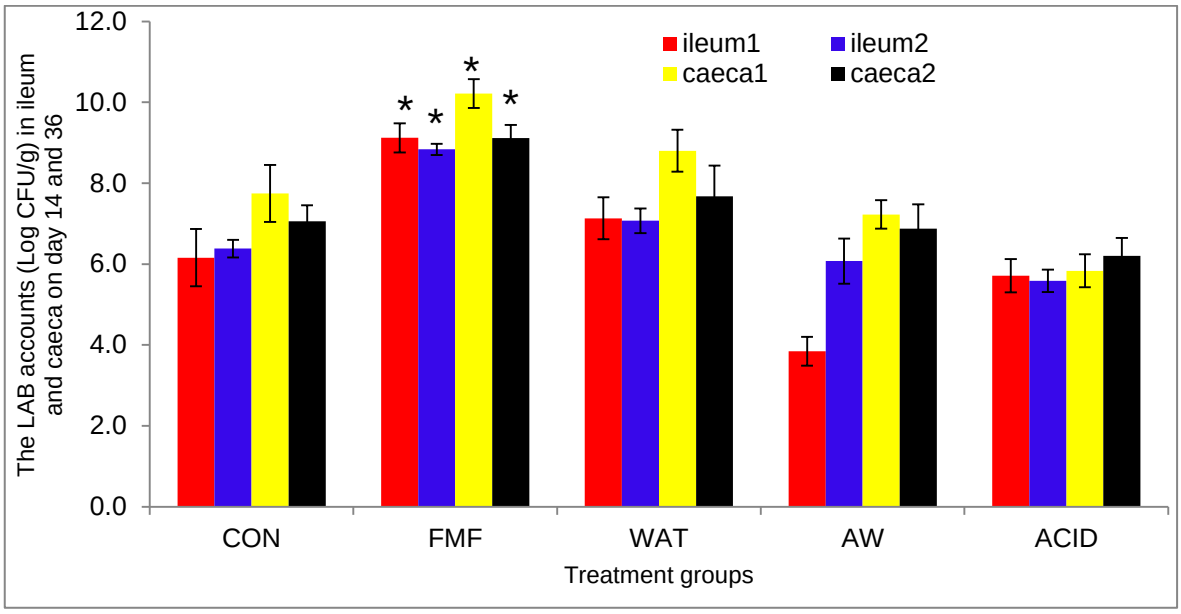

Figure 5. 4. LAB numbers (Log CFU/g) in caeca and ileum before and after inf. C= caeca, il= ileum, 1= day14 and 2= day36. (*)significantly different with the same organ in all treatments (P>0.05). (n= 30).

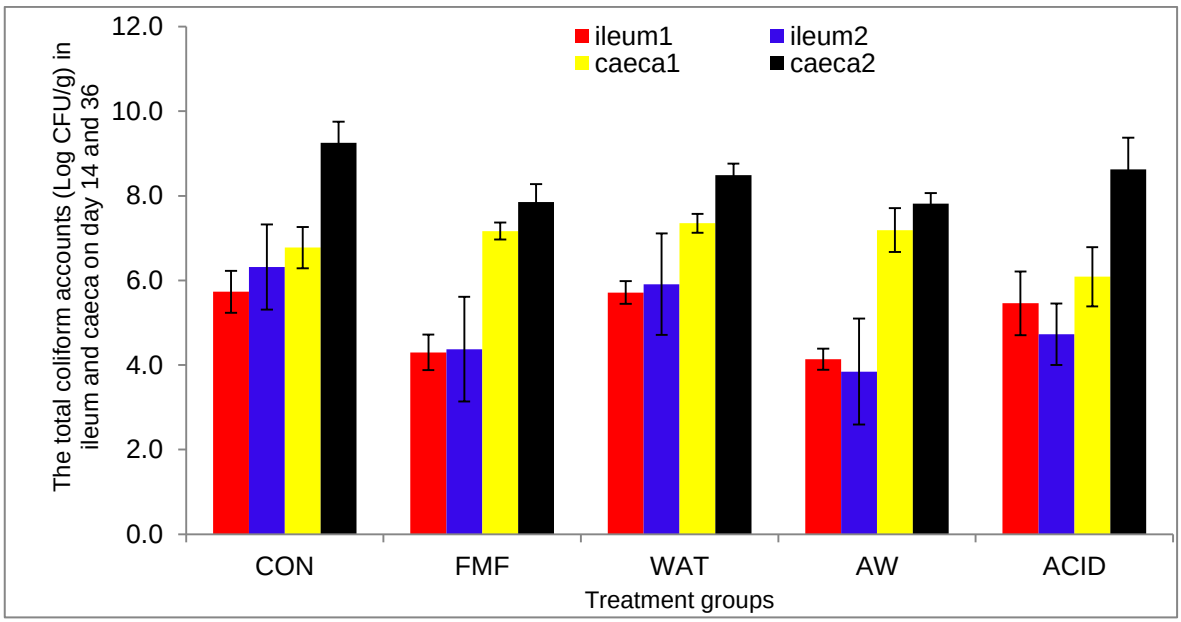

Figure 5. 5. Total Coliform counts (Log CFU/g) in caeca and ilea before and after infection in all treatment groups. (n= 30).

Table 5. 3. Bacterial composition of chicken caeca and ileum in different treatments at the end of the trial 36 days (Log CFU/g).

Bacteria counts (Log CFU/g) & ratio	CONTROL		FMF		WATER		Acid-Water		Acid		P. value between groups		p. value within groups				
	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	Caeca	Ileum	CON	FMF	WATER	AW	Acid
LAB	7.7 ±0.6^{c1}	6.2 ±0.3^{bc2}	10.2 ±0.2^{a1}	9.1 ±0.2^{a2}	8.8 ±0.2^{b1}	7.1 ±0.4^{b2}	7.2 ±0.1^{c1}	3.8 ±0.6^{d2}	5.8 ±0.6^{d1}	5.7 ±0.3^{c1}	0.00	0.00	.50	.01	.01	.00	.86
E. coli	9.1 ±0.7^{c1}	6.2 ±0.5^{bc2}	7.9 ±0.4^{a1}	4.1 ±0.5^{a2}	8.5 ±0.5^{b1}	5.9 ±0.3^{b2}	7.7 ±0.4^{c1}	3.8 ±0.3^{d2}	8.6 ±0.4^{d1}	4.6 ±0.8^{c1}	0.26	0.01	.50	.01	.01	.00	.86
Enterobacteriacae other than *E. coli*	6.1 ±1.5^{a1}	4.9 ±0.5^{a1}	2.7 ±0.0^{b1}	2.9 ±0.2^{b1}	6.5 ±0.8^{a1}	4.5 ±0.3^{a2}	5.6 ±0.9^{a1}	2.7 ±0.0^{b2}	2.7 ±0.0^{b1}	2.7 ±0.0^{b1}	0.01	0.00	.47	.43	.04	.01	NA
Total coliform	9.3 ±0.7^{a1}	6.3 ±0.5^{a2}	7.9 ±0.4^{a1}	4.4 ±0.4^{ab2}	8.5 ±0.5^{a1}	5.9 ±0.3^{a2}	7.8 ±0.4^{a1}	3.8 ±0.3^{b2}	8.6 ±0.4^{a1}	4.7 ±0.8^{ab2}	0.23	0.01	.03	.01	.00	.00	.85
LAB : T. coliform	0.8	1.0	1.3	2.1	1.0	1.2	0.9	1.0	0.7	1.2							
LAB : *E. coli*	0.85	1.007	1.29	2.22	1.04	1.20	0.94	1.00	0.67	1.24							

$^{(a, b, c, d)}$ data with the same superscript in the same raw and same organ are not significantly different (P>0.05), $^{(1, 2)}$ data with same number in same group are not significantly different. (n=30).

5.2.4 Molecular microbiology (DGGE and RISA)

5.2.4.1. DGGE analysis of bacterial community at day 14

DGGE fingerprints give qualitative data and do not represent the real numbers of bacteria in the chick's gut. The band locations in the gel were different according to their molecular weight.

The samples of caeca from chicks at the age of two weeks before infection with *Salmonella* Typhimurium were investigated. The results were compared within the same treatment group and other groups. DGGE image showed the bands or operation taxonomy unit (OTU) in each sample Figure 5.6.

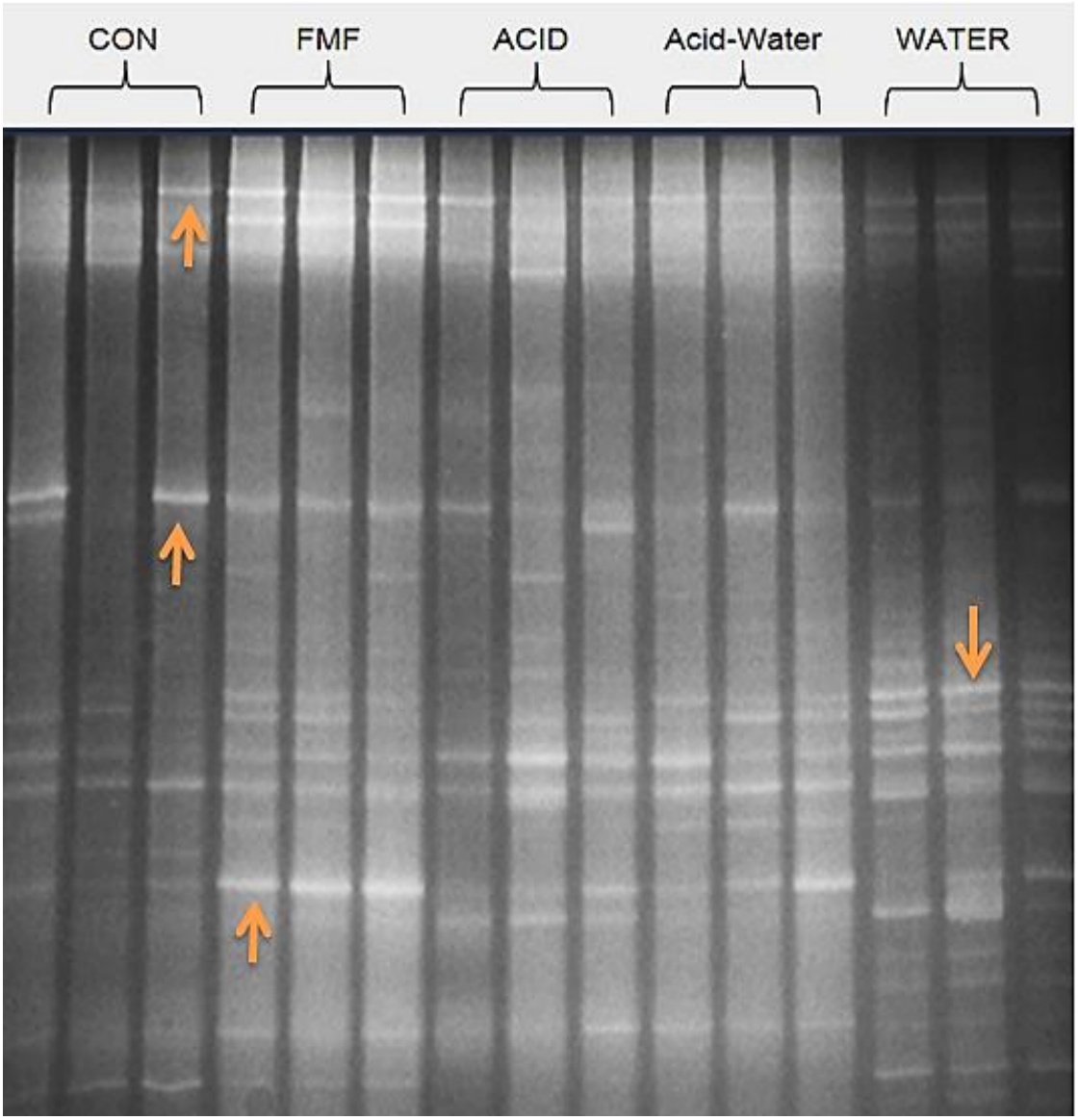

Figure 5. 6. The chicken caeca DGGE image of cyber gold stained 40-60% DGGE gel, analysis of the intestinal contents of caeca using a banding pattern, showing a banding pattern of PCR- amplified bacterial 16Sr RNA fragments. Each lane represents a one replicate sample pooled from two chicks. Each band or operational taxonomic unit (OTU) (arrows) represents one species of bacteria present in the each sample of ileum of all treatments. CON= control, FMF= fermented moist feed group. (n=15).

Using PRIMER v.6 software revealed the half matrix similarity of caeca DGGE fingerprints as shown in Table 5.4 indicates the average similarity within the control group is 91%, FMF 86%, Acid group 85%, Acid water group 91% and finally the water group 68%. The average bacterial population similarity between the groups was lesser than within the same group. The average bacterial population similarity was ranged from 66% (lower) between acid and water groups to 89% (higher) between acid and acid water groups.

Table 5. 4. The average similarity of bacterial populations groups in caeca before infection with *Salmonella* Typhimurium day 14. CON = control, FMF = fermented moist feed and AW = acid water groups. (n=15).

Groups	Average similarity
Control x FMF	**80**
Control x Acid	**85**
Control x Acid water	**84**
Control x Water	**66**
FMF x Acid	**84**
FMF x Acid water	**88**
FMF x Water	**72**
Acid x Acid water	**89**
Acid x Water	**66**
Water x Acid water	**72**

Table 5. 5. The half matrix similarity of caeca DGGE fingerprints showed the similarities between the replicates of the groups.

	CON1	CON2	CON3	FMF1	FMF2	FMF3	Acid1	Acid2	Acid3	AW1	AW2	AW3	WAT1	WAT2	WAT3
CON1															
CON2	93														
CON3	94	88													
FMF1	81	76	84												
FMF2	75	70	78	93											
FMF3	83	87	84	84	80										
Acid1	85	87	84	76	71	87									
Acid2	87	83	90	88	84	89	83								
Acid3	83	79	87	92	88	86	79	92							
AW1	88	83	91	88	84	91	83	96	93						
AW2	80	75	83	95	92	82	75	89	94	89					
AW3	85	81	88	91	88	84	79	91	97	92	93				
WAT1	67	62	70	78	83	65	60	71	76	71	81	77			
WAT2	64	59	67	75	78	60	57	66	71	66	76	72	94		
WAT3	70	65	70	73	74	59	58	64	68	64	72	69	89	89	

CON = control, FMF = fermented moist feed, AW = acid water, WAT = water group. 1-3 refers to replicate number in each case. (n=15).

Adding probiotics to feed or water increased species richness in the caeca as indicated by Shannon's diversity index Figure 5.7.

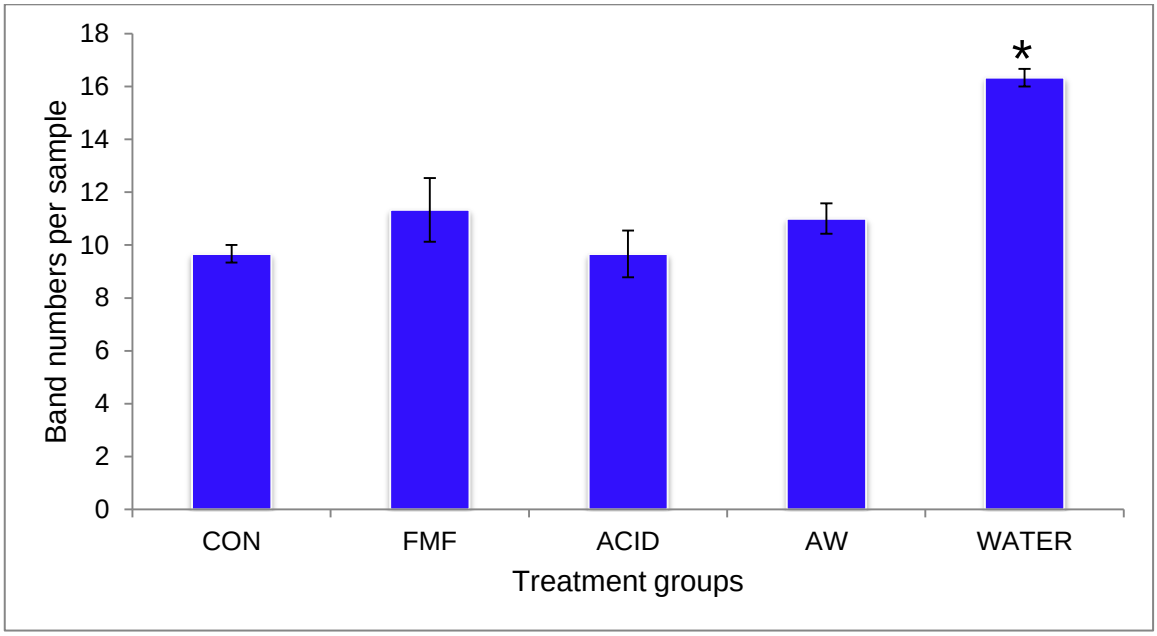

Figure 5. 7. Average DGGE fingerprints band numbers of chicken caeca before infected with *Salmonella* Typhimurium. (*) significantly higher different with all treatments (P>0.05). (n= 15).

The ileum of the chicks at age two weeks and before infecting with *Salmonella* Typhimurium was investigated using the same procedures as the caeca. The sequences of bands of the gel which selected depending on its locations as common band for more than one group or as a unique for its group revealed many different type bacteria Figure 5.8 and Table 5.6.

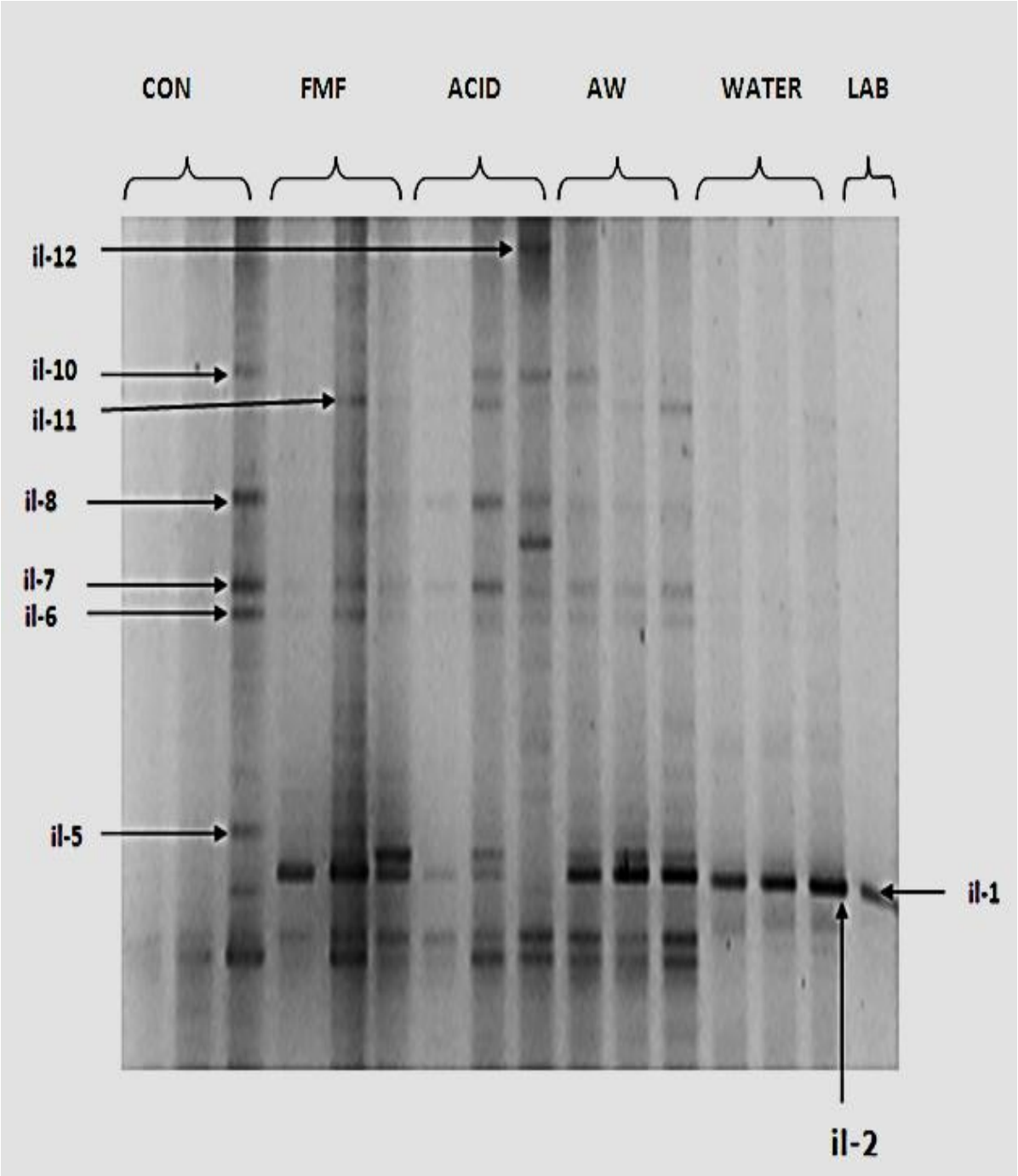

Figure 5.8 Chicken ileum DGGE image of cyber gold stained 40-60% DGGE gel, the marked bands are successfully sequenced. Each lane represents one sample pooled from two chicks except the LAB lane which is DNA of pure culture *Lb. plantarum*. (n=15).

