Application of *Lactobacillus* Species in the Development of Probiotic Poultry Feed as an Alternative to Antibiotic Growth Promoters

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DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis, entitled "Application of *Lactobacillus* species in the Development of Probiotic Poultry Feed as an Alternative to Antibiotic Growth Promoters" submitted by me, Saifa Sharmin, has been carried out under the co-supervision of Professor Naiyyum Choudhury, former Coordinator of Biotechnology and Microbiology Programmes, MNS Department, BRAC University and Monzur Morshed Ahmed, Research Coordinator & Principal Scientific Officer, Industrial Microbiology Division, IFST, BCSIR in the Industrial Microbiology Lab of the Institute of Food Sciences and Technology division at the Bangladesh Council of Scientific and Industrial Research. It is further declared that the research work presented here is original and suitable for the submission for the partial fulfilment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

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My Beloved

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Abstract

The increased use of antibiotics in animal feed for growth promotion may cause antibiotic resistance in animal and humans; due to this, alternative measures are searched to maintain production performance of poultry without the use of antibiotic. One such alternative could be use of probiotics. Probiotics are living organisms which when administered in adequate amounts give a beneficial health effect on its host. One of the most common species known to have probiotic effects are the *Lactobacillus* species. The objective of this study was to develop probiotic poultry feed using *Lactobacillus* species which were isolated from the gastrointestinal tract of local healthy chickens. Four isolates of *Lactobacillus* was obtained from Industrial Microbiology lab, BCSIR. The acid and bile tolerance of the four isolates were investigated at pH of 3, 4, 5 and 6 and salt tolerance at the concentration of 0.5%, 1.0%, 1.5% and 2.0%. The cell surface hydrophobicity was determined using microbial adhesion to hydrocarbon assay with xylene. The antimicrobial activity of the isolates was determined against *Salmonella Typhi*, *E. coli*, *Shigella*, and *S. aureus* by agar well diffusion method. The isolate nos. 10 and 14 gave more promising results and was grown in large numbers at 37°C in anaerobic condition in a medium formulated at Industrial Microbiology lab. The *Lactobacillus* cells were then freeze dried to a powder form where skim milk was used as the cryoprotectant and mixed with poultry feed. The viability of the freeze dried probiotic supplement was determined for a period of four months. A field trial was carried out at BLRI where one day old chicks were fed with the probiotic nos. 10 and 14 for a period of five weeks. Results of this study have shown that the isolates had a good tolerance to acidic pH and were tolerant to bile salts. Isolates 10 and 14 showed cell surface hydrophobicity greater than 40%, with 48% and 46% respectively and also showed good antimicrobial activity towards *S. typhi*, *Shigella* and *S. aureus*. Both the probiotic supplement showed good cell counts of log 10 after four months of storage at 4°C. At the end of the field trial, Antibiotic fed chickens showed the maximum weight gain with 1.93% increase followed by chickens fed with probiotic 14 and then 10 with an increase of 1.76% and 0.96% respectively. The control showed the least weight gain. This study concludes that the probiotic supplement developed is quite promising but further field studies are required to give more conclusive results before these can be used to replace antibiotic growth promoters.

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Chapter 1

Introduction

1.1 Introduction

Bangladesh is one of the densely populated countries in the world. About eighty percent of the population of this country still live in villages and are poor. According to a annual report of Bangladesh Rural Advancement Commission, now called Building Resources Across Community (BRAC) 70% of rural households are involved in poultry keeping but they face constraints as the mortality rate of poultry is said to be as high as 25% (Ali & Hossain, 2012). Poultry such as chicken is one the main sources of animal protein for Bangladeshi people (Kamal & Shafiullah, 2016). Although government introduced improved breeds of poultry in government poultry farms for multiplication and distribution to village people, but commercial poultry production has started from mid 1980s in Bangladesh (Raha, 2007). Due to increasing population, there is an increasing demand for meat and eggs which led to commercialization of poultry production, with a large number of farms now operating across the country (Raha, 2013). One of the major challenges this industry faces is the spreading of diseases among the poultry population due to bacterial pathogens which results in serious economic losses (Huque *et al.*, 2011). As a result the use of antimicrobial agents and growth promoters is substantially increasing in the poultry industry to prevent diseases and to promote faster growth (Islam *et al.*, 2016).

An assortment of substances, such as growth promoters is added to the feed and the drinking water of poultry to improve its production and reduce or prevent the spread of diseases (Diarra & Malouin, 2014). These are substances used to increase the feed efficiency, average daily gain, eggs and meat production. The most common growth promoter agents added are antibiotics and they are referred to as Antibiotic growth promoters (Castanon, 2007). The use of antibiotic growth promoters began when Stokstad and Jukes added dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues to chicken feed half a century ago. Stokstad and Jukes added it with the objective that it would serve as a source of vitamin B_{12} but this addition caused a growth acceleration that was far too large to be explained as the effect of vitamin hence the obvious cause was due to the antibiotic residue. Soon afterwards this observation was extended to other antibiotics and to other animal species which lead to the widespread use antibiotic growth promoters in animal feeds (Falcão-e-Cunha *et al.*, 2003). The term antibiotic growth promoter is used to describe any medicines that kill or inhibits the growth of bacteria and is administered at sub therapeutic levels (Hao *et al.*, 2014). In therapeutic usage of antibiotics, it is usually used in high dosage

for a short time and administered via injection or through feed or water whereas in growth promoting usage of antibiotic is quite opposite, it is administered in low dosage for a long period of time usually via feed (Chowdhury *et al.*, 2009).

The usage of antibiotics in animal feed improved feed efficiency and animal growth and reduced morbidity and mortality due to clinical and subclinical diseases (Philips *et al.*, 2003). In the past it was acceptable for the use of antibiotics in poultry for disease prevention and the usefulness of antibiotics as growth promoter is seldom being contested; it is their relatedness to antibiotics used for human medicine is causing concern (Kiilholma, 2007). Presently there are concerns about their use in poultry feed due to fear that this may lead to development of antibiotic resistant pathogens in poultry which may then transfer to humans (Hughes & Heritage, 2007). The European Union recently reported that due to infection from drug-resistant bacteria 25,000 people died each year (Salim *et al.*, 2013). In many countries, the use of antibiotics as growth promoters is being restricted to avoid the harmful effects on human health. Due to these consequences, animal growers are looking for an effective alternative to antibiotic growth promoters (Barton, 2000).

A promising candidate for the replacement of antibiotic growth promoter could be probiotics. The term probiotic is composed of the Latin preposition “pro” meaning for or in support and the Greek “biotic” from the noun “bios” meaning life hence the term probiotic meaning “for life” or “in support of life”, it thus means the exact opposite of antibiotics. According to World Health Organization's 2001 definition, probiotic is defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. Elie Metchnikoff, the father of probiotics, hypothesised that the long, healthy lives of Bulgarian peasants were the result of their consumption of fermented milk products and was convinced that yoghurt contained the organisms necessary to protect the intestine from the damaging effects of harmful bacteria (Parvez *et al.*, 2006).

The most persuasive effect of probiotic on the gut microflora in resistance to diseases was shown by Collins and Carter in 1978. Their study showed that a germ-free guinea-pig was killed by 10 cells of *Salmonella enteritidis* whereas a conventional grade animal with complete gut microflora it took 10^9 cells to kill. Thus this shows that animals have an intestinal population of microorganisms that protects them against diseases. Though all animals naturally have a population of microorganisms in the GI tract there is a need for

probiotics. In the wild the young rapidly acquires a protective flora from the mother and its environment, however in modern methods of animal rearing there is limited contact with the mother and it's young. Especially in poultry industry where after the egg is laid then the chick is permanently separated from its mother. This results in the GI tract being deficient in some microorganism able to resist diseases (Nowroozi *et al.*, 2004).

The most extensively studied and widely used probiotics are the Lactic acid bacteria, particularly the *Lactobacillus* and *Bifidobacterium* species (Parvez *et al.*, 2006). The *Lactobacillus* genus includes a large number of species which have many important applications especially in fields of food and probiotics (Felis & Dellaglio 2005). *Lactobacilli* are gram positive, non spore forming; rod or even sometimes is coccobacilli in shape and facultative anaerobic bacteria. The genus *Lactobacillus* falls under the group of Lactic acid bacteria (LAB) hence these defines that the bacteria are catalase negative and are able to produce lactic acid as an end product of the fermentation of carbohydrates (Bergey *et al*., 1974). The bacterial species of *Lactobacillus* is widely applied to the food and feed industry especially in the fermentation of vegetable, sourdough bread, and dairy and meat products, although some species may cause food spoilage (Avall-Jaaskelainen & Palva 2005). *Lactobacilli* are GRAS organisms, i.e. they are generally regarded as safe due to their lack of pathogenecity. *Lactobacillus* species also confer health benefits to the host and are utilised as probiotics (Patterson & Burkholder, 2003). There is two possible mechanism in which LAB gives a beneficial effect to the host: 1) it produces antimicrobial substance such as lactic acid and bacteriocins and 2) Adherence to the mucosa forming a barrier and preventing colonization by pathogens (Ehrmann *et al.*, 2001). Nurmi and Rantala in 1973 first reported the oral dosing of poultry with native gut microorganisms to prevent infection with *Salmonella*. Watkins *et al.,* reported that inoculation of germ-free chicks with *Lactobacillus acidophilus* was shown to reduce shedding of pathogenic *Escherichia coli* from 100 to 47%. Also there was an increased weight gain in chicks after an oral dosage of *Lactobacillus acidophilus* as reported by Tortuero in 1973.