The band number (il-1) was *Lb. plantarum* and it is found in treated groups FMF, AW and Water groups only. *Lb. plantarum* (il-1) was absent in the control group but some traces of the band in an acid treatment group and this may be contaminated from the other groups during the field trial. This result confirms the survival of *Lb. plantarum* in chicken GIT. The control group has a unique band (il-5) which

is not appearing in the other treatment groups and this was identified as *Bacillus amyloliquefaciens* subsp. plantarum strain Kt7-3. The acid group also had a unique band (il-12) which was an uncultured Clostridiaceae bacterium clone MFC-G7. Other bands appear in both acid and control treatment group only were il-10 and il-8, they were *Curtobacterium oceanosedimentum* strain Kb28 and *Bacillus amyloliquefaciens* subsp. *plantarum* strain Kt10-12, respectively. The control and water treatment group had no band il-11which represents a *Bacillus spp*. TMNR3.3. The band number il-6 and il-7, *Bacillus amyloliquefaciens* subsp. *plantarum* were absent from the water group only.

The sequencing results of selected bands were illustrated in Table 5.6.

Table 5. 6. Summary results of sequencing analysis bands of DGGE fingerprints of poultry ileum samples. A= before infection, B= after infection.

Band number	NCBI Accession number	Query coverage	Max. Identity	NCBI BLAST matches
Ileum1	**AB617651.1**	61%	100%	*Lactobacillus plantarum* gene
Ileum5	**JF460753.1**	100%	100%	*Bacillus amyloliquefaciens* subsp. *plantarum* strain Kt7-3
Ileum6	**JF460755.1**	100%	100%	*Bacillus amyloliquefaciens* subsp. plantarum
Ileum7	**JF460758.1**	100%	100%	*Bacillus amyloliquefaciens* subsp. plantarum
Ileum8	**JF460758.1**	100%	100%	*Bacillus amyloliquefaciens* subsp. plantarum strain Kt10
Ileum10	**JF460771.1**	100%	100%	*Curtobacterium oceanosed-imentum* strain Kb28
Ileum11	**JN596245.1**	92%	100%	Bacillus sp. TMNR3.3
Ileum12	**HM043229.1**	100%	86%	Uncultured *Clostridiaceae* bacterium clone MFC-G7

The average similarity within the control group is 39%, FMF 65%, Acid group 68%, Acid water group 52% and finally the water group 48%. The average bacterial population similarity ranged from 33% between acid and water groups to 71% between acid and acid water groups as shown in Table 5.7.

Table 5. 7. The average similarities of bacterial populations in ilea of all treated in day 14. (n=15).

Groups	Average similarity
Control x FMF	**52**
Control x Acid	**57**
Control x Acid water	**56**
Control x Water	**43**
FMF x Acid	**67**
FMF x Acid water	**70**
FMF x Water	**44**
Acid x Acid water	**71**
Acid x Water	**40**
Water x Acid water	**33**

DGGE fingerprint analysis of non-metric multidimensional scaling (MDS) and cluster analysis of the ileum fingerprints are shown in Figure 5.8. Both analyses indicate the similarity within the group is higher than the similarities between the groups.

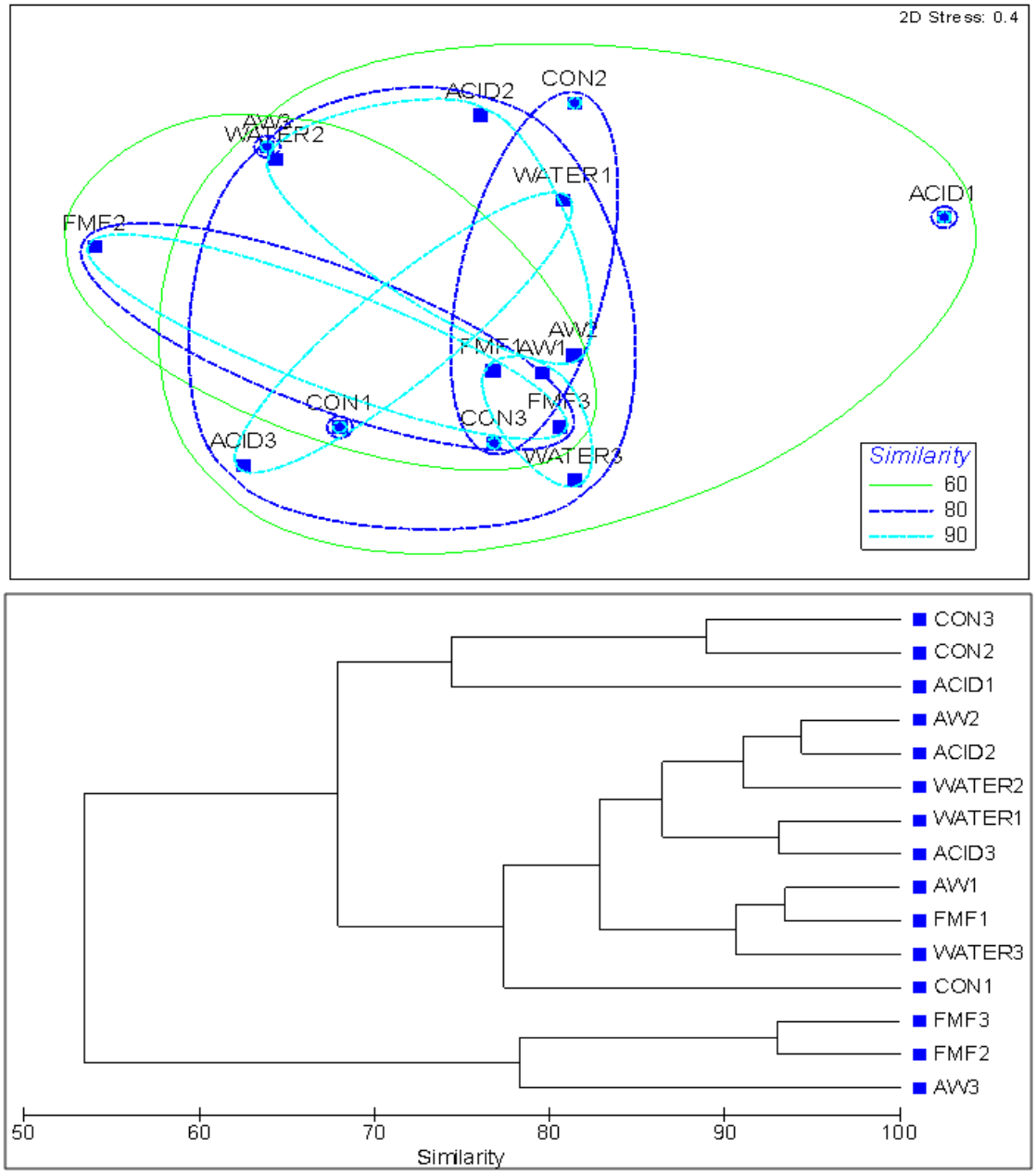

Figure 5. 9. Multidimensional scaling (MDS) (above) and cluster analysis (bottom) of DGGE fingerprints showing similarities of different treatment groups bacterial communities in poultry ileum.

5.3.5.1 DGGE analysis of bacterial at end of trial (day36)

At day 36, six birds per treatments were killed and aseptically the caeca contents were investigated. DGGE results were compared within same group and other groups.

The average similarity within the control group is 72%, FMF 67%, Acid group 50%, Acid water group 72% and finally the water group 47%. Table 5.8 displays the average similarities between the ileum groups

Table 5. 8. The average similarity of bacterial populations of caeca in day 14. (n=15).

Groups	Average similarity
Control x FMF	**72**
Control x Acid	**67**
Control x Acid water	**50**
Control x Water	**72**
FMF x Acid	**47**
FMF x Acid water	**68**
FMF x Water	**62**
Acid x Acid water	**72**
Acid x Water	**62**
Water x Acid water	**63**

The ileum DGGE image shows many different bands in all treatment groups.

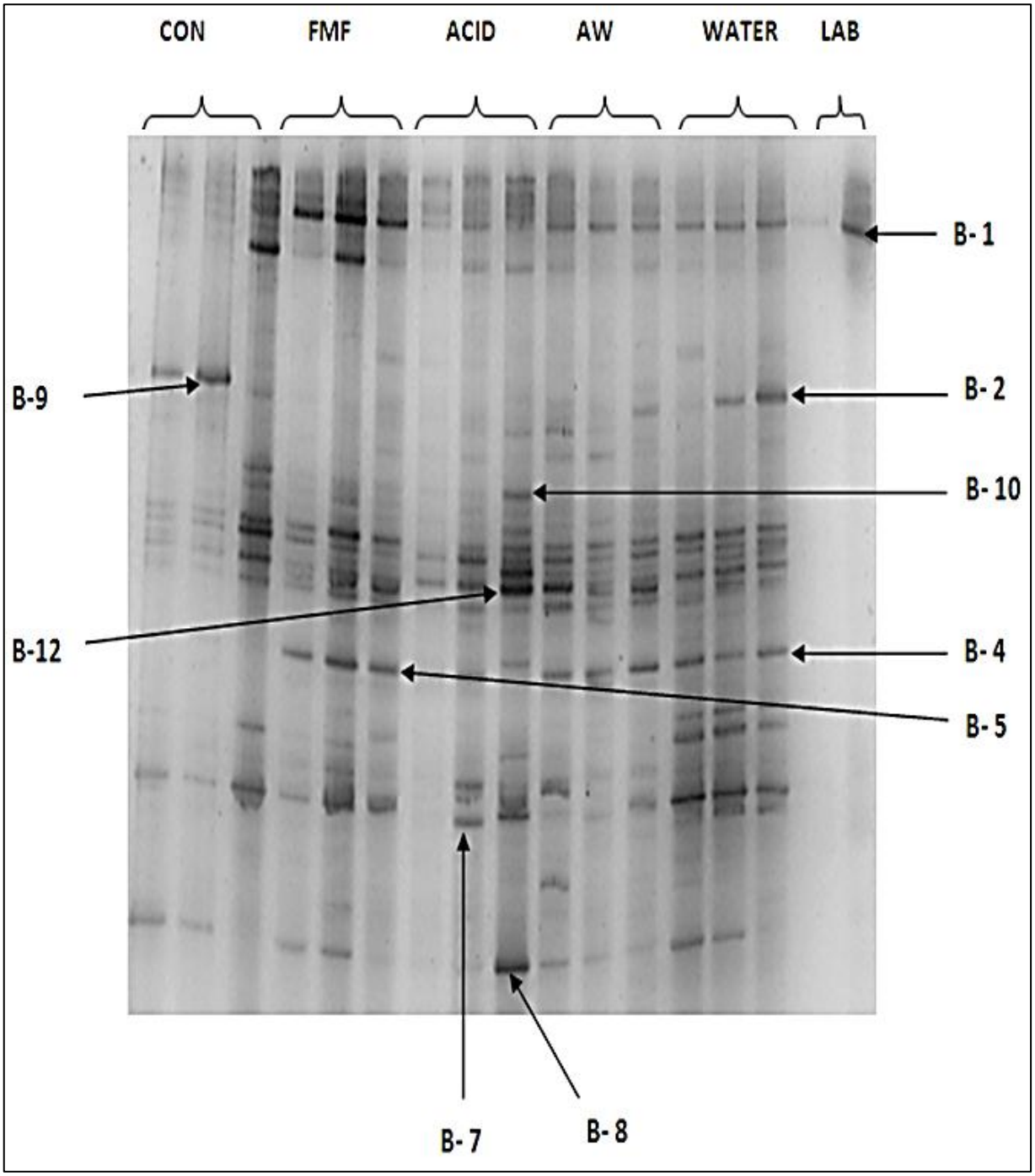

Figure 5. 10. The DGGE of the chicken ileum at age 35 shows the band (OTU) numbering of successful sequencing results.

The selected bands sequencing results showed in Table 5.9.

Table 5. 9. Sequencing results of the chicken ileum contents at age 36 days from NCBI BLAST match results.

Band No.	NCBI Accession number	Query coverage	Max. identity	NCBI BLAST match
Ileum B-1	GU430841.1	100%	98%	*Lactobacillus plantarum* clone WWC_C3MLM108
Ileum-B-2	JF460755.1	100%	100%	*Bacillus amyloliquefaciens* subsp. plantarum strain Kt8-11
Ileum B-4	JF460753.1	100%	100%	*Bacillus amyloliquefaciens* subsp. plantarum strain Kt7-12
Ileum B-5	JN021842.1	100%	97%	Uncultured bacterium clone VDRd28bio58b
Ileum B-7	JN255028.1	95%	90%	Uncultured *Firmicutes bacterium* clone Firm-D-10-
Ileum B-8	JN409252.1	100%	82%	Uncultured actinobacterium clone HG-
Ileum B-9	NR042111.1	96%	93%	*Lactobacillus gallinarum* strain ATCC 33199
Ileum B-10	FR852788.1	100%	100%	Uncultured *Lachnospiraceae bacterium*
Ileum B-12	AB661436.1	100%	100%	*Roseburia inulinivorans* gene

The DGGE fingerprint gel shows many different bands (OTU) which present in each lane. The sequencing results of band number B-1 were *Lactobacillus plantarum* which is presented in all treatment groups except the control group. The acid group and one sample of control group have may be contaminated by *Lb. plantarum*. The band (B-2) *Bacillus amyloliquefaciens* subsp. *plantarum* strain Kt8-11 was not present in acid and control groups only. The band numbers (B-4 & B-5) were present in all treatments except control group and which *Bacillus amylolique-*

faciens subsp. *plantarum* strain Kt7-12 and Uncultured bacterium clone VDRd28bio58b. The band number B-7 and B-8 (Uncultured *Firmicutes bacterium* and Uncultured *Actinobacterium*) were present in all treatment groups. A unique band (B-9) was present in the control group only and resulted was *Lactobacillus gallinarum* strain ATCC 33199. The band number B-10 (Uncultured Lachnospiraceae bacterium) was present in acid and control groups only. The band B-12 (*Roseburia inulinivorans* gene) was present in all groups.

In general the richness of the microbial population was significantly increased in ileum after infection in all treatments including the control group. However, the caeca results were different; the richness of microbial populations was decreased after infection including the control group.

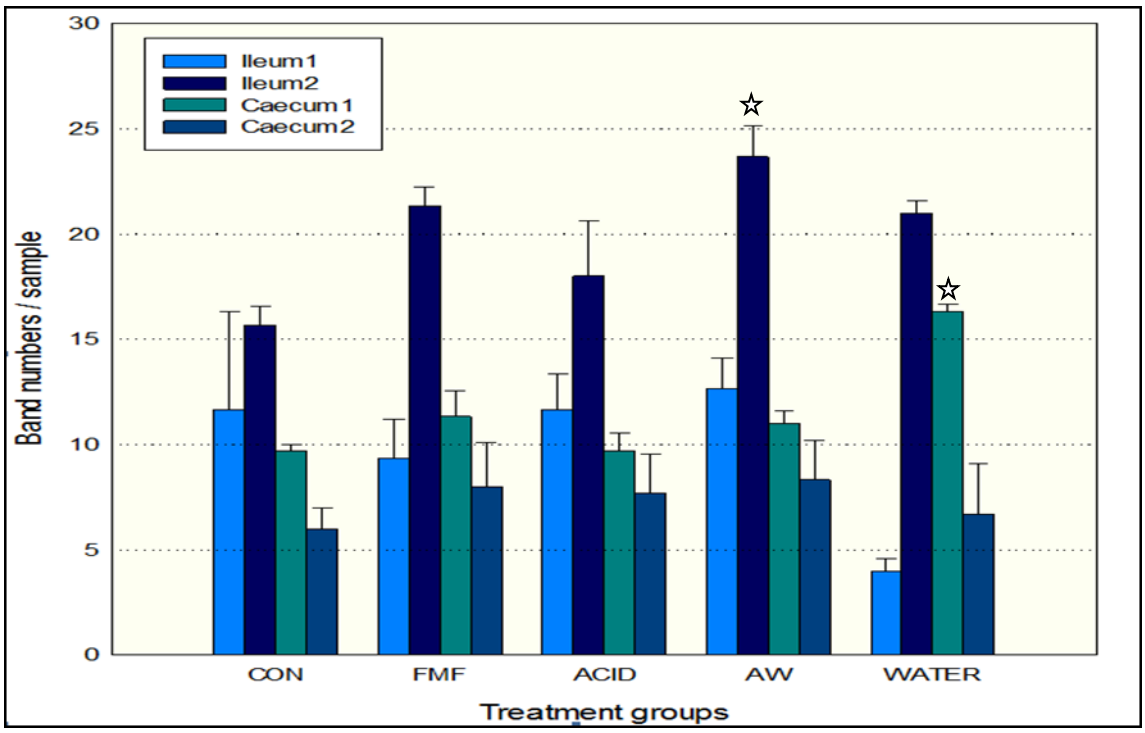

Figure 5.11. Comparison of average Shannon's diversity indices detected in DGGE fingerprints of ileum and caeca before and after infection samples (n=15). (*) significantly different with the same organ in all treatments.

5.2.5 RISA analysis of bacterial community profiles

Agarose-RISA results before and after infection revealed few numbers of bands. (OTU) 1- 3 per each sample.

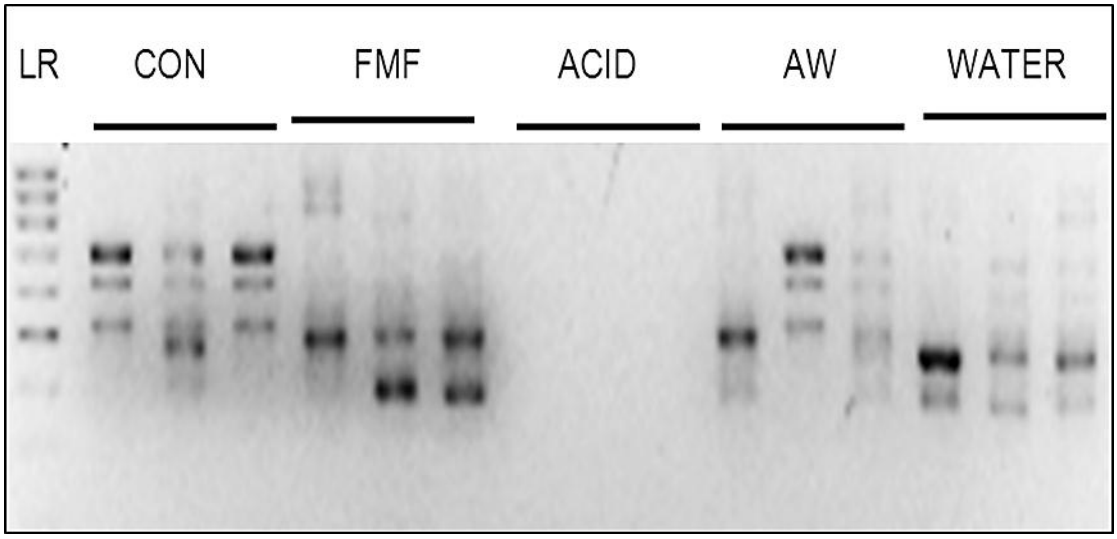

Figure 5.12. The RISA - Agarose image of chicken ilea of all groups after infection by *Salmonella* Typhimurium, LR= 100 bp ladder, CON= control, FMF= fermented moist feed, AW= acid water group.

However, PAGE-RISA (Poly Acrylamide Gel Electrophoresis) analysis revealed more microbial communities present in all replicates from all intestinal samples Figure 5.13. Some of the bands (OTU) were common to all samples and all replicates. There was a difference in band numbers and distribution within the same treatment as well as between treatments. The diversity was different according to the age and diet and can assess by species richness and evenness.

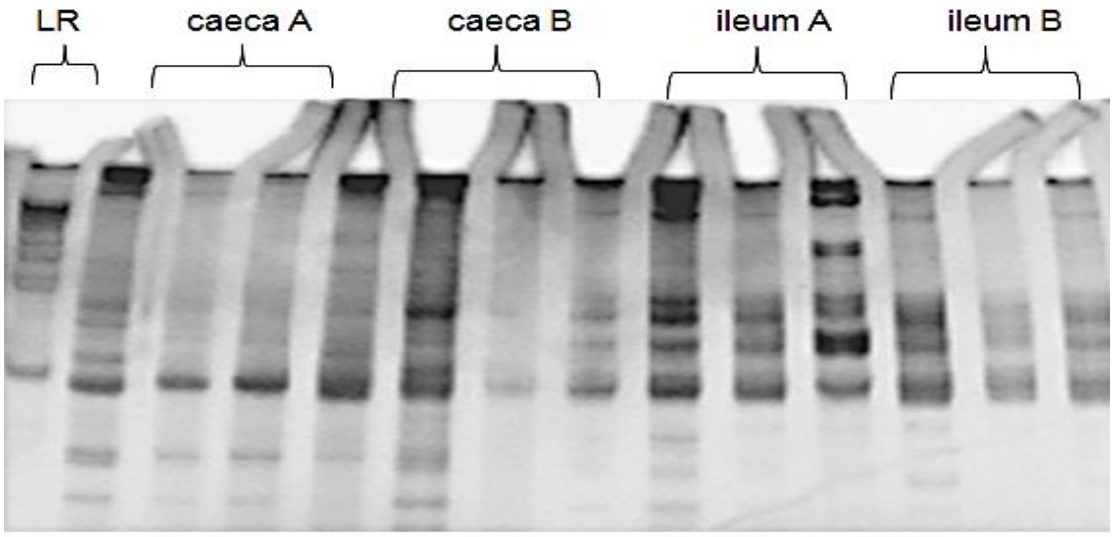

Figure 5. 13 PAGE-RISA image of control group both caeca and ileum before and after infection, banding patterns represent fingerprints of the bacterial communities present in the samples. LR= 100 bp ladder, (n = 12).

The half matrix similarity of caeca of control treatment before and after infection is shown in Table 5.10 and indicates the average similarity within the caeca control group before the infection is 77% and 90% after infection. The average bacterial population similarity between the caeca before and after infection was 71%.

Table 5. 10. Half matrix similarity of caeca of control group before the infection (A) and after infection (B) shows the similarities between replicates.

Control caeca	CON A1	CON A2	CON A3	CON B1	CON B2	CON B3
CON A1	**100**					
CON A2	**88**	**100**				
CON A3	**73**	**69**	**100**			
CON B1	**84**	**74**	**65**	**100**		
CON B2	**77**	**66**	**59**	**91**	**100**	
CON B3	**81**	**71**	**67**	**92**	**88**	**100**

In general, from RISA fingerprints results of caeca showed the number of bacterial population before infection was less than after infection in all treatments and vice versa for the ileum Figures 5.14 & 5.15.

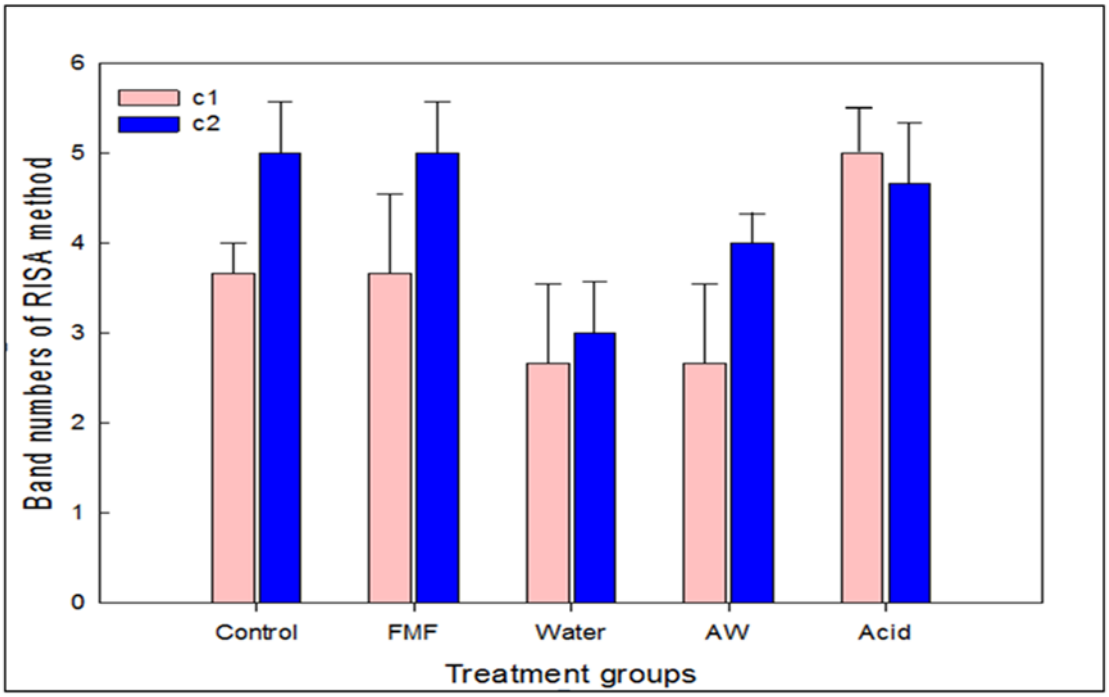

Figure 5. 14. PAGE-RISA gel band numbers of chicks caeca day 14 (C1) and day 36 (C2).

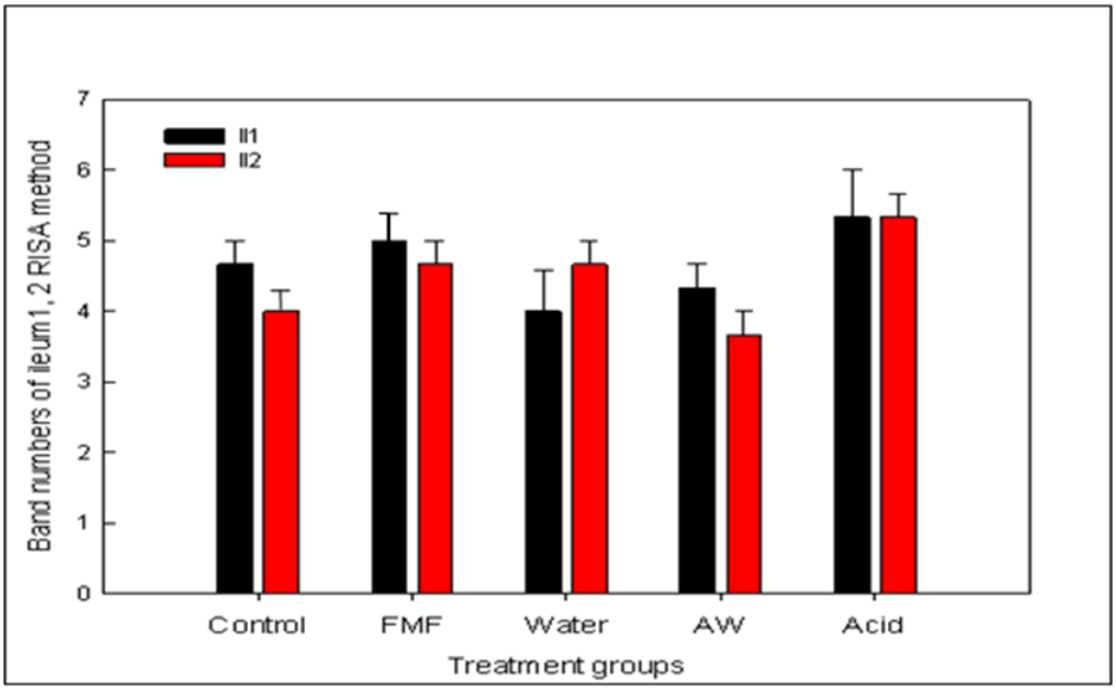

Figure 5. 15. PAGE-RISA gel band numbers of chicks ilea day 14 (il1) and day 36 (il2).

5.2.6 Sequence analysis

The sequence analysis of the pure culture (MS18 & lyophilized bacteria) confirmed the identification of *Lactobacillus plantarum* which is used in this study. The sequencing results attached in appendix 1.

5.2.7 Haematology

The analysis of variance indicates no significant difference ($P<0.05$) in white blood cell counts before and after infection. There are decreases in lymphocyte percentage after infection in FMF and acid groups but these were not significant. Heterophil percentages were increased in all groups except the acid group but again these were not significant and there was no change in the control group.

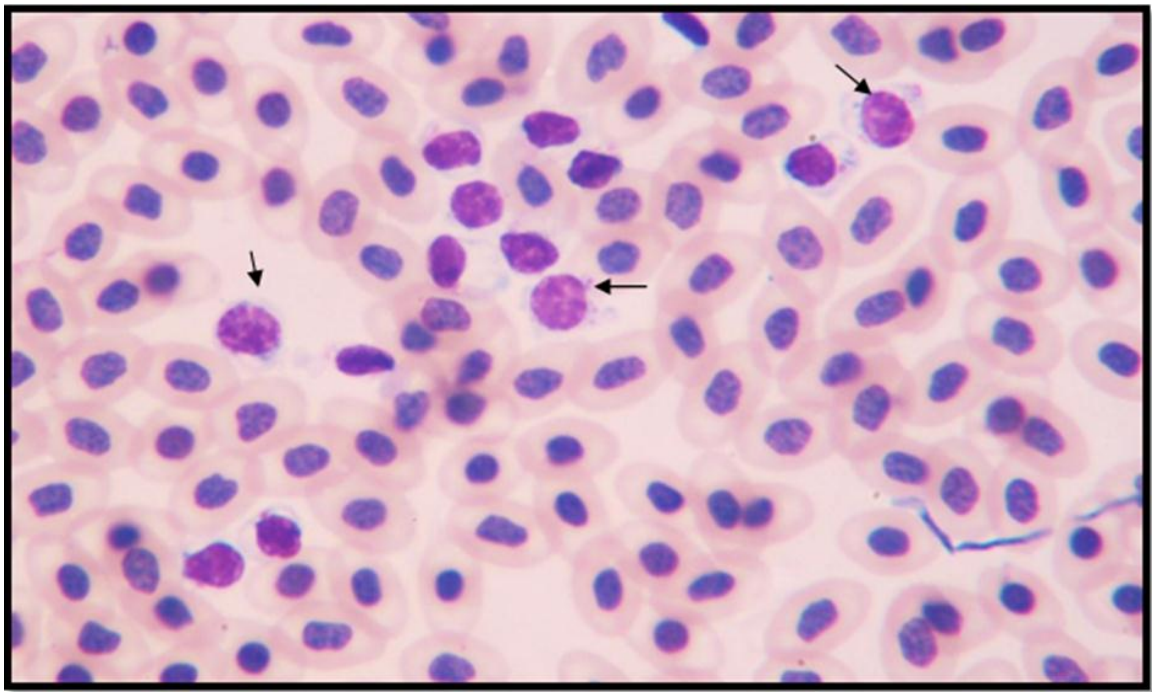

Figure 5. 16 Blood film of chicken showed the red blood cells have a nucleus and many lymphocytes. Arrows are leukocytes, Giemsa stain used with magnification X100.

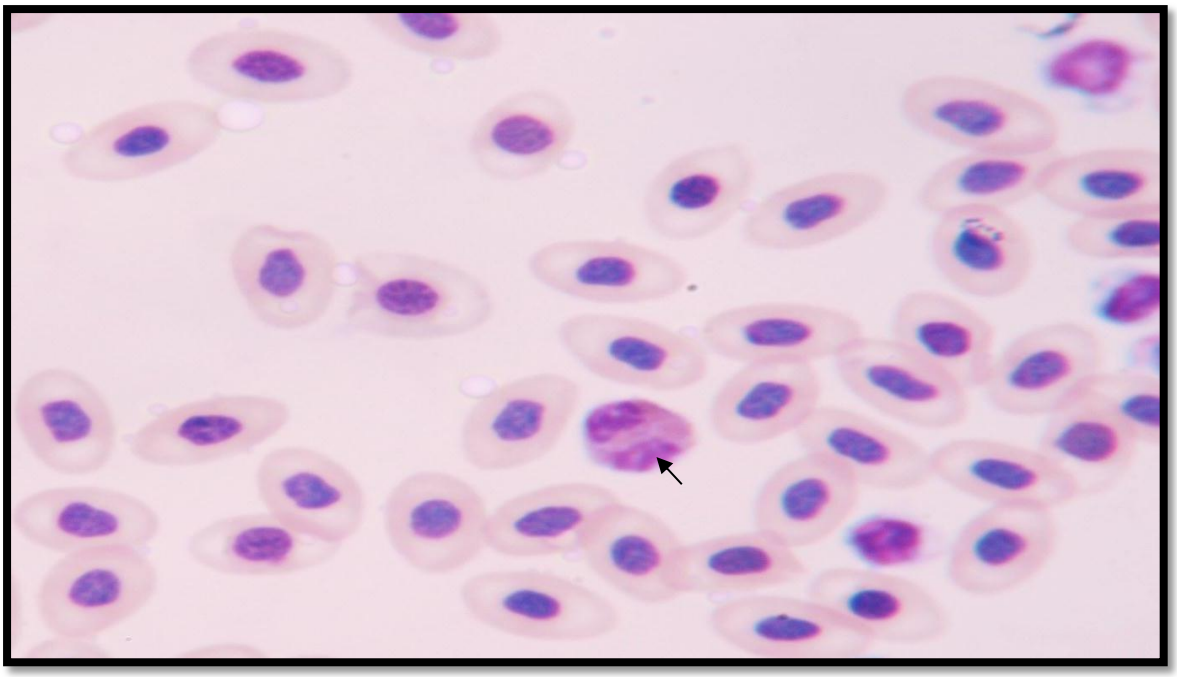

Figure 5.27 Blood film of chicken showed red blood cells (RBCs). The arrow is leukocyte, Giemsa stain used with magnification X100.

Table 5. 11. Results of WBC's counts and heterophil/ lymphocyte ratio in all treatment groups on day 14 and 36 of trial and.

	Control		FMF		ACID		Acid water		WATER	
Day	**14**	**36**	**14**	**36**	**14**	**36**	**14**	**36**	**14**	**36**
Lymphocyte	85	85	87	83	90	85	90	90	90	91
Heterophil	10	10	8.3	9	7	11.7	6	7.3	6.3	7
H/L ratio	0.12	0.12	0.095	0.11	0.078	0.14	0.067	0.081	0.070	0.08

5.2.8 Histology

- ***Scanning electron microscope (SEM)***

SEM confirmed a number of cocci shape bacteria and some rod shapes with 1.5 - 2 µm diameter at the top and between the microvilli which cover the villi of the FMF group. These bacterial populations were not present in control group chicken. The finger shaped villi Figures 5.18 and 5.19 have broad bases tapering to blunt points and between 0.5 – 0.9 mm height. The shapes of villi are different from the circular, flattened or tongue shaped villi Figures 5.20 & 5.21. At X1000 magnification the outlines of individual epithelial cells are seen. They are flattening topped or gently convex Figure 5.22 & 5.23 and they vary in size. At higher magnification (X1000 to X3000) microvilli can be seen clearly.

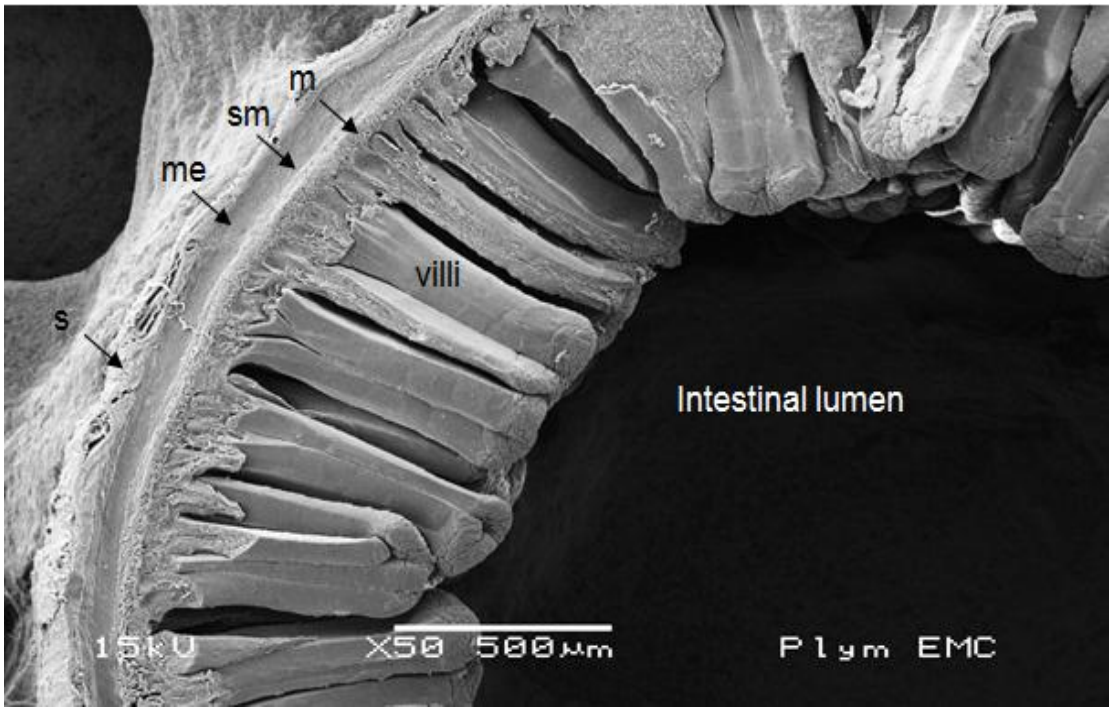

Figure 5. 18 SEM micrograph of control chicken ileum layers, m= mucosa, sm= sub mucosa, me= muscularis externa and s= serosa layer.