1.2 Literature Review

Many studies have been carried out over the years to isolate the various *Lactobacillus* species and study its various physiology and biochemical properties. Studies have also shown its effectiveness as probiotics in humans as well as in animals. Although there is a wide range of literature which cover various aspects of *Lactobacillus* such as its utilisation in food production as a starter culture in for e.g. yoghurt and cheese making, its clinical applications, its metabolism, taxonomy, its physiological properties and so on. This literature review will focus on certain characteristics of *Lactobacillus* such as its isolation, characterization, its probiotic potential and its efficacy as probiotic in animal feed. It will also view the antimicrobial properties and the viability of *Lactobacillus* as a freeze dried product with different cryoprotectants.

1.2.1 Isolation and Identification of *Lactobacilli*

Isolation is often a long drawn out process since it requires repeated subculturing to obtain a pure single strain colony. In case of *Lactobacillus,* the isolates are frequently grown and subcultured in MRS media. MRS named so by the inventors De Man, Rogosa and Sharpe in 1960. They developed an improved growth medium for *Lactobacilli* which supports good growth of it and also useful for strains which grow poorly in other media. In this media the use of tomato juice which is a highly variable material wasn't required. They also reported that in a slightly modified form it can be used as a basal medium for fermentation tests (de Man *et al*., 1960).

A study carried out for the development of a PCR method for the identification of *Lactobacillus* at the genus level was accomplished by Sègolenè Dubernet *et al.,* in 2002 they designed one specific primer, LbLMA1-rev by analyzing similarities between the nucleotide sequence of the spacer region between the 16S and 23S rRNA genes in a number of *Lactobacillus* strains. Their results showed that amplification with the primer LbLMA1-rev and R16-1 generated a PCR product for 23 *Lactobacillus* species. They also carried out PCR using *Escherichia coli*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *Carnobacterium pissicola*, *Pediococcus pentosaceus*, *Bifidobacterium bifidum*, *Weissella confusa*, *Enterococcus hirae*, *Staphylococcus aureus* and *Listeria monocytogenes* DNA as a template. After carrying out electrophoresis using these PCR products, they did not reveal any discrete bands. They concluded that PCR primers amplified DNA only from

bacteria belonging to the *Lactobacillus* group of LAB and did not amplify other strains closely related to *Lactobacilli*. LbLMA1- rev/R16-1-based PCR could be a useful tool for identification of the members of the *Lactobacillus* genus (Dubernet *et al*., 2002).

Nasrin Noohi *et al.,* in 2014 isolated *Lactobacillus* species from poultry and evaluated the phenotypic characteristics and its probiotic potential. They isolated a total of 168 Lactic acid Bacteria from twenty-one day old chickens and feed samples. They identified the isolates by morphological, biochemical, and molecular tests including PCR and 16S rRNA gene sequencing. Biochemical fingerprinting with Phene Plate system (Ph-P) was done and the acid and bile resistant *Lactobacilli* were subjected to the antibiotic susceptibility test. Their results showed that amongst the entire examined LAB, 30.3% were resistant to bile and acid. Most of the isolated LAB (57.1%) belonged to the genus *Lactobacillus* with *Lactobacillus brevis* (78.1%) as the dominant species followed by *L. reuteri* (16.6%), *L. plantarum* (3%), and *L. vaginalis* (2%). The remaining isolates were identified as *Pediococcus* spp. (42.9%). The Ph-P cluster analysis of 75 *L. brevis* and 16 *L. reuteri* strains showed high phenotypic diversity. Whilst the results of Ph-P typing from *L. reuteri* strains showed low phenotypic variations especially among the strains sensitive to acid and bile salts (Noohi *et al*., 2014).

Isolation of *Lactobacilli* species was also done by Ro Osawa and his colleagues in 2000 in Japan. They isolated *Lactobacilli* species with tannase activity from human faeces and fermented foods. The tannase activity was determined by growing the isolates on tannin-treated brain heart infusion agar. Colonies with distinct clear zones were the ones having tannase activity. The identification of the isolates was then determined by biochemical tests with commercially available kit API 50 CHL. This revealed that faecal isolates were *Lactobacillus plantarum* and the food isolates were *Lactobacillus plantarum* and *Lactobacillus pentosus*. This was further confirmed by PCR- based method that amplifies species specific sequences in the 16S-23S ribosomal DNA spacer regions of *Lactobacillus* species. The PCR results correlated with results obtained from the commercial kit API 50 CHL, however four isolates that were tentatively identified as *L. plantarum* with the API system were found to be *L. paraplantarum* by PCR. This study was the first to report occurrence of *Lactobacilli* capable of degrading tannin in human gut microflora and foodstuffs. Though humans don't rely much on tannin-rich diets, but there are beverages and teas that are consumed regularly that have hydrolysable tannins with marked pharmacological

activities. Hence the presence of these *Lactobacilli* in the gastrointestinal tract may have a significant effect on medicinal properties of tannins (Osawa *et al*., 2000).