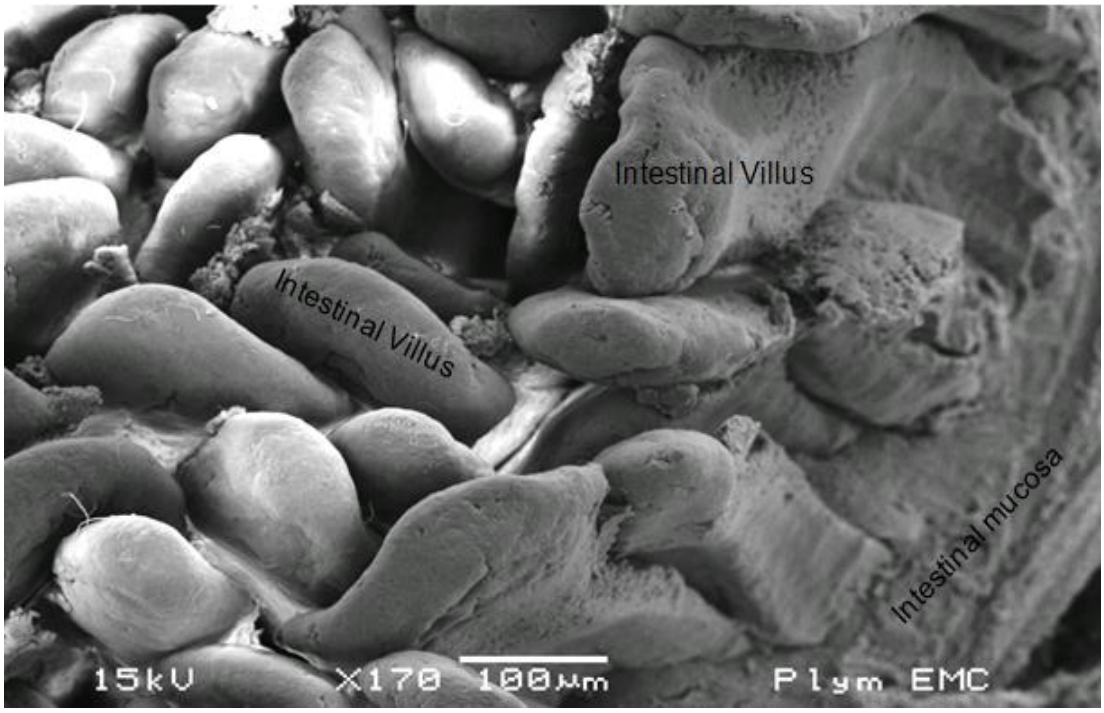

Figure 5. 20. SEM micrograph of gut of chicken control group showed a top view of the intestinal villi density and different shapes of villi.

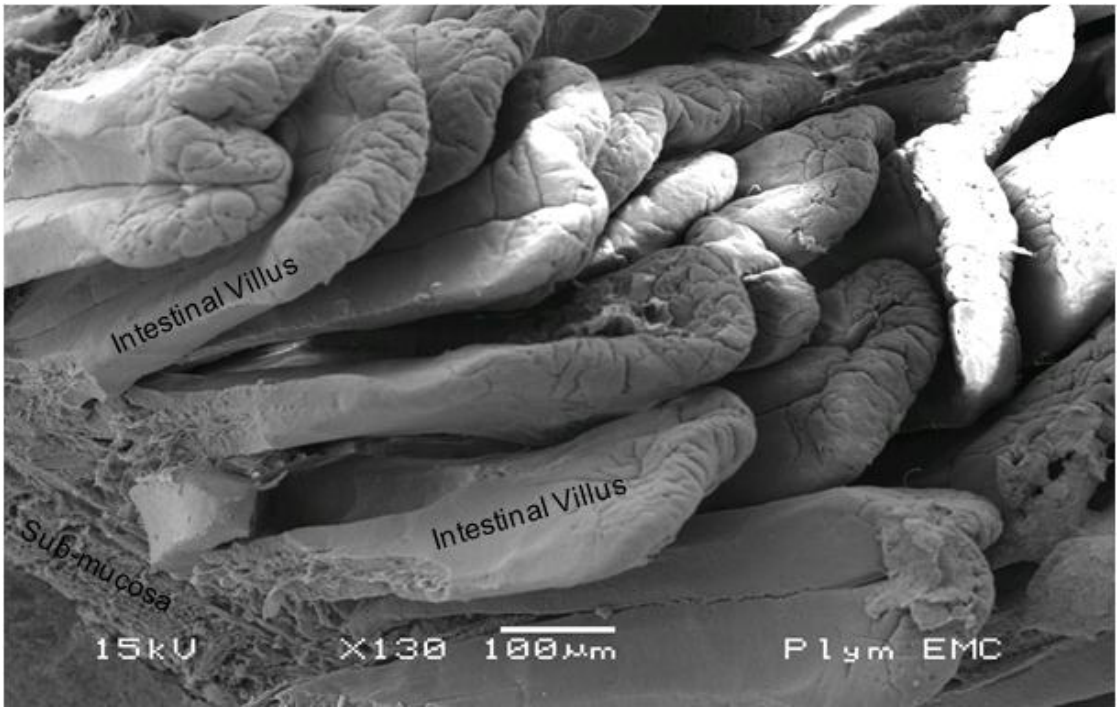

Figure 5. 21. SEM micrograph of top side view of the intestinal villi of FM treatment group showed the density and tongue shapes of villi.

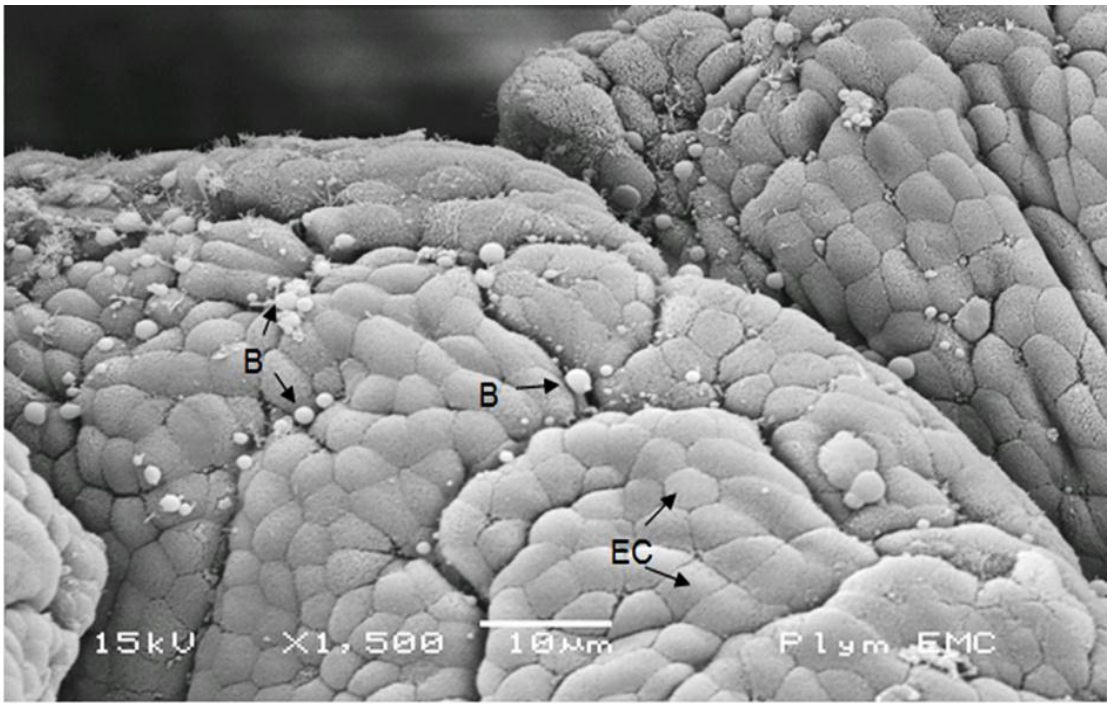

Figure 5.22. SEM micrograph of bacterial colonisation in the ileum of the chicken fed FMF. B= bacteria and EC= epithelial cells.

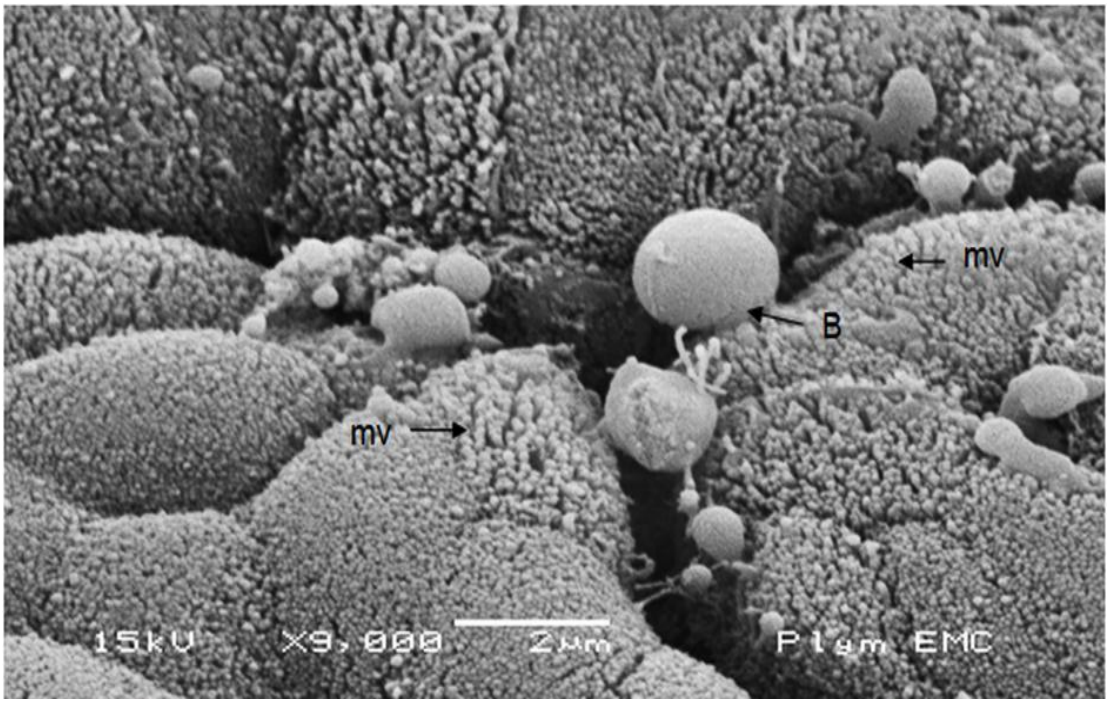

Figure 5. 23. SEM image with bigger magnification showed more details of the microvilli and two types of bacteria. B= bacteria and mv= microvilli.

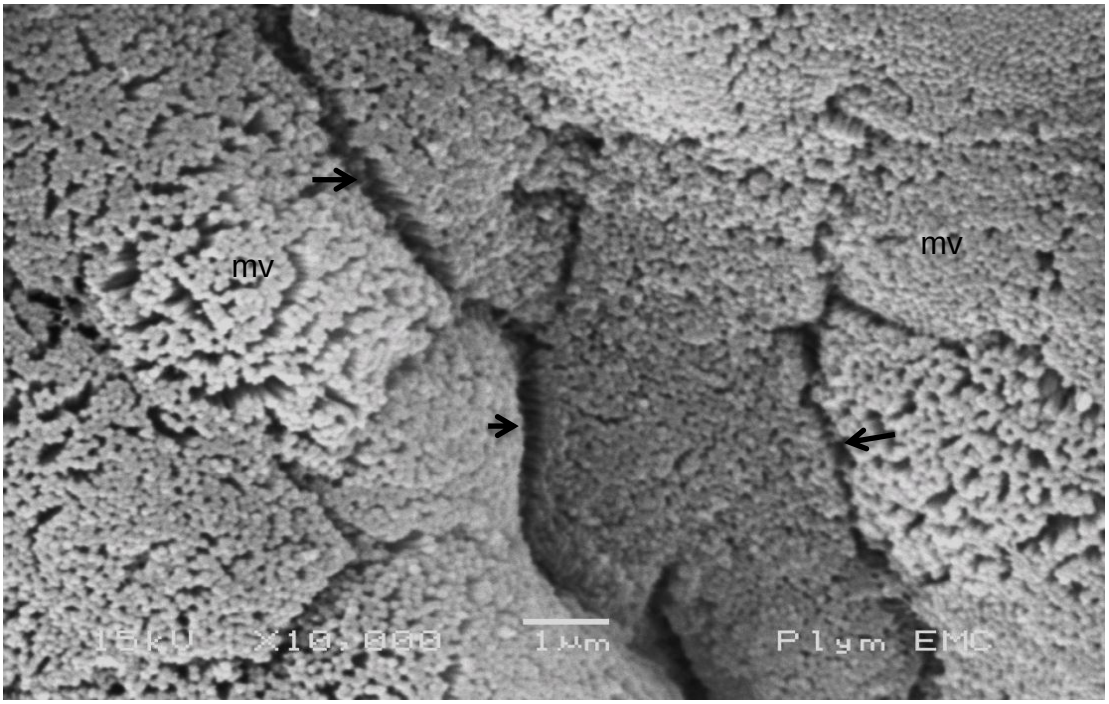

Figure 5. 24. SEM image of the ileum of chicken fed FMF showed the microvilli with a regular distribution (mv) and the edge of the enterocytes (arrow).

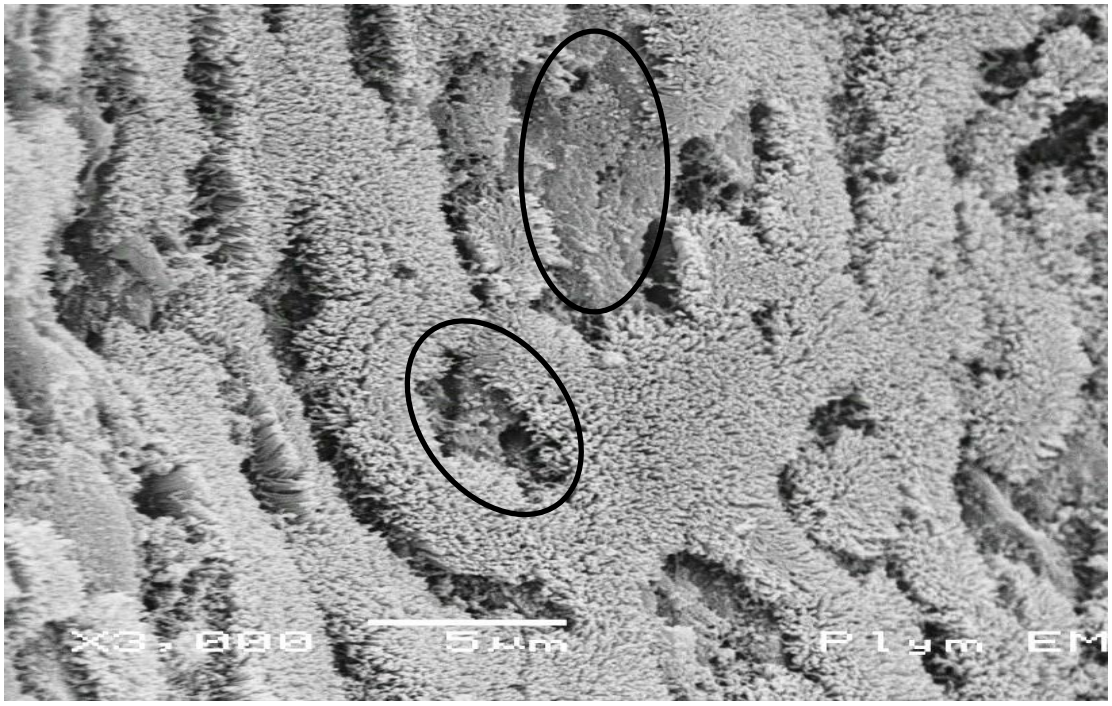

Figure 5.25. SEM image of the control ileum chicken group showed the deformed, irregular distribution of microvilli (oval mark) on the top of villi.

The rod shaped microvilli appear as a separate structure giving the cell surface a feathery texture particularly in an FMF group while in the control group there are some patches of missing microvilli. All villi of treated groups were significantly higher than control group ($p < 0.05$). These results suggest a benefit of administration of probiotic and organic acids (lactic acid) to the chicken. Microvilli are very important indicators of intestinal health as an increase in the number and size of the microvilli mean the absorption capacity of the gut is increasing.

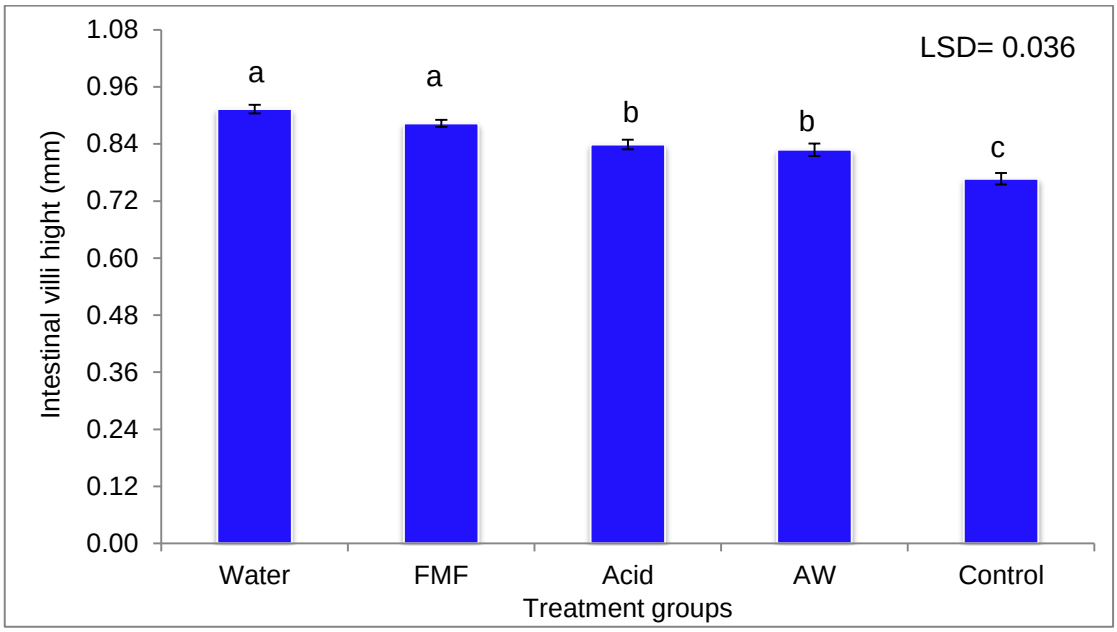

Figure 5. 26. Intestinal villi heights (mm) mean ±SE for all treated groups.

Light microscopy

The results of classical histology stained by Haematoxylin Eosin stain (HE) and examined by light microscope confirmed the results of SEM regarding the villi height. The enterocytes nuclei were stained with Haematoxylin with blue colour while the cytoplasmic contents stained by Eosin stain. Figures 5.27 & 5.28 of the

cross section of intestine showed all the layers of and villi and the top of villus stained by (HE).

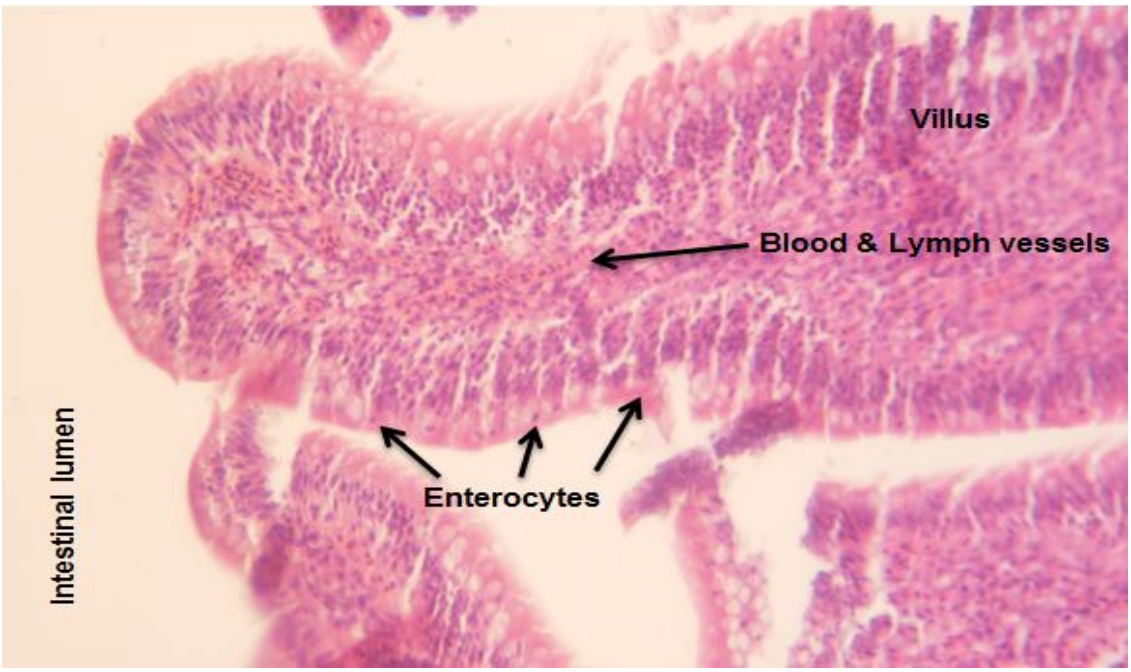

Figure 5.27. Cross section of chicken ileum showing Blood and lymph vessels, enterocytes, goblet cells which composed the villus.

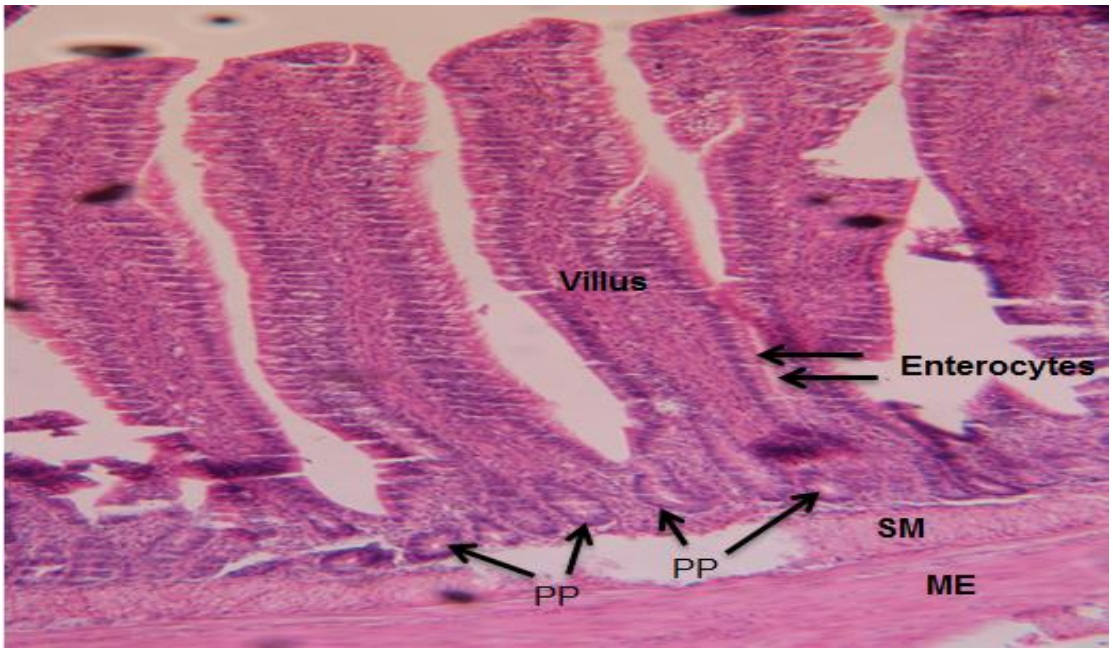

Figure 5. 28. Chicken ileum using HE. Two important components of the intestinal immune system are the enterocytes that form a physical barrier of one single cell layer and the gut-associated lymphoid tissue (GALT) system consisting of various immune cells (T-cells, B-cells, dendritic cells & macrophage cell) in the small intestine clustered in follicles known as Peyer's Patches (PP).

- ***FISH of pure culture bacteria results***

The FISH technique used with pure cultures of LAB and *Salmonella* Typhimurium with specific fluorescent probes designed from their oligonucleotide sequencing and labelled with fluorescent dyes (FITC for LAB and TRITC HEX for *Salmonella*). FITC fluorescent gave brilliant green colour while HEX fluorescent gave a red colour Figure 5.29. A general bacterial probe EUB338 showed both *Lb. plantarum* (small brilliant green rods) and *Salmonella* Typhimurium (larger brilliant green rods) as showing in Figures 5.30.

- ***FISH of tissue sections results***

A biofilm of LAB on the edge of intestinal villi was shown as a brilliant white Figure 5.34 and yellow brilliant colour in all layer technique image Figure 5.33, DAPI was used to stain all tissue nuclei DNA including the bacterial cell's DNA Figure 6.32. The image results were negative for *Salmonella* species.

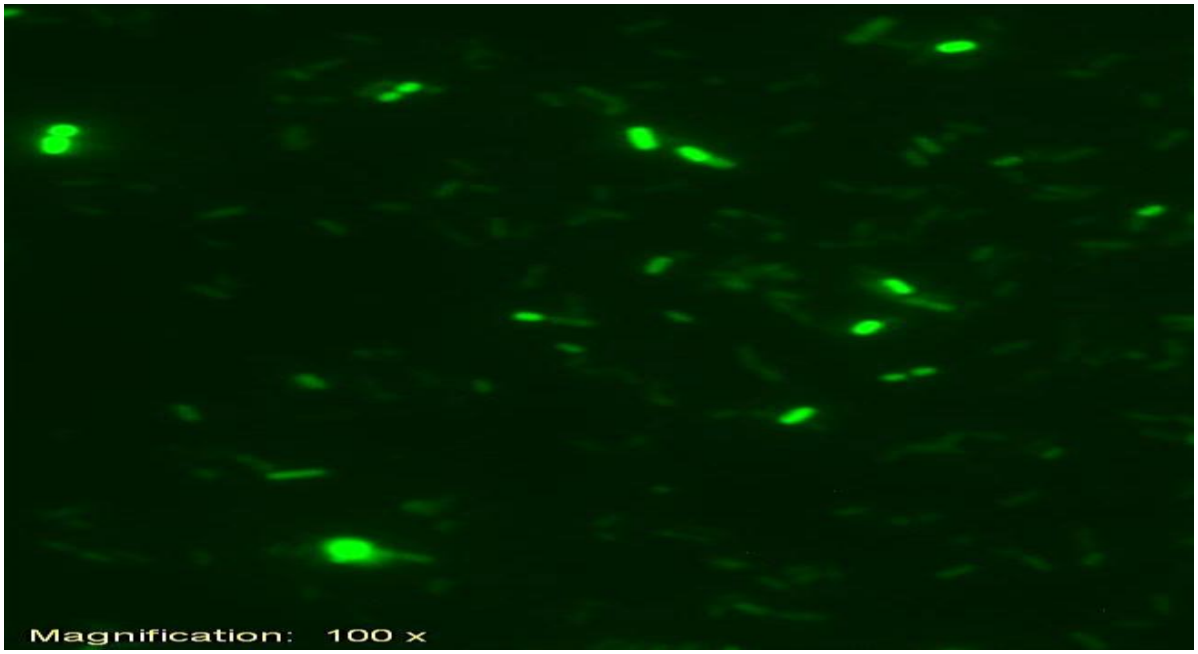

Figure 5. 29. The FISH analysis of LAB bacteria culture (the brilliant green) using specific probe visualised by epiflourecence microscope.

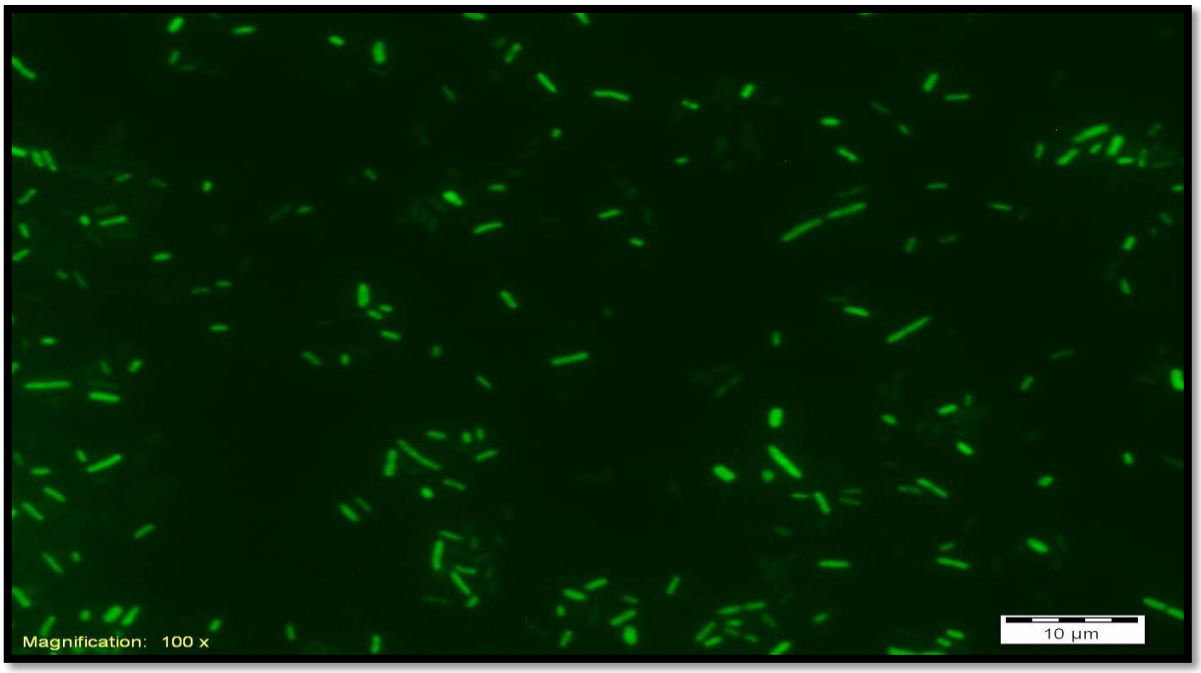

Figure 5. 30. The FISH analysis of both LAB and *Salmonella* bacterial culture using general bacterial probe EUB 383.

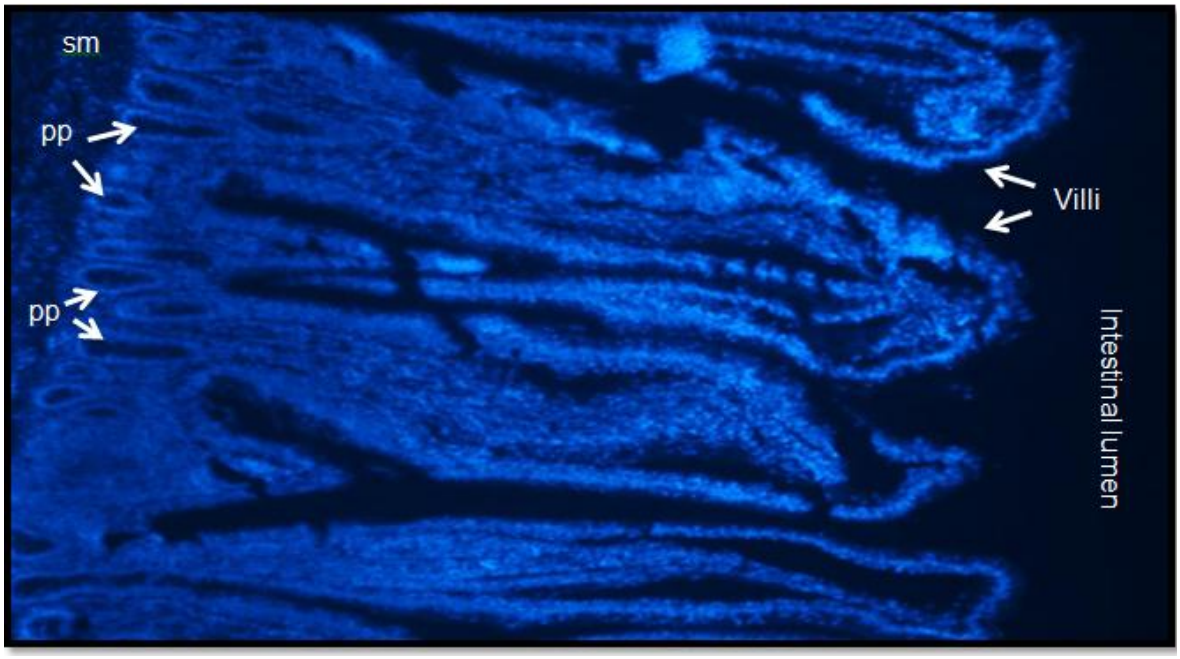

Figure 5. 31. The FISH analysis image of the ileum of chicken fed FMF using DAPI dye visualised by epiflourecence microscope. The image showed nucleus of the all intestinal cells including enterocytes cells (blue) of intestinal villi and Peyer's patch (pp).

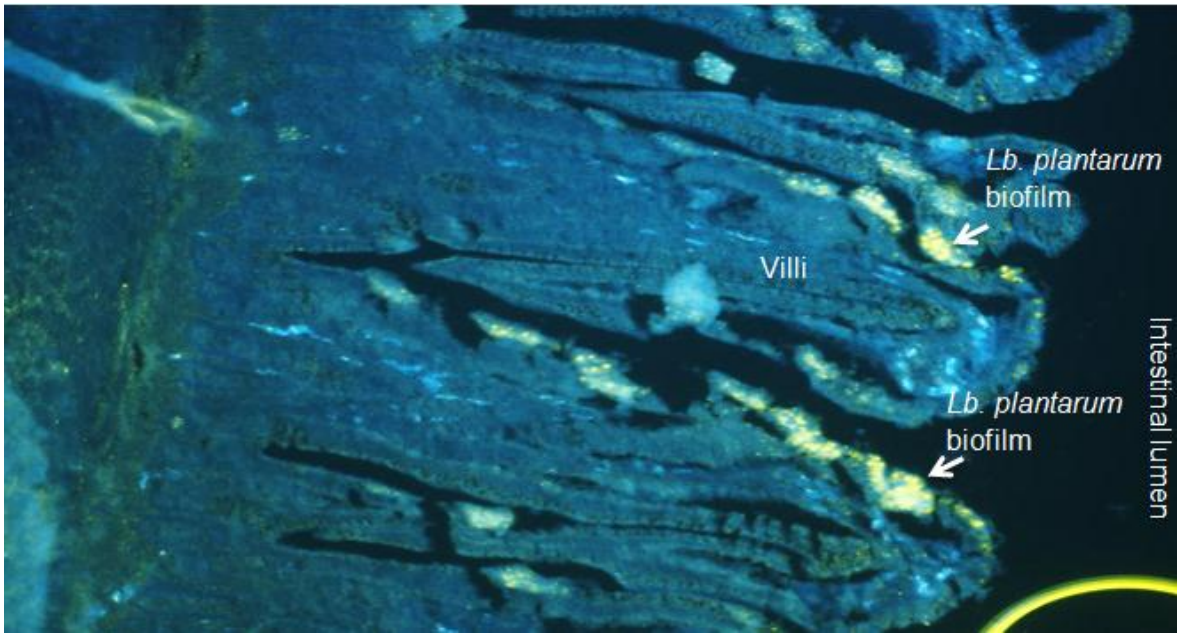

Figure 5.34. The FISH analysis merged image of the ileum of chicken fed FMF using *Lb. plantarum* probe and visualized by epiflourecence microscope. The image showed the LAB biofilm like (brilliant green-yellowish) on the intestinal villi which stained with DAPI (blue).

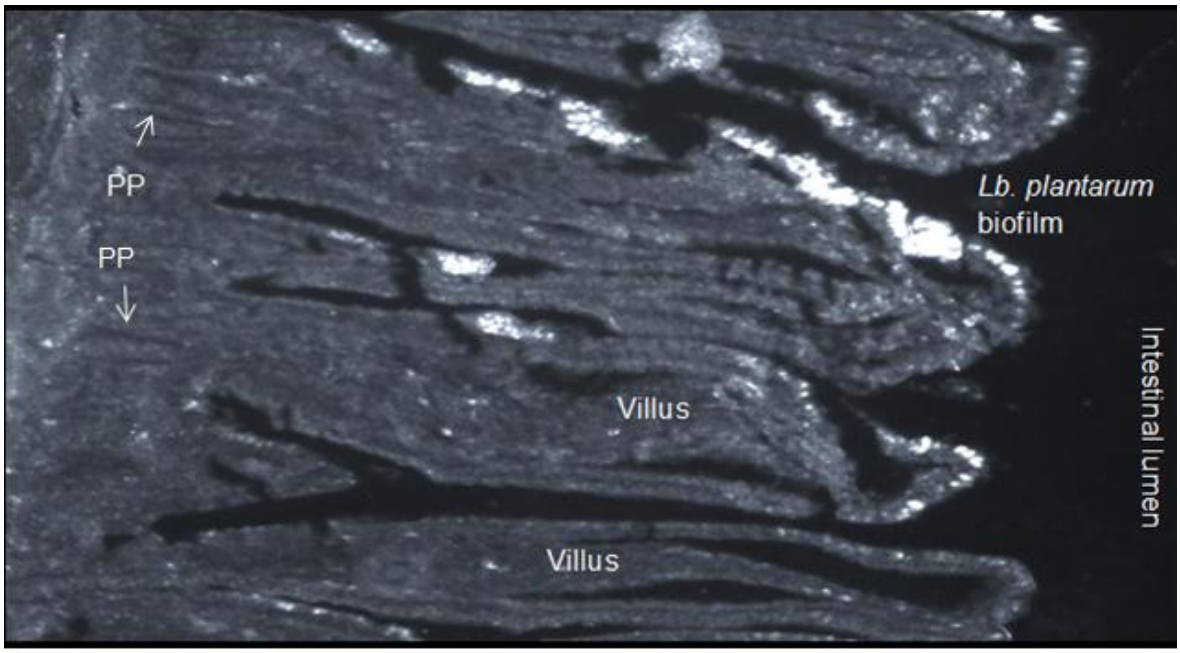

Figure 5.33. The biofilm of Lactobacilli in the ileum of chicken fed FMF which analysed by FISH using a specific LAB probe visualised by epiflourecence microscope. The image showed the LAB biofilm like (white brilliant colour) on the villi of intestine.