1.2.2 Acid and Bile Tolerance of *Lactobacillus* species

In many studies the results of probiotics are contradictory; most of them show that they are beneficial while some study suggest that they have no beneficial effects (Jin *et al*., 1998). The reason for the difference in results could be due to the strain of the microorganism used. Hence emphasis has been placed on certain factors for the preparation and selection of *Lactobacillus* strains as probiotics (Havenaar *et al*., 1992). Certain factors such as the bile tolerance and tolerance to low pH have to be considered for selecting probiotics which will give beneficial results.

A study carried out by Jin *et al.*, in 1998 with 12 *Lactobacillus* strains isolated from the chicken intestine was used to investigate acid and bile tolerance in vitro. Their results showed that 10 out of the 12 strains were slightly affected by 0.3% bile salts showing a delay of growth as compared with the growth in the control cultures. Two strains were not affected by the bile salts. Of the 12 strains, seven were classified as resistant and five as tolerant. Their results showed that *Lactobacillus* strains from the caecum showed better tolerance to acid than those from the ileum. The survival of ileal strains was very low at pH 1.0 and 2.0 and moderate at pH 3.0. In contrast, the caecal *Lactobacillus* strains could survive at pH 1.0 for upto 2 hours of incubation. Growth was moderate at pH 2.0 and good at pH 3.0 and 4.0 (Jin *et al*., 1998).

In another study by Boonkumklao *et al*., in 2006 five strains of novel thermotolerant lactic acid bacterium, *Lactobacillus thermotolerans* was isolated from the chicken faeces and were assayed for acid and bile tolerance. Cultures of *L. thermotolerans* were grown anaerobically at 42°C in four different conditions. They were grown in MRS broth at pH 6.5 and pH 3.0, MRS broth supplemented with 0.3% ox gall and MRS broth supplemented with 7mM sodium taurocholate. Their results showed that all strains showed an acid tolerance when incubated at pH 3.0 over a period of 4 h. *L. thermotolerans* demonstrated significant changes in absorbance from the control when cultured with 0.3% bile concentration. The strain JCM11427 showed the highest tolerance to bile. The conjugated bile salt (sodium taurocholate) at physiological concentration (7mM) had no effect on the growth of the bacterial strains (Boonkumklao *et al*., 2006).

W.K. Ding and N.P. Shah carried out a study on eight strains of probiotic bacteria including *Lactobacillus rhamnosus*, *Bifidobacterium longum, L. salivarius, L. plantarum*, *L. acidophilus*, *L. paracasei*, *B. lactis* type Bl-O4, and *B. lactis* type Bi-07, for their acid, bile, and heat tolerance. Microencapsulation in alginate matrix was used to enhance the survival of the bacteria in acid and bile as well as brief exposure to heat. The acid tolerance of probiotic organisms was tested using HCl in MRS broth over a 2-h incubation period. Bile tolerance was tested using 2 types of bile salts, oxgall and taurocholic acid, over an 8-h incubation period. Heat tolerance was tested by exposing the probiotic organisms to 65 °C for up to 1 h. They used free probiotic organisms as a control. Their results showed that microencapsulate probiotic bacteria survived better than the free probiotic bacteria in MRS containing HCl. When they exposed free probiotic bacteria to oxgall, viability was reduced by 6.51-log CFU/ml, whereas only 3.36-log CFU/ml was lost in microencapsulated strains. At 30 min of heat treatment, microencapsulated probiotic bacteria survived with an average loss of only 4.17-log CFU/ml, compared to 6.74-log CFU/ml loss with free probiotic bacteria. However, after 1 h of heating both free and microencapsulated probiotic strains showed similar losses in viability. Overall their results suggested that microencapsulation improved the survival of probiotic bacteria when exposed to acidic conditions, bile salts, and mild heat treatment (Ding and Shah, 2007).

1.2.3 The probiotic potential of *Lactobacillus*

Gusils, Gonzàlez and Oliver in 1999 reported the probiotic properties of chicken *Lactobacilli*. *Lactobacillus* is beneficial due to its ability to colonise the gastrointestinal tract. In their work, they determined the adhesion assays with three *Lactobacillus* strains and intestinal fragments obtained from chicken. They reported that *Lactobacillus animalis* and *L. fermentum* were able to adhere to three kinds of epithelial cells i.e. crop, small and large intestines with predominance to small intestine. They characterised the adhesion of *L. animalis* and it indicated that lectin-like structure of this strain has glucose/mannose as specific sugars of binding. They also reported that *Lactobacillus fermentum* was effective in reducing the attachment of *Salmonella pullorum* by 77%, while *L. animalis* was able to inhibit the adhesion of *S. pullorum* by 90%, *S. enteritidis* by 80% and 78% of *S. gallinarum* to host-specific epithelial fragments. Their final results suggested that these *Lactobacilli* are able to block the binding sites for *Salmonella* adhesion (Gusils *et al*., 1999).