5.3 Discussion

The aim of this study was to investigate the inclusion of feed additive (probiotics or lactic acid) on *Salmonella* Typhimurium and microbial population of the chicken gut and on growth performance. There was no effect of treatments on FCR but the total feed intake of the wet feed (FMF and AMF) was significantly (P> 0.05) less than dry feed of control and water groups. Adding probiotic through feed in this study did not affect body weight and this agreed with the results of many researchers (Al-Zenki, Al-nasser & Al-Saffar, 2009; Eckert et al., 2010; Jin *et al.*, 1998; Kabir et al., 2004; Kalavathy et al., 2008; ; Olnood, Choct & Iji, 2007; Opalinski *et al.*, 2007). However, other researchers reported significant effects on increasing body weight (Apata, 2008; Awad *et al*., 2009; Eckert *et al.*, 2010; Engberg *et al*., 2009; Faria Filho *et al*., 2006; Jin *et al.*, 1998; Kabir *et al.*, 2004; Kalavathy *et al.*, 2008; Khaksefidi & Ghoorchi, 2006; Niba, 2008; Samanya & Yamauchi, 2002; Vicente *et al.*, 2007b; Wang & Gu, 2010).

At day 14 of the trial, all the chicks were infected with *Salmonella* Typhimurium. At day 36 there was no *Salmonella* detected in all groups including the control group. Many researchers have reported a reduction of *Salmonella* by probiotics *in vivo* (Higgins *et al.*, 2008; Niba, 2008; Savvidou, 2009; Vandeplas *et al*., 2009b; Wolfenden *et al*., 2007). Mead (2000) proposed four mechanisms includes competition for nutrient or receptor sites or production of bacitracin or SCFA. In this trial *Lb. plantarum* used which produced lactic acid and this may increase the pH value in the intestine chyme which may be inhibiting the mechanism of *Salmonella* (Canibe *et al.*, 2005). In this study, the pH was not measured, so no conclusion can be made. Another option that the reduction of *Salmonella* may be due to inherit ability of this bird type to resist (Al-Murrani, Al-Rawi & Raof, 2002).

The numbers of LAB and Coliform were higher in caeca than ileum. These results agreed with earlier studies that show the numbers of viable bacteria were higher in the caeca than ileum (Niba, 2008; Salanitor, Blake & Muirhead, 1978; Savvidou, 2009). The numbers of LAB and Coliform in both caeca and ileum after infection (day 36) were more than before infection (day 14), this result may be because the chicks age and not associated with the stress of infection.

Many researchers have used one dose of probiotic with no reduction of *Salmonella* in chicken (La Ragione *et al*., 2004; Zhang, Ma & Doyle, 2007). Nor did the continuous supply of probiotic in this study prevent infection by Salmonellae. The suggested level of probiotic to have an antibacterial effect is between 10^7 – 10^9 CFU/g feed (La Ragione *et al.*, 2004; La Ragione & Woodward, 2003; Tellez *et al*., 2001; Vicente *et al.*, 2008; Zhang, Ma & Doyle, 2007). In this study *Lb. plantarum* was supplied continuously at a rate of 10^8 -10^9 CFU/g in FMF and water treatment groups.

In general, the viable LAB bacteria in caeca were more than ileum but not significantly different ($p > 0.05$). These results are agreed with (Mountzouris et al., 2007) but Jin et al. (1998) claims there is no significant effect of probiotics on population profiles.

The LAB produces short chain fatty acids, lactic acid and acetic acid, during fermentation which decrease the pH of feed and that might act against *Salmonella*. FMF confers some health benefit by increasing beneficial microbes and decreasing pathogens (Patterson & Burkholder, 2003). Savvidou (2009) and Niba (2008) claim that prophylactic treatment of chicken by FMF protected the chicks from infection by *Salmonella*. A significant reduction in susceptibility of broilers to coloni-

sation by *Campylobacter jejuni* in chicken fed the FMF was reported by Heres *et al*. (2003a). In their studies, they claim that improvement of the barrier function of the crop and gizzard accorded by FMF consumption protected chickens against *Salmonella* but the lower intestinal compartment did not show a substantial effect reduction in *Salmonella*. Heres *et al*. also claim that broiler chickens fed FMF are less susceptible to a single oral administration of *Salmonella* than chicken fed normal dry feed, and that chickens fed FMF needed a higher challenge dose of *Salmonella* to establish infection. In this study, all chickens on all treatments became infected after one dose of *Salmonella* by the oral route (Log $1X10^6$ CFU/chick). However, all chickens including control were clear of infection two weeks later.

The AMF treatment was designed with the same concentration of lactic acids which produced by FMF and the aim was to reveal whether the effect of FMF treatment was due to the production of lactic acid alone or whether higher concentrations of LAB had an effect. Some commercial poultry products are available as acidifiers, such as Bio-Add liquid (formic acid, total formic acid, ammonium formate and propionic acid) to control *Salmonella*. Heres *et al*. in (2004) used 5.7% of lactic acid or 0.7% acetic acid in a chicken trial and reduced *Salmonella* to an undetectable level. The high concentration of lactic acid reduced the pH in the crop and gizzard, which reduced the numbers of *Salmonella* passing into the lower digestive system. The mechanism of action of the acids against *Salmonella* may be due to act their ability to down-regulate invasion genes in *Salmonella* (Van Immerseel *et al.*, 2006) or lactic acid acts as a permeabiliser of the *Salmonella* outer membrane which may augment the effect of other antimicrobial substances (Heres *et al.*, 2003a). The numbers of LAB and coliform were less than other treated groups.

In the AW group, the LAB numbers in the caeca and ileum were higher than acid group. The administration of the probiotics via water is the easiest way to deliver probiotics to the chicken. The numbers of LAB at the end of the trial in the WAT group were significantly higher ($P< 0.05$) compared with control, acid and AW groups but not FMF group.

In general, analysis of microbial populations detected by traditional microbiology of this study showed an increase in the number of LAB and Coliform in the caeca after infection (end of trail) and a decrease in the ileum except the FMF group and that may be due to age. The replica plating of rifampicin-MRS was an easy technique to identify *Lactobacillus plantarum*. The rifampicin was a good marker of detection the survival *Lactobacillus plantarum*. About 80% of LAB detected in the caeca and ileum were *Lactobacillus plantarum*.

Traditional microbiological techniques have limited use for classification and identification of the bacterial population. Most bacteria cannot be isolated from their habitats by routine culturing methods. 90% of bacteria isolated from modern molecular work were uncultured bacteria previously (Apajalahti, Kettunen & Graham, 2004). Bacterial morphological classification also is very poor because the very small size of bacteria (Muyzer, 1999). A modern technique of molecular microbiology of DGGE, RISA and FISH was applied in this study.

In this study, the author has not followed the normalization procedure when applying the PCR products into the DGGE gel. Therefore, the DGGE fingerprints represent a qualitative analysis of bacterial population in the different organs. The DGGE analysis revealed complex microbial communities present in all replicates from all intestinal samples. To obtain objective interpretation of complex DGGE

fingerprints, Image J software or Quantity One software of Bio Rad Company and PRIMER6 V.6 software were used. DGGE is a useful tool and may help to evaluate the overall intestinal microbial profile(Muyzer, 1999) The complexity of a single sample can be expressed by diversity indices. Diversity indices can used to assess the diversity of any population in which each member of a unique species. The diversity was different according to the age and diet and can assess by species richness and evenness. Studies suggest that higher 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.

In general, the richness or microbial population was increased in ileum after infection in all treatments including the control group and this may be due to age (Hume *et al.*, 2003). These results agree with the results of (Hume *et al.*, 2003; Lu *et al*., 2003), but disagree with Nakphaichit *et al*. (2011).

In this trial, the caeca results were different before and after infection, the richness or microbial populations were decreased after infection including the control group at day 36 and these results are agree with the results of Lu *et al*. (2003). The DGGE gel band numbers were different within the same group and these results agree with results of Zhu *et al*., (2002) who reared the chickens under very similar conditions.

The sequence analysis of DGGE bands was helpful to know the types of the bacterial population profile. Each sample has different numbers and species (subspe-

cies) of bacteria. The band sequencing results confirm the traditional results of survival of *Lactobacillus plantarum* via the conditions of chick's GIT.

Future work requires more bands be sequenced to confirm all types of present bacteria inside the chicken gut by using this method because cheap or using new techniques of next generation sequencing method.

The RISA results were not very useful concerning microbial population analysis due to the limited number of bands. The RISA analysis was displayed by two methods RISA- Agarose and RISA- PAGE method. Five percent poly acrylamide gel electrophoresis (PAGE) had more bands than agarose gels. This may be due to the PAGE gel pore size is smaller than the agarose pore size and that's allow the small size molecular weight to penetrate via smaller pore sizes of PAGE and separated from the big molecular weight fragments. Limited numbers of bands may be due to the length of the intragenic spacer region of different species being relatively similar.

Blood leukocytes are widely used as an indicator of stress, such as infection, heat or transportation. The (Heterophil/ lymphocytes) H/L ratio is a more reliable indicator of mild to moderate stressor than corticosteroid levels in plasma (Maxwell, 1993). Leukocytes are known to be affected by stress. A decrease in the numbers of lymphocytes and monocytes and an increase Heterophil numbers were reported Al-Murrani, Al-Rawi & Raof, (2002). Al-Murrani claims the selection for general resistance to *Salmonella* based on H/L ratio should conducted under stressor such as under prevailing rearing condition or heat to allow the H/L ratio to express itself normally.

The H/L ratio decreased after infection in all treated groups compared to control group but not acid group. This reduction of H/L ratio maybe because the adding of probiotics and these results agreed with Khajali *et al*., (2008). The H/L ratio is increased under stress and infection but using probiotic decreased the ratio. The H/L ratio is under the control of few genes and is changed depending on different stressor such as bacterial infection, heat and viral infection (Al-Murrani, Al-Rawi & Raof, 2002).

The histological examination of the intestine was done by scanning electron microscopy (SEM) and light microscopy (LM). SEM is a useful tool for demonstrating mucosal colonisation in the digestive system. The procedure was not complicated but the chance of determiner images with bacteria was not easy possibly because sample size compared to the long size of the intestine or the numbers of bacteria were not sufficient to determine. SEM images showed the bacteria cells on the enterocytes cells of the intestine and other intestinal epithelial cells that may play a role in host immune stimulations. The histology by light microscope using HE stain allowed differentiation between the cells, lumen villus height to be used as an indicator of intestinal change in the chicks. These modifications in intestine morphology influence gut function and health and may change nutrient uptake. Both methods of SEM and LM show significant difference of the villus height compared with the control group of chicken. These results agree in general with the results of Samanya and Yamauchi (2002). They indicated that chicks fed *Pedicoccus subtilis* var nantto for period of 28 days had a tendency to prominent villus height than control group, Awad *et al*., (2009) claims the adding probiotic (*Lactobacillus* species) 1Kg/ ton of starter feed for 5 weeks to 600 chicks results increase in villus height compared with the control group.

The modified FISH procedure which was developed in the present study allows specific identification of LAB in both culture and tissue samples. FISH analysis confirmed the presence of LAB in the gut of the chicken fed FMF. The presence of LAB biofilm was not detected in samples from chickens fed other treatments by this method. This because the concentration of LAB or the limitation of numbers and size of the sample used compared to the size of the intestine. The probe was designed from the sequence results of the *Lactobacillus plantarum* of this study. This FISH analysis showed that *Lactobacillus plantarum* biofilm can occur *in vivo* in the ileum of chicken and this is an important criteria for microorganism to be a probiotic.

FISH-based methods have been demonstrated to be applicable to the identification of bacteria in their natural environment (Bojesen *et al*., 2003; Lebeer *et al*., 2011; Waines, 2011). Moreover, many studies remarked that bacterial cultivation is the least sensitive method for the detection of bacteria in tissue in comparison to *in situ* hybridisation and PCR, underlining the importance and necessity of alternatives to traditional detection methods (Tegtmeier, Angen & Ahrens, 2000). Persistence of ingested probiotic bacteria in the intestines is a condition for their beneficial effect, and some data in humans indicated that orally administered *Lactobacillus* can survive transit, but efficient colonization was not demonstrated (Sheen *et al*., 1995).

A number of key issues could arise from these trial results. Adding *Lb. plantarum* increased the microbial diversity in the treated chicks, which leads to decrease the possibility for colonisation of pathogens.

High percentages of *Lb. plantarum* surviving in the ileum and caeca suggests that these bacteria pass the acidic barrier of the proventiculus and gizzard and this was supported by *in vivo* and FISH technique used in this study. However, to demonstrate that the *Lb. plantarum* colonises the gut its presence would need to be detected after feeding the organism stopped. Adding probiotics or acids to chicken feed did not prevent infection with high dose of pathogens like *Salmonella* Typhimurium. The normal clearance of *Salmonella* Typhimurium by the control group suggests that using Ross chicken is not a favourable model for this type of study as they may be resistant to *Salmonella* infection. The rifampicin biomarker showed *Lb. plantarum* delivered to chickens could be retained in the digestive system. The specific pathogen free (SPF) white Leghorn chick which used in previous studies is favoured in this aspect.

CHAPTER 6

CHAPTER 6: Concluding discussion

Since 1950's antibiotics were used as growth promoters (AGP) and as prophylactic treatments. There is a link between risk of zoonotic diseases such as Salmonellosis and antibiotic growth promoters (AGP) usage in livestock and poultry. The banned use of AGP in animals in 2006 in Europe forced researchers to search for alternatives. Probiotics are one of the choices because they are natural pathogen antagonists in the intestinal tract and ultimately decreased the risk of poor health in humans, they are also generally recognised as safe (GRAS). Probiotics have the ability to balance and maintain the intestinal microflora in poultry. Probiotics can help to improve natural defences against pathogenic bacteria and reduce bacterial contamination (Ghareeb *et al*., 2012).

The study was divided into two parts, *in vitro* and *in vivo*. The *in vitro* laboratory work was designed to investigate the potential characteristic of *Lactobacillus plantarum* and the potential to use rifampicin as a biomarker.

The *in vitro* work (chapter 3), *Lactobacillus plantarum* (NCIMB 41607) was found to inhibit the growth of *Salmonella* Typhimurium and *Salmonella* Enteritidis as well as the co-aggregate with *Salmonella* and high degree of auto-aggregation. *Lactobacillus plantarum* has ability to survive in water after 24h over a range of temperatures. Adding *Lactobacillus plantarum* to wet feed has produced fermented feed with low pH (<4.5) that contains more than 150 mmol/L lactic acid and $1x10^9$ CFU/g during a 24h fermentation at 30°C.

The application of *Lactobacillus plantarum* in a simulated chicken digestive system (FMF & DW) showed that the level of both serotypes *Salmonella* were reduced in the acidic parts of the simulated digestive tract but as later they progressed in the

intestine, *S.* Typhimurium level increased in case of DW group. On the other hand, both types of *Salmonella* were in not detected level (Log 2.7 CFU/ml) in case of FMF group and it is likely that lactic acid present in FMF increased inhibitory effect of *Lactobacillus plantarum*. The detection of *Lb. plantarum* by using replica plating technique on MRS rifampicin agar shows the possibility of using rifampicin as a marker for the *Lactobacillus plantarum* for *in vitro* research.

The second part of the study (chapters 4 and 5) consisted of the *in vivo* application of *Lactobacillus plantarum* and this was done by three trials. The first trial was with 14 specific pathogen free (SPF) chicks, where the results confirmed the significant reduction of *Salmonella* Typhimurium numbers in the infected chicks from the fermented moist feed (FMF) which is consistent with the *in vitro* outcome. The results also indicate that *Lactobacillus plantarum* NCIMB 41607 survives well in the conditions of the gastrointestinal tract of chickens. The replica plating method used to detect *Lactobacillus plantarum* was successful. The microbial population diversity in the gastrointestinal tract of chickens changed due to feed treatment. These results led to a second trial using *Lactobacillus plantarum* with an increase in the numbers of chicks as well as using three groups of treatments, control group, *Lactobacillus plantarum* mixed with dry feed and with water. The second trial was conducted with 21 SPF chicks. It was revealed that the feed and water groups contained high levels of LAB and total coliform than the control group.

The total bacteria level detected in the caeca of the feed group were higher than other groups in all organs. In addition, the total Coliforms were not detected in a control group, which is same result, obtained from swabbing in day 7 of the trial. The difference in the bacterial numbers in 10 and 20 days may be due to age or the effect of continuing addition of LAB.

The molecular technique of DGGE was used because the of the limitation of clas-

sical microbiology to detect all types of bacteria in the gut because the selective enrichment cultures fail to mimic the condition for all bacteria to grow. Waynes *et al*. (1987) claims that only 20% of bacteria have been isolated and characterized so far.

The DGGE analysis revealed that the numbers of bacterial OTUs (bands) in the caeca at the age of 10 and 20 days in the feed and water groups were higher than all organs in all other groups. In general, the DGGE gel band numbers in the treatment groups were higher than the control group. These results agree with the traditional microbiology results. The high species richness in gut microflora is associated with decreased ability of pathogens to colonize the gut (Dillon *et al.*, 2005). Yu *et al*., (2010) reported that using lincomycin (antibiotic) significantly reduced the richness of bacterial population indicated by the reduction of band numbers in DGGE. DGGE as a technique was a very helpful tool to study the bacterial population diversity and for following up sequencing. DGGE analysis has a high sensitivity for detecting sequence differences and the understanding of the genetic diversity of complex microbial population. One of the limitations of using DGGE is that organism making less than 1% of the population may be unnoticed (Muyzer, De Waal & Uitterlinden, 1993). The bacteria counted less than 10^8 CFU/g in a total microbial population to be not visualized by DGGE (Simpson *et al*., 1999).

RISA technique was also used with two methods, Agarose-RISA and PAGE-RISA. The PAGE-RISA was more sensitive and revealed more bands and that indicates the 5% polyacrylamide was higher sensitive than agarose. The caeca results of feed and water in 10 days was more than other organs in all groups. The ileum gel bands of control group of 20 days were more than others.

The sequence analysis of DGGE bands revealed the most family BLAST results were Bacillaceae, including *Lactobacillus plantarum*.

The last trial was conducted with 103 broiler Ross chicks. It designed to understand the differences between five treatments were used of lactic acid and LAB in water, fermented feed by LAB, combination LAB in water and acidified feed and control groups. Many parameters used including growth performance, traditional and molecular microbiology, histology including scanning electron microscopy, lymphocyte-heterophil ratio and the modern molecular technique of fluorescent *in situ* hybridisation (FISH). The aims of this trial were to assess the effect of probiotic LAB or lactic acid or feed fermented by LAB on the microbial population of caeca and ileum of the chicks as well as their ability to reduce *Salmonella* Typhimurium in infected chicks. The main results of this trial revealed the possibility of using the FISH technique to determine the bacterial species (biolfilm of *Lactobacillus plantarum*) in the ileum of FMF group chick. This was a novel work of determining LAB in the chick's ileum by FISH technique in Plymouth University.

The length size of the intestinal villi were increased may be because adding probiotics to the feed led to an increase the numbers of intestinal enterocytes which is the main composition of the villi. These results agreed with the results of Dunham *et al*., (1993b) and Pelicano *et al*., (2005).