A study carried out by Layton *et al*. in 2013 reported the effect of *Lactobacillus* probiotic in the control of necrotic enteritis (NE) caused by *Clostridium perfringens* (CP) in poultry. This disease causes serious economic implications. They studied the effect of commercially available *Lactobacillus*-based probiotic (FM-B11) and carried out the study by observing one day hatched chicks for 29 days placed randomly in three groups. Group 1 was the control, group 2 was challenged with CP and group 3 was challenged with CP and treated with the probiotic FM-B11. Their results showed that chicks treated with FM-B11 had significantly higher body weight gain after challenge when compared to control challenge chickens. Total mortality was higher in group 2 which was 48.8% when compared to group 3 which was 12.7%. Also, the group treated with the probiotic had a lower CFU/ml number of CP recovered from ileal mucosa when compared to the challenged group. The result of this study showed that the commercially available *Lactobacillus*-based probiotic FM-B11 was able to reduce the severities of NE, as a secondary bacterial infection, in an experimental NE challenge model (Layton *et al*., 2013).

The probiotic potential of *Lactobacilli* species isolated from the intestinal tract of chickens was also carried out by Behira Belkacem and his fellow researchers in 2009. They isolated thirteen thermotolerant *Lactobacilli* from the small intestine of domestic chickens. The isolates were characterised and identified but only the isolate that survived and grew in acidic medium and resisted bile salts were retained. Their results showed that among thirteen isolates five isolates that responded to these criteria and were identified as *Lactobacillus gallinarum*, *Lactobacillus crispatus*, *Lactobacillus dulbreuki*i, *Lactobacillus acidophilus* and *Lactobacillus jonhsonni*. These strains were homofermentatives and produced more than 0.6% lactic acid (w/v) after 24 h of incubation and the nature of the isomers was confirmed by the High Performance Liquid Chromatographic. All the strains were proteolytic. They also investigated antimicrobial activity against *salmonella* and results showed that anti-*salmonella* activity was detected *in vitro* against the species of *Salmonella enteriditis* and *Salmonella infantis* (Belkacem *et al*., 2009).

1.2.4 Antimicrobial activity of *Lactobacillus* and its Bacteriocin

Probiotic *Lactobacillus* can also be used to reduce the colonization of pathogenic bacteria in food animals, and therefore reduce the risk of foodborne illness to consumers. In a model system, it was examined the mechanism of protection conferred by *Lactobacillus* species to

inhibit *C. jejuni* growth in vitro and reduce colonization in broiler chickens. Possible mechanisms for the reduction of pathogens by *Lactobacilli* include 1) stimulation of adaptive immunity; 2) alteration of the cecal microbiome; and, 3) production of inhibitory metabolites, such as organic acids. In a study it was shown that *Lactobacillus* species produced lactic acid at concentrations sufficient to kill *C. jejuni* in vitro. It was determined that lactic acid produced by *Lactobacillus* disrupted the membrane of *C. jejuni*, which was judged by biophotonics. The spectral features obtained using Fourier-transform infrared (FTIR) and Raman spectroscopy techniques was used to accurately predict bacterial viability and differentiate *C. jejuni* samples according to lactic acid treatment. FT-IR spectral features of *C. jejuni* and *Lactobacillus* grew in co-culture revealed that the metabolism was dominated by *Lactobacillus* prior to the killing of *C. jejuni*. Based on these results, the development of future competitive exclusion strategies could include the evaluation of organic acid production (Neal-McKinney *et al.*, 2012).

Antifungal and biopreservation potentials of *Lactobacillus plantarum* YML007 isolated from Korean kimchi were analysed in a study. The biopreservative efficacy of the bacterium was analysed using maize grains. Maize was divided into 3 groups and treated with a 5× concentrated cell-free supernatant of *L. plantarum* YML007, organic acids, and a control group lacking treatment. All groups were stored for 30 days. Maize was tested for moisture and crude fat contents, mould growth, and aflatoxin production. The moisture content, mould count, and toxin production were higher in the control maize. The YML007 and acid treated maize remained uninfected after 30 days. Animals fed with YML007 treated maize showed more weight gain and less feed consumption. Hence the study concluded that YML007 can be used to preserve the nutritional value of stored grain and to ensure better quality feedstuffs that are necessary for improving animal health and performance (Rather *et al.*, 2014).

A study carried out by Ogunbanwo *et al.* in 2003 characterised bacteriocin produced *L. plantarum* F1 and *L. brevis* OG1. *L. plantarum* F1 and *L. brevis* OG1 which was isolated from Nigerian fermented food products. The bacteriocins produced had a broad spectrum of inhibition against both pathogenic, food spoilage organisms and various lactic acid bacteria. The test organisms exhibited activities against *Escherichia coli* NCTC10418 and *Enterococcus faecalis* EF1 but did not inhibit *Candida albicans* ATCC10231 and *Klebsiella* sp. UCH15. Their study showed that bacteriocin produced by *L. brevis* OG1 was the most heat stable at 121°C for 60 min, while that of *L. plantarum* F1 was stable at 121°C for 10

min. The bacteriocins produced by the test isolates maintained full stability after storage for 60 days at – 20°C; partial stability after storage for 120 days at 4°C; while activity was not detected after storage for 80 to 120 days at 37°C. Bacteriocin produced by *L. brevis* OG1 was stable at pH range of 2.0 to 8.0 while, that of *L. plantarum* F1 was found to be stable at pH 2.0 to 6.0. They found that the activity of bacteriocin was not affected by mitomycin C and UV light but extracting it with chloroform destroyed its activity. They concluded that the ability of bacteriocins produced by the test isolates inhibited a wide-range of bacteria and it could be of potential interest for food safety and applications as a food preservative (Ogunbanwo *et al.*, 2003).

1.2.5 Viability of *Lactobacillus* Species after freeze drying

A study was carried out by Sofia Carvalho *et al.* in 2002 to investigate the survival of freeze dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of protectants. Their results showed that no significant differences were observed in the viability of both the species cells during freeze drying in the presence or absence of inositol, sorbitol, fructose, trehalose, monosodium glutamate and propyl gallate. However, their results showed that survival of *Lactobacillus* was higher during storage when drying took place in the presence of these compounds. Sorbitol produced more significant effects than the other compounds toward maintaining the viability of freeze dried *L. plantarum* and *L. rhamnosus* (Carvalho *et al.*, 2002).

In another similar study by Michele Savini *et al.* in 2010 determined the viability of freeze dried probiotic bacteria using different protective agents. They analysed the viability of freeze dried *Lactobacillus rhamnosus* IMC 501 and *Lactobacillus paracasei* IMC 502 using glycerine, mannitol, sorbitol, inulin, dextrin and Crystalean as protective agents and were compared with semi skimmed milk (SSM) as the control. Their results showed that no significant differences were observed between the tested protectants and the control SSM when under storage in refrigerated conditions. Their results also showed that in storage during room temperature conditions only glycerine was found to stabilize better that the other tested protectants (Savini *et al.*, 2010).

Freeze drying is one of the methods to preserve probiotic cultures. A study was carried out with the aim to compare survival rate of probiotic bacteria *Lactobacillus rhamnosus* during spray and freeze drying in aqueous two- phase systems by Katarzyna Leja *et al.* in 2009.

These were compared with survival rate of cells dried under same conditions but suspended in skim milk, 6% solution of PVP or 6% solution of dextran. The bacteria *Lactobacillus rhamnosus* GG were suspended and spray or freeze dried in various types of aqueous two-phase emulsions which were: PVP/dextran, PEG4000/dextran and PEG8000/dextran. These emulsions consisted of different types of polymers and had a varying ratio of polymers in dispersed (dextran) and dispersing (PEG and PVP) phases. The research demonstrated that survival rate of bacteria directly after drying depended mainly on protective reagent, rather than on drying method. After 30-day-storage of the dried bacteria cell specimens, the highest survival rate was noted in the case of freeze dried cells in milk. In the case of spray drying the highest cell survival rate was observed when emulsion PVP3.6%/dextran2.4% was used as a drying medium. They concluded the study with results indicating that cell survival rate was not strongly influenced by the storage temperature of the powder but it depended on the drying medium (Leja *et al.*, 2009).

1.3 Objective of the Study

The main objective of this study was to:

- Develop probiotic supplement to poultry feed using *Lactobacillus* species isolated from the gastrointestinal tract of local healthy chickens.
- Determine the probiotic potential of the isolated *Lactobacillus* species by using in-vitro analysis.
- Observe the shelf life and viability of the freeze dried probiotic supplement in poultry feed.
- Determine the efficacy of the probiotic poultry feed by in-vivo analysis through a field trial conducted for a period of five weeks.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Research Location

This study was carried out in Industrial Microbiology Laboratory of the Institute of Food Sciences and Technology (IFST) division at the Bangladesh Council of Scientific and Industrial Research (BCSIR) located at Dr Qudrat-I-Khuda Road, in Dhaka. The Field Trial part of this study was carried out at Bangladesh Livestock Research Institute (BLRI), located in Savar, Dhaka.

2.1.2 Isolates

In this study, four isolates were used which were obtained from Industrial Microbiology lab and were isolated from the intestinal tract of locally bred chickens and were identified with morphological and biochemical tests to be as species of *Lactobacillus*.

2.2 Media and Solutions

MRS broth and Agar: Bacterial growth medium and it is named after its inventors: de Man, Rogosa and Sharpe. This medium favours the luxuriant growth of *Lactobacilli* for lab study. It contains sodium acetate, which suppresses the growth of many competing bacteria.