Salmonella Typhimurium counts were reduced in all groups including control birds after 10 days of infection and this indicates the normal clearance of *Salmonella* by Ross broiler chicks. However, adding probiotic or lactic acid did not prevent high dose infection by *Salmonella* Typhimurium.

In this study, culture-independent techniques have shown that the chicken intestinal bacterial population is more complex. The bacterial viable numbers (LAB and total coliform) were higher in the caeca than ileum, the viable numbers were higher at the end of the trial than before infection and that maybe is related to the age or stress or may be the addition of probiotics. The LAB numbers are significantly

higher ($P<0.05$) in the FMF group than in other groups. LAB level was higher than total coliform in the caeca and ileum within the same group. On the other hand, adding probiotics and lactic acid had no effect on the ratio of LAB: total coliform. Generally, adding probiotics led to increase in the numbers of the microbial population in the chicks gut and this case will reduce the space for pathogens to colonization.

Lactic acid group weight gain was less than other groups; even though the numbers of LAB and total coliform were the lesser than other groups. The simplest way to use probiotics for chicken was via the drinking water. In the WAT group, the LAB numbers were higher than the other groups except the FMF group.

Adding probiotic or lactic acid in this trial had no significant effect on lymphocyte counts.

The classical microbiology is very good to track probiotic colonization level but including molecular microbiology enhanced the results of probiotic studies particularly using DGGE, electron microscope and FISH technique.

The engineering problem of providing of FMF or AMF is still not solving for the big poultry house but it may be easier for small producers, particularly in villages.

Future works

The main areas to be highlighted are:

There is a lot of knowledge accumulated on the application of probiotic in the poultry industry but this is still limited and the research should continue. For example, little are known about the immunity response of chicken to the probiotics. There is a possibility of using real time PCR to investigate the *Salmonella* virulence gene expression with an application of probiotics (Haghighi et al., 2008).

The effective dose of probiotics and frequency of feeding is still largely unknown. This study and those of Niba and Savvidou demonstrate that FMF was most effective. In FMF, the dose of probiotics is about 10^{11} because FMF contains at least 10^{9} CFU/g. The method of administration of fermented moist feed to chickens is still not solved. Several practical problems relating to the engineering of production and delivery of FMF to chickens remains such as accumulation of sticky feed on the tray which may lead to spoilt feed because of contamination with yeasts. This may also make the feed a source of infection. Feed fermentation could be a better way of reducing *Salmonella* infection and this worth further investigation. Further studies on the effect of probiotics on microbial community in the chicken gut using genomics, metabolomics, and short chain fatty acid production in the gut to show the effect of probiotics on bacterial community.

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Appendices

• Appendix I: Buffers and FD LAB sequences

Buffers
Buffer solutions and acrylamide mixtures used during PCR-DGGE analyses as described in Chapter 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

6 x Gel loading buffer
40% glycerol, 0.25% Bromophenol Blue
Made up in 1x TAE buffer

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 mls with distelled water

Store refrigerated in the dark

Lysis Buffer
50 mM Tris/Cl, 25 mM EDTA pH 8.0, 3% SDS, 1.2% PVP

Extraction Buffer
10 mM Tris/Cl, 1 mM EDTA pH 8.0, 0.3 M sodium acetate, 1.2% PVP

The *Lactobacillus plantarum* DNA sequence resulted of 1000 nucleotide letters.

The sequencing result was *Lactobacillus plantarum*:

ACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTgaTGAGTGGCGAACTGGTGAG-

TAACACGTGGGAAACTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATAC-

CGCATAACAACTTGGACCGCATGGTCCGAgCtTGAAAGATGGCTTCGGCTATCACTTTT-

GGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCA CCATGGCAATGA-

TACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCC-

TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAAC-

GCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATA-

TCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGG CTAACTAC-

GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA-

GCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTG-

CATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTG-

TAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCT

GTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAG-

TCCATACCGTAnACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTG-

C A G C T A A C G C A T T A A G C A T T C C G C C T G G G G A G T A C G G C C G -

C A A G G C T G A A A C T C A a A G G A A T T G A C G G G G G G C C C G C A C A A G C

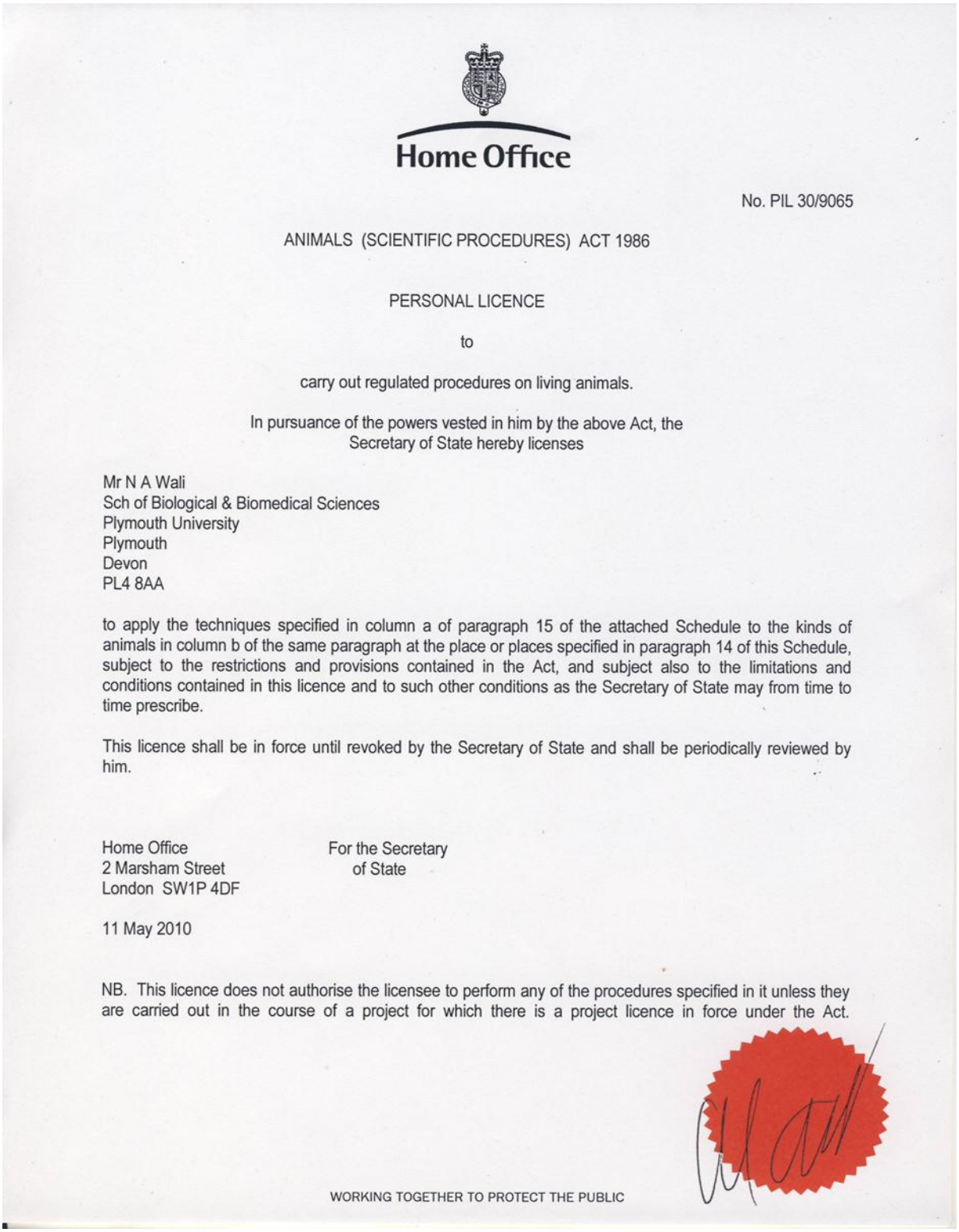
Sequencing analysis results of LAB Freeze dried (FD)and pure culture MS18 which used for the trial.

Sample number	NCBI Accession number	NCBI BLAST match	Maximum Identity
FD1	AB617650.1	*Lactobacillus plantarum* strain: SCP53	**99%**
FD2	AB617650.1	*Lactobacillus plantarum* strain: SCP53	**99%**
MS18	AB617650.1	*Lactobacillus plantarum* strain: SCP53	**98%**

```
L.plantarum5.LY-78                A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 179
Lactobacillus.species8.SCA39      A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 171
L.plantarum4.SCP15                A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 153
L.durans9.R03-16                  A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 192
L.plantarum7.CCZZ2                A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 168
L.plantarum3.SCP21                A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 190
L.plantarum1_AB617650.1           A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 188
L.plantarum2.SCP49.AB617649.1     A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 188
L.plantarum.Freezed.dry.          A--------CAACTTGGACCGCA----TGGT----CCGAGCTTG--AAAG 178
L.plantarum6.WCFS1                AACGAAACTCAACCCGAAATACACTTTTGATACTTTCGTGATCGGTAAAG 397
                                  *        ****  * *   **    ** *     ** * * *  ****

L.plantarum5.LY-78                TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 276
Lactobacillus.species8.SCA39      TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 268
L.plantarum4.SCP15                TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 250
L.durans9.R03-16                  TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 289
L.plantarum7.CCZZ2                TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 265
L.plantarum3.SCP21                TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 287
L.plantarum1_AB617650.1           TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 285
L.plantarum2.SCP49.AB617649.1     TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 285
L.plantarum.Freezed.dry.          TCACC--ATG----------GCAATGATACGT----AGCCGACCTGAGAG 275
L.plantarum6.WCFS1                CCACCTAATGCACGCTATCGGTAACAAATTGTTAGAAACCGATCCGACTA 547
                                   ****  ***          * **  *   **    * **** * **
```

Nucleotide sequences multiple alignment of 16S rRNA gene sequencing of *Lactobacillus plantarum* rifampicin resistant evaluation and relationship with other *Lactobacillus* species, " * "mean that all nucleotides in the column are identical in all sequence in the alignment. The alignment made using Clustal W2 software of EMBI-EBI and BLAST NCBI.



Home Office

No. PIL 30/9065

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 N A Wali
Sch of Biological & Biomedical Sciences
Plymouth University
Plymouth
Devon
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

11 May 2010

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

Personal licence to work with the project of chicken in Plymouth University

- **Appendix II: Conferences contributions and courses attended**

Conferences contributions and published papers

Platform (oral) presentations

- Survival of *Lactobacillus salivarius* NCIMB 41606 in an *in vitro* model of the chicken digestive process and its effect on the survival of *Salmonella* Typhimurium Nal^r SAL 1344. Published paper in International Journal of Probiotics and Prebiotics/ USA. IJPP (2011). Volume 6, Number 4, Page 193-196.
- Effect of moist food fermented with *Lactobacillus plantarum* on *Salmonella* Typhimurium infection in chicken. XV^{th} International society of animal health congress, Vienna, Austria. July 2011, proceeding of the congress book, Volume 1, Page 81- 83, ISBN 978-80-263-0008-3, Brno, 2011.
- Effect of lactobacillus salivarius on *Salmonella* Typhimurium and *Salmonella* Enteritidis in an *in vitro* digestive system of chicken. Society of Environmental Toxicology and Chemistry–Environmental Pollution in a Changing World. 13th–14th September 2010, London, UK. Abstract SETAC15: p. 20.

- *Effect of probiotics on Salmonella in broiler chicken, PG society, Plymouth University, March 2011.*
- *In vitro* reduction of *Salmonella* Typhimurium in digestive system of chicken by probiotic XV^{th} International society of animal health congress, Vienna, Austria. July 2011, proceeding of the congress book, Volume 1, appendix, ISBN 978-80-263-0008-3, Brno, 2011.
- Reduction of *Salmonella* in chicken by *Lactobacillus salivarius. An international scientific conference on probiotics and prebiotics / Slovakia. June 2010. The conference proceeding ISBN 978-80-970168-4-5, Page 79.*
- *Application of molecular methods to study bacterial diversity in the chicken gut samples. 2012, CARS-Duchy college, Cornwall- UK. 05/07/2012.*
- Effect of probiotics on *Salmonella* in *in* vitro chicken gut. PG society conference, Plymouth University June 2011

Poster presentations

- Global environmental change in the Marine environment conference University of Plymouth, MBA, 03/06/10.
- Plymouth Postgraduate society conference 18/11/10.
- Marine Biology conference, MBA, University of Plymouth 20/12/10.
- CUC Conference, Cornwall-UK, European Centre for Environmental and Human Health, proceeding book p.23. 16-17/09/10.

- Plymouth PG conference, Plymouth –UK, PG society 29/06/201.
- Plymouth PG conference, Plymouth –UK, PG society 23/11/11.
- Plymouth PG conference, Plymouth –UK, PG society 16/03/2012.
- Plymouth PG conference, Plymouth –UK, PG society 26/06/2012.
- CRTB/ Annual research day, Plymouth-UK, CRTB, 4/07/12.
- ISAPP international conference, Cork-IRELAND, ISAPP, 29/09 – 02/10/12.
- Altech UK, Altech Young Scientist Graduate competition 2012.
- Plymouth PG conference, Plymouth –UK, PG society 21/11/2012.

Courses attended

- English Language Summer School, Academic writing (April 2009 to September 2009), University of Plymouth.
- Postgraduate Research Skills and Methods in Biology (October 2009 to January 2010), University of Plymouth.
- Supporting English Language Classes (October 2010 to November 2010), University of Plymouth.
- General Teaching Associates Course (10/02 – 18/03/2010), University of Plymouth.
- Laboratory Based Teaching and Methods Practice (Env 5101) (10/11/ 2009 to 18/12/2009), demonstration exam, University of Plymouth.
- Student Associate Scheme Training, organized by the Faculty of Education (03/03-10/06/2010), Prince Rock Primary School, Plymouth.
- Application and Principles in Electron Microscope (Bio 5102), 18/10/2009-18/12/2009, full attendance. Course work report
- Molecular and Cellular Marine Biology, MBAM 5102, 02/11/2009-18/12/200, Full Attendance.
- Microbial life Biol 2409, 10/10/09-20/11/09, partial attendance
- Microbial life and biotechnology, Bio 3321, 18/01/2010-18/03/2010.
- Small Animal Module1, 2 and 3 (31/03 -01/04/2010), Personal license.

- Chicken Module1, 2 (31/03 - 01/04/2010), Personal license.
- Laboratory based teaching methods and practice, ENV 5101, 28/10 - 02/12/2010.
- Practical Molecular workshop, PG Society, 16–19/07/12, Full Attendance.

Taught sessions attended

- Impact factors (19th September 2008).
- Endnote for beginners (7th November 2008).
- MS Excel Introduction to essential features (21/09/2009).
- Getting Started in MS Office 2007 (13/10/2009).
- Preparing effective poster presentation (11/10/2009).
- SPSS State clinic (12/10/2009, 22 and 27/04/2010).
- MS Excel simple Formulas and Charts (16/10/2009).
- MS share point designer (20/10/2009).
- MS Word Structuring your Thesis (23/10/2009).
- MS Power Point is enhancing your presentation (27/10/2009).
- Introduction to my site (29/10/2009).
- Creating a graphic using paint shop (04/11/2009).
- Internet for PGS (10/11/2009).
- Presenting to an Audience (13/11/2012).
- MS Word Proofing and Tracking (25/11/2009).
- MS Word Introduction (26/11/2009).
- EndNote (01/12/2009).
- MS Excel Introduction to essential features (16/01/2009).
- Plagiarism (20/01/2010).
- EndNote Clinic (21/01/2012 and 18/02/2012 and 18/03/2010).
- Going global presenting a conference paper (19/02/2010).
- Preparing to transfer (19/03/2010).
- Oral Presentation part 2 (24/03/2010).
- Corel Paint shop X2 (25/03/2010).
- Microsoft Excel 2007 (03/10/2010).
- Active reading (20/02/2010).
- Professional writing skills (28/03/2012).
- FISHER scientific event, Wembley London (19/04/2012).
- Master document (19/04/2012).
- Alpha laboratories pipetting techniques workshop (03/05/2012).
- Cryogenic gases Safety Awareness workshop (25/06/2012).
- Safe handling gases workshop (26/06/2012).

4. Professional membership

- Member, Wold Poultry Science Association (WPSA).
- Member, Society of Applied Microbiology (*sf*am).
- Member, Society of Experimental Biology (SEB).

6. Awards

- Veterinary Medicine University of Vienna, PHP scholarship for International Society of Animal Health conference, Vienna - Austria, July 2011, $350.
- International Society of Probiotics and Prebiotics (USA), Travel grant for ISAPP conference in Cork –Ireland, September 2012. $500.

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SURVIVAL OF *LACTOBACILLUS SALIVARIUS* NCIMB 41606 IN AN *IN VITRO* MODEL OF THE CHICKEN DIGESTIVE PROCESS AND ITS EFFECT ON THE SURVIVAL OF *SALMONELLA* TYPHIMURIUM *NAL*^{R} SALL344

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[Received February 5, 2011; Accepted June, 2011]

ABSTRACT: ***Probiotics may be a viable alternative to antibiotic growth promoters in controlling gastrointestinal infections in chickens. In order to be effective probiotics must be able to survive conditions in the gastrointestinal tract. This study investigated the survival of Lactobacillus plantarum NCIMB 41606 in an in vitro model of the chicken digestive process and the effect on the survival of Salmonella*** Typhimurium ***nal^{r} Sal1344. Lb. salivarius was administered either as a dry feed treatment (DF) or as fermented moist feed (FMF). Lb. salivarius survived passage through the digestive process with no loss of viability for control and dry feed. The presence of Lb. salivarius administered in dry feed had no significant effect on the survival of S.*** Typhimurium ***compared with the control, in both cases there was a 1.3 log reduction in S.*** Typhimurium ***numbers over the course if the digestive process. However, with FMF no S.*** Typhimurium ***were detectable from the end of the gastric stage. FMF contained 175mmol/l lactic acid and it is likely that this contributed to its anti-Salmonella activity.***

KEY WORDS: *Lactobacillus salivarius*, Probiotic, Poultry, *Salmonella* Typhimurium

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INTRODUCTION

The gastrointestinal microflora have an important role in nutrition, growth performance and protection against infection (McCracken and Lorenz, 2001). The European ban using antibiotics as growth promoters in the poultry industry in 2006 promoted the search for alternative additives for use in poultry production. One of these alternatives is probiotics. Lactic acid bacteria (LAB) are widely used as probiotics for humans and animals. Requirements of LAB strains as probiotics include their tolerance to the acid and bile conditions in the host gastrointestinal tract, their capabilities to
adhere to the host intestinal epithelium, and also their antagonistic effect against pathogenic bacteria as well as their immune-modulating activity in hosts (Delgado *et al*., 2007; Wu and Chung, 2007). S*almonella* Typhimurium and *Salmonella* Enteritidis along with *Campylobacter jejuni* are common bacterial isolates from poultry faeces and poultry products worldwide and lead to considerable economic loss in the poultry industry. Furthermore, poultry and poultry products are the main sources for the dissemination of these diseases to humans (Dunkley *et al*., 2009). Recently, the *in vitro* and *in vitro* antagonistic effect of certain LAB strains such as *Lactobacillus salivarius* and *Lactobacillus plantarum* against *S.* Typhimurium was demonstrated (Niba, 2008; Savvidou, 2009).