Ringer's Solution: Solution of several salts dissolved in water for the purpose of creating an isotonic solution, and named after Sydney Ringer. Ringer's solution typically contains sodium chloride, potassium chloride, calcium chloride and sodium bicarbonate. In this study, it was used for the serial dilution of bacterial cultures.

Synthetic Media for *Lactobacillus* growth: The mass culture medium was a synthetic media consisting of (%, w/v): yeast extract 0.5%, beef extract 0.75%, Proteose peptone 0.5%, Dextrose 1%, NaCl 0.3%, K_2HPO_4 0.1%, $(NH_4)_2SO_4$ 0.1% and $MgSO_4 \cdot 7H_2O$ 0.5%, in distilled water, it had a pH of 6.5±0.2.

PBS buffer: Abbreviated for Phosphate buffered saline is a water based salt solution containing disodium hydrogen phosphate, sodium chloride, potassium chloride and potassium dihydrogen phosphate. It is isotonic and non-toxic to most cells. In this study, PBS was used for the rinsing of cells during centrifugation and also used in the process of determining the cell surface hydrophobicity.

2.3 Methods

2.3.1 Acid Tolerance Test

The acid tolerance of the microorganisms was studied by modifying the pH of the MRS broth. The pH of the MRS broth was modified by using 1N HCl and the pH was adjusted to pH of 3, 4, 5 and 6 and was inoculated with 1% of fresh culture of the isolates and incubated for 18 hours at 37°C.

2.3.2 Bile Tolerance Test

The *Lactobacillus* isolates were grown overnight in MRS broth and 1% of this fresh culture was used to inoculate MRS broth containing bile salts at the concentration (w/v) of 0.5%, 1.0%, 1.5%, and 2.0%. The one without bile salt acted as the control. The inoculated tubes were incubated at 37°C and kept for 18 hours.

2.3.3 Antimicrobial Activity

Agar well Diffusion Assay

The antimicrobial activity was determined by agar well diffusion assay (Khay *et al*., 2011). The *Lactobacilli* fresh cultures were grown overnight in 10ml of MRS broth and incubated at 37°C under anaerobic conditions. The indicator strains were also grown overnight in 10ml of BHI and incubated at 37°C. Using a borer, wells were cut onto Mueller Hinton Agar (MHA) laid plates. The indicator microorganisms were lawn plated onto the MHA agar plates, they were inoculated onto the plate by using a sterile cotton swab dipped into the BHI broth containing the indicator microorganisms. The overnight *Lactobacilli* culture was centrifuged to obtain the supernatant. Then 30µl of the supernatant was transferred to the wells cut into the MHA plates. The plates were then incubated at 37°C for 24 hrs and the inhibition zones were measured to determine antibacterial activity.

2.3.4 Cell surface Hydrophobicity

Determination of cell surface hydrophobicity is based on the ability of the cells to partition into the hydrocarbon from the phosphate buffered solution. The test was performed as described by Rosenberg *et al.* in 1983. The bacterial isolate was grown in MRS broth at 37°C

for 24 hrs in anaerobic conditions. After that 3ml of the broth were centrifuged at 100×100 rpm for 5 minutes to obtain the cell pellet. The pellet was washed twice with PBS buffer and then the pellet resuspended in 5ml of PBS buffer and the absorbance measured at 540nm. Then 1ml of hydrocarbon in this case xylene was added to the cell suspension and vortexed vigorously for 30 sec. After that, it was kept for phase separation approximately 30 minutes. Then the aqueous phase was carefully collected and the optical density was measured and compared with the initial value. The hydrophobicity was calculated according to the equation:

$$(A_{540}\ \text{initial} - A_{540}\ \text{aqueous phase})/A_{540}] \times 100 = \%\text{hydrophobicity.}$$