Probiotics are commonly added to poultry diets and it is suggested that they may promote gut health by increasing the balance of a beneficial microflora and reducing pathogenic bacteria. In order to achieve this; any potential probiotic must have the ability to survive the conditions in the digestive tract as well as show their capability to reduce pathogenic bacteria. In this study an *in vitro* model of the poultry digestive process was used to determine the survival of *Lactobacillus salivarius* NCIMB 41606 in such conditions and its inhibitory effect on *Salmonella* Typhimurium. There are several ways of administering probiotics to chickens. One of the novel methods recently investigated by Niba 2008 and Savvidou 2009 was the use of fermented moist feed (FMF). In this *in vitro* study, FMF as a vehicle for administering *Lb. salivarius* was compared with administration via dry feed.

Effect of probiotic on Salmonellas in Chicken model

Nabil Ali Wali, Victor Kuri and Jane Beal

University of Plymouth, Devon, England

Introduction

Salmonellosis is a food born diseases. Poultry and poultry eggs are the main source of diseases for human. Reduction of Salmonella in chicken will reduce the disease in humans. One of the ways of protecting chickens from Salmonella infection may be to use probiotics in the feed. In this study probiotic bacteria, *Lactobacillus salivarius*, was used. An *in vitro* model of the chicken digestive tract was used to determine if the probiotic had any effects on *Salmonella enterica Typhimurium* in the gastrointestinal tract of the chicken.

Methods

I. Antagonistic activity to *Salmonella*: to determine the inhibitory effect of probiotic to pathogens,2 methods selected
- A- Well Diffusion Assay
- B- Co-aggregation

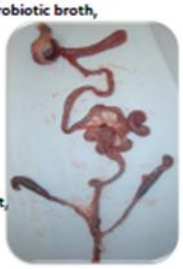
II. Delivery method

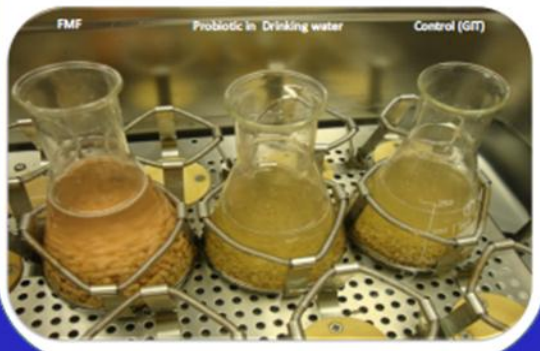
A. Preparing Freeze Dry(FD) *L.salivarius* : making probiotic as Lyophilized (FD) freeze dryer device from washed broth

B-Preparing Fermented Moist Feed(FMF) by *L.salivarius*: 100 gm food:120mlwater (1:1.2) 1 ml Probiotic broth, incubated at 30 °C for 24 hrs

III. *In vitro* Chicken model: (10 gm food, 70 ml water, Shaking water bath 41 °C) divided to 3 stages:

Stage 1	Crop:	pH4.5, 45 min
Stage 2	Gizzard:	pH2.4, 90min, Pepsin
Stage 3	Intestine:	pH 6.2, 120min, Bile salt, NaHCo3, Pancreatin

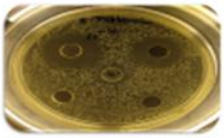




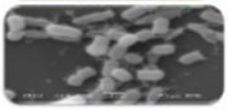
Results

I.

A- Well diffusion assay
Broth culture showed more inhibition effect than Lactic acid



B- Coaggregation with Salmonella.
Aggregation between both bacteria was high.

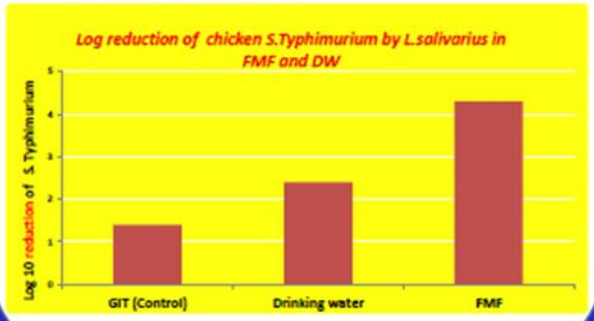


II.

A- Freeze Dry: The concentration of lyophilized *L.salivarius* in 1 gm was $1x10^{12}$ CFU

B- FMF: 1 gm of FMF was $1x10^{9}$ CFU. With pH4.5 and about 150 mmol of lactic acid

III. Salmonella reduction by FMF better than reduction by probiotic in water



Conclusions

Probiotic have a high effect on reductions of *Salmonella Typhimurium* infection in the Chicken digestive system model. Probiotic may be can used as alternative to Antibiotic in poultry productions. The study showed high percentage surveillance of *lactobacillus salivarius* passed through the simulated chicken gut. The study will continue with *in vivo* application in chickens.

Effect of probiotics on *Salmonella typhimurium infection* and microbial population changes in Chicks

Nabil Ali Wali, Victor Kuri and Jane Beal

University of Plymouth, Devon, England

Nabil.wali@plymouth.ac.uk

Introduction

Salmonellosis is a food born diseases. Poultry and poultry eggs are the main source of diseases in human. Reduction of Salmonella in chicken will reduce the disease in humans. Banned using of antibiotic as prophylactic treatments in animals in 2006 in EU pushed the researcher to search for alternative. In this study probiotic bacteria, *Lactobacillus plantarum* was used to ferment moist feed to produce FMF. Two groups of one day old chicks control and fermented moist feed (FMF) were used to determine the effect on reduction of *Salmonella typhimurium* infections and microbial population changes in the gastrointestinal tract.

Methods

I. Day 1

Two Specific pathogen free chicks groups

A- Control group : normal chicks feed

B- Fermented moist feed group:

L. plantarum : moist feed (1:1.2) for 24hr incubated in 30 ºC to produce pH 4.4, 175 mmol Lactic acid and 10^9 CFU/g *L. plantarum*

II. Day 3

Infected chicks

by Salmonella typhimurium $1X10^6$ CFU/chick by oral gavage

III. Day 5 & 8

Cloaca swabs

IV. Day 14

All ilea & caeca removed and examined by

A- Traditional microbiology using culture methods

B- Molecular microbiology :

200 mg of caecum and ileum contents for

- DNA extraction,
- Polymerase chain reaction (PCR)
- Denaturing Gradient Gel Electrophoresis (DGGE)
- Gene Sequencing

Figure 1: The digestive system of chicken showing ileum and both caeca

Results

I. High palatability and acceptability of FMF for the chicken

Figure 2: High acceptability of FMF to all chicks

II- All chicks were positive for salmonella in day 5 & 8 by cloaca swabs

II. Day 14

A- Traditional method was negative Salmonella in treated chicks and positive in control group

High survival of *L. plantarum* and caecum of FMF group chick has highest numbers of *L. plantarum*.

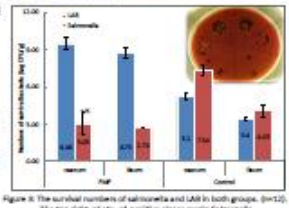


Figure 3: The survival numbers of salmonella and LAB in both groups. (n=12). The top right photo of positive cloaca swabs Salmonella

B- DGGE band numbers of FMF were higher than control group in ileum and caecum.

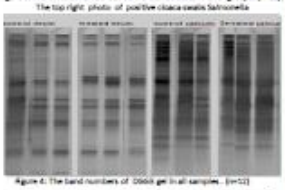


Figure 4: The band numbers of DGGE gel in all samples. (n=12)

The band numbers in caecum of FMF were higher than other organs

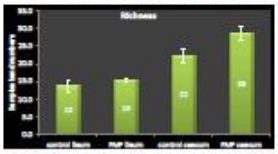


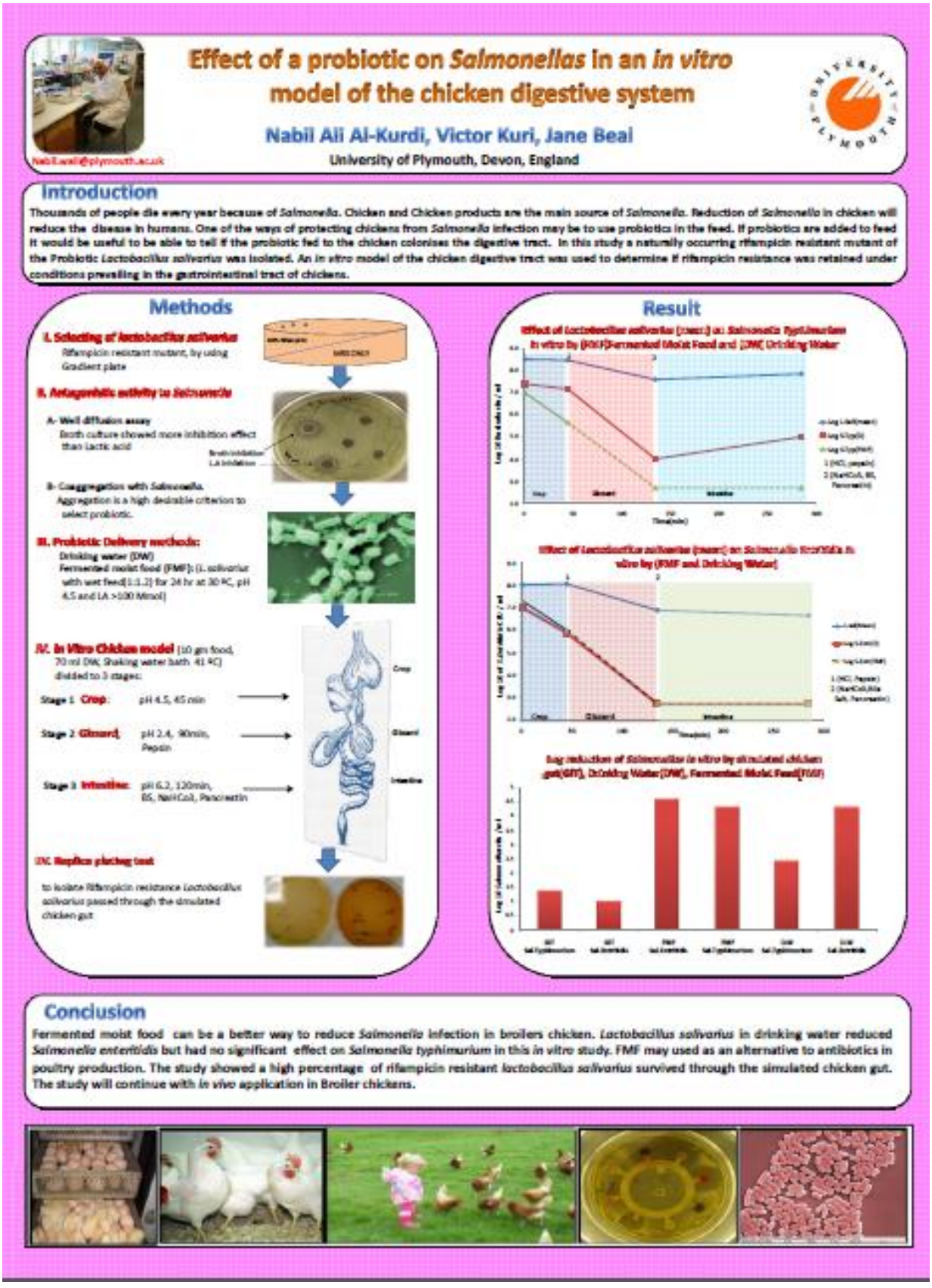
Figure 5: The band numbers of FMF caecum significantly higher (P< 05) than other organs in both groups

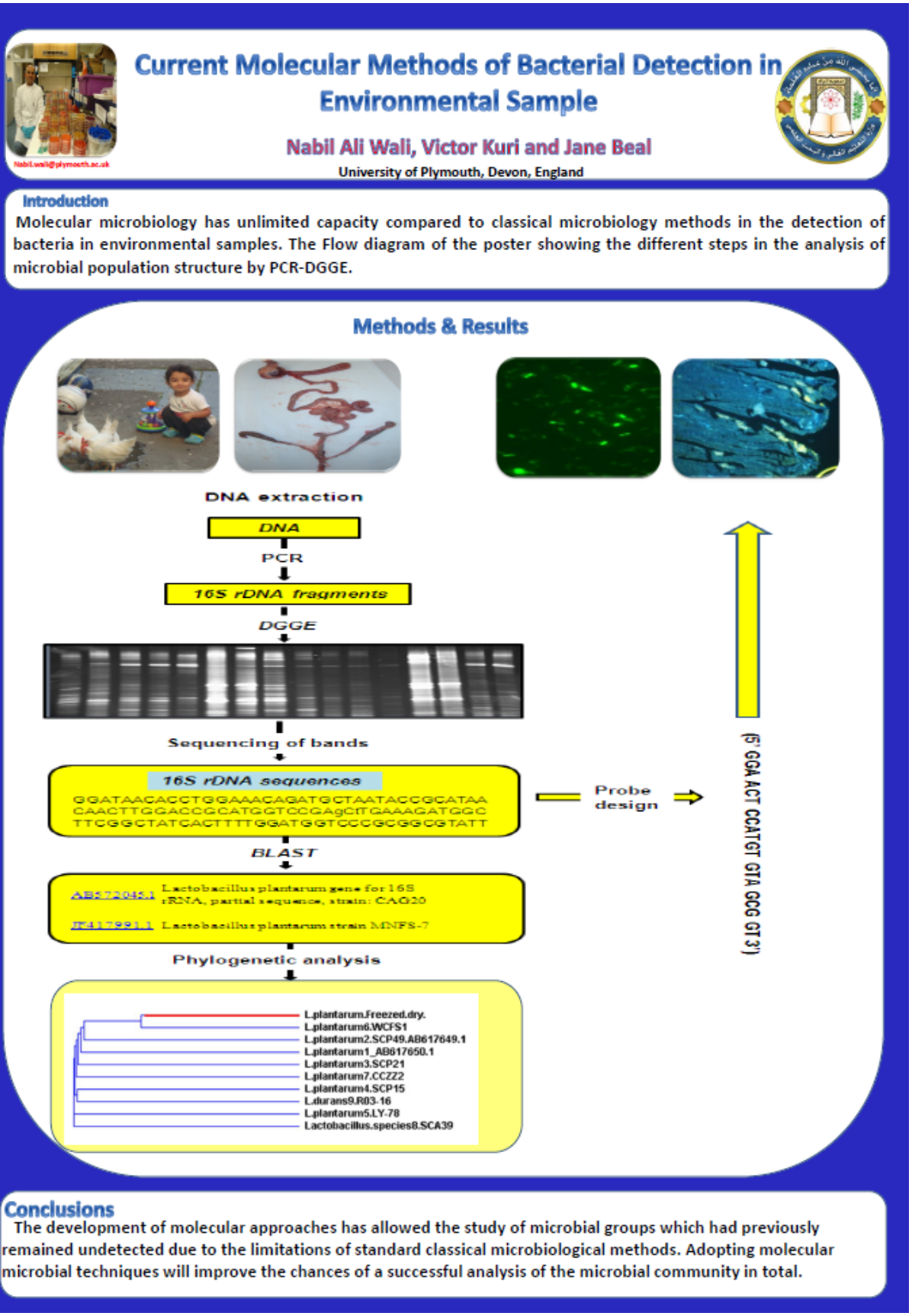
C- Sequencing revealed many types of uncultured bacteria were found in the gastrointestinal tract of chicks

Figure 6: The NCBI match results of selected band with accession numbers of each band

Conclusions

FMF by *Lactobacillus plantarum* has a high effect on reductions of *Salmonella typhimurium* infection in the chicken digestive system. FMF may be can use as alternative to Antibiotic in poultry productions. The study showed high percentage surveillance of *lactobacillus plantarum* and high acceptability of FMF by chicks and successful using DGGE for profiling the bacterial population of the chicks gut.


Effect of a probiotic on Salmonellas in an in vitro model of the chicken digestive system
Nabil Ali Al-Kurdi, Victor Kuri, Jane Beal
University of Plymouth, Devon, England
Introduction
Thousands of people die every year because of Salmonella. Chicken and Chicken products are the main source of Salmonella. Reduction of Salmonella in chicken will reduce the disease in humans. One of the ways of protecting chickens from Salmonella infection may be to use probiotics in the feed. If probiotics are added to feed it would be useful to be able to tell if the probiotic fed to the chicken colonises the digestive tract. In this study a naturally occurring rifampicin resistant mutant of the Probiotic Lactobacillus salivarius was isolated. An in vitro model of the chicken digestive tract was used to determine if rifampicin resistance was retained under conditions prevailing in the gastrointestinal tract of chickens.
Methods
I. Selecting of Lactobacillus salivarius
Rifampicin resistant mutant, by using Gradient plate
II. Antagonistic activity to Salmonella
A- Well diffusion assay
Broth culture showed more inhibition effect than Lactic acid
B- Coaggregation with Salmonella.
Aggregation is a high desirable criterion to select probiotic.
III. Probiotic Delivery methods:
Drinking water (DW)
Fermented moist food (FMF): (L. salivarius with wet feed(1:1.2) for 24 hr at 30 ºC, pH 4.5 and LA >100 Mmol)
IV. In Vitro Chicken model (10 gm food, 70 ml DW, Shaking water bath 41 ºC) divided to 3 stages:
Stage 1 Crop: pH 4.5, 45 min
Stage 2 Gizzard: pH 2.4, 90min, Pepsin
Stage 3 Intestine: pH 6.2, 120min, BS, NaHCo3, Pancreatin
Crop
Gizzard
Intestine
IV. Replica plating test
to isolate Rifampicin resistance Lactobacillus salivarius passed through the simulated chicken gut
Result
Conclusion
Fermented moist food can be a better way to reduce Salmonella infection in broilers chicken. Lactobacillus salivarius in drinking water reduced Salmonella enteritidis but had no significant effect on Salmonella typhimurium in this in vitro study. FMF may used as an alternative to antibiotics in poultry production. The study showed a high percentage of rifampicin resistant lactobacillus salivarius survived through the simulated chicken gut. The study will continue with in vivo application in Broiler chickens.


Current Molecular Methods of Bacterial Detection in Environmental Sample
Nabil Ali Wali, Victor Kuri and Jane Beal
University of Plymouth, Devon, England
Introduction
Molecular microbiology has unlimited capacity compared to classical microbiology methods in the detection of bacteria in environmental samples. The Flow diagram of the poster showing the different steps in the analysis of microbial population structure by PCR-DGGE.
Methods & Results
DNA extraction
DNA
PCR
16S rDNA fragments
DGGE
Sequencing of bands
16S rDNA sequences
GGATAACACCTGGAAACAGATGCTAATACCGCATAA
CAACTTGGACCGCATGGTCCGAgCTTGAAAGATGGC
TTCGGGCTATCACTTTTGGATGGTCCCGCGGCGTATT
Probe design
(5' GGA ACT CCATGT GTA GCG GT 3')
BLAST
AB572045.1 Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: CAG20
JF417991.1 Lactobacillus plantarum strain MNFS-7
Phylogenetic analysis
L.plantarum.Freezed.dry.
L.plantarum6.WCFS1
L.plantarum2.SCP49.AB617649.1
L.plantarum1_AB617650.1
L.plantarum3.SCP21
L.plantarum7.CCZZ2
L.plantarum4.SCP15
L.durans9.R03-16
L.plantarum5.LY-78
Lactobacillus.species8.SCA39
Conclusions
The development of molecular approaches has allowed the study of microbial groups which had previously remained undetected due to the limitations of standard classical microbiological methods. Adopting molecular microbial techniques will improve the chances of a successful analysis of the microbial community in total.