2.3.5 Developing the Probiotic Additive

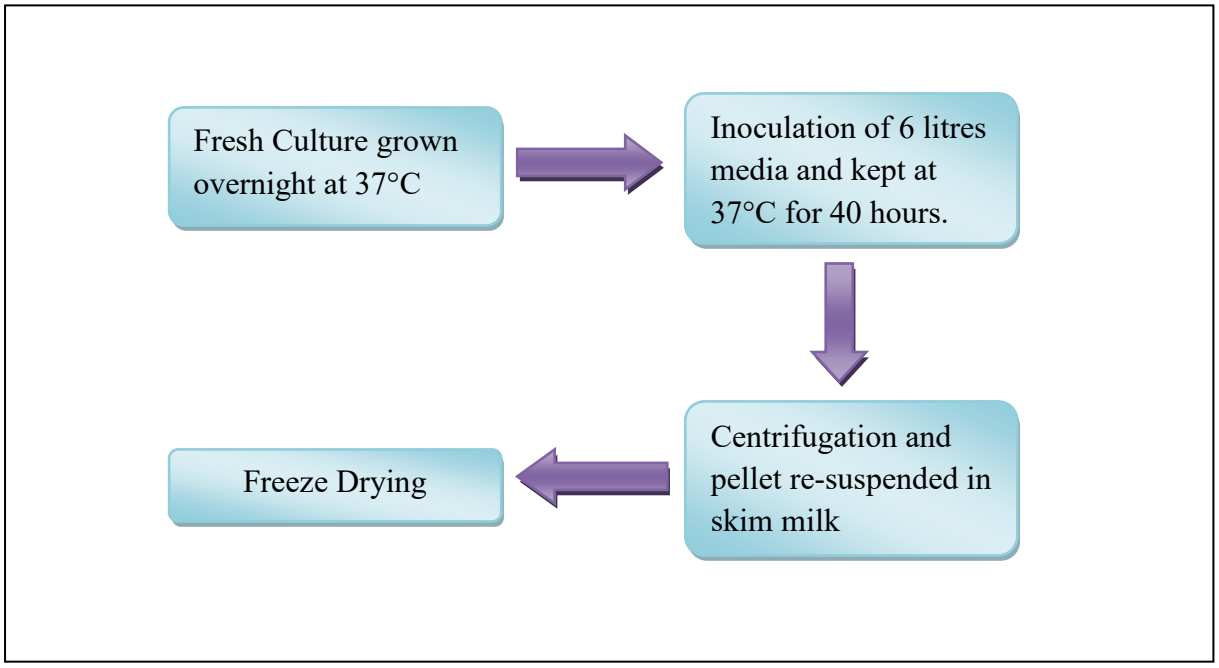

Figure 2.1: Outline of the Probiotic additive development.

2.3.5.1 Bacteria and Growth conditions

The isolates used for mass culture were isolate 10 and isolate 14. The fresh culture was grown overnight in MRS broth and incubated at 37°C under anaerobic conditions. The fresh culture was used to inoculate the mass culture media broth.

2.3.5.2 Mass Culture

The mass culture media was prepared according to the composition as previously described. A total of 6 litres of media was prepared for each isolate at a time. This media was autoclaved at 121 °C for 15 minutes. The media was inoculated with 2% of fresh culture and were incubated at 37 °C without agitation in anaerobic conditions (CO_2 incubator with 5% CO_2) and kept for 40 hours.

2.3.5.3 Centrifugation and Freeze drying

After the mass growth of isolates the media was centrifuged to separate the broth and cells to obtain the pellet. It was centrifuged at 50×100 rpm, for 10 minutes at a temperature of 4°C. After centrifugation the pellet was re-suspended in 10% of skim milk and kept overnight at -20°C. After that, the freeze drying process was started and carried out for 48 hours straight to obtain a dry powder form containing the probiotic microorganisms.

2.3.5.4 Cell Count

The cell count was taken of the mass culture after its incubation period and after the freeze-drying process. 1ml of media broth after incubation period was taken and transferred to 9ml of ringer's solution. From here it was serially diluted from 10^{-1} to 10^{-15}. After serial dilution the last 3 dilutions i.e. 10^{-13} to 10^{-15} was spread plated by transferring 100µl onto MRS agar plates and then incubated at 37°C in anaerobic conditions. The cell count was also taken after the freeze drying process. The freeze dried isolate, in its powder form was diluted in ringer's solution. 1g of powder was diluted in 9ml of ringer's solution. It was serially diluted from 10^{1} to 10^{-15}. After serial dilution, the last 3 dilutions i.e. 10^{-13} to 10^{-15} was spread plated by transferring 100µl onto MRS agar plates and then incubated at 37°C in anaerobic conditions. The count was taken by counting the number of colonies as CFU/ml.

2.3.6 Determining the Growth Curve of *Lactobacillus*

The growth curve of one of the isolates of the *Lactobacillus* was observed to determine the log, lag and the stationary phase of the isolate. Using a fresh culture grown overnight the isolate 10 was inoculated into 14 McCartney bottles each containing 10ml of MRS broth. Starting from the zero hours after every two hours the cell count were taken by removing a McCartney from the incubator containing the inoculated media and serial diluting the MRS broth culture into ringer's solution and transferring 100µl of the ringer to be spread plated

onto plates laid with MRS agar. The plates were then incubated at 37°C under anaerobic condition for 24hrs and then the number of colonies was counted. The graph was plotted cell count against time to observe the growth pattern.

2.3.7 Heat Stability of *Lactobacillus*

The heat stability of *Lactobacillus* was determined at 80°C for a couple of minutes. The heat stability was determined by keeping the *Lactobacillus* species in MRS broth, re-suspended in skim milk and the freeze dried powder in a water bath at a temperature of 80°C for 0.5 min, 1 min, 1.5 min and 2 minutes. For the *Lactobacillus* in MRS broth, 1 ml of fresh culture grown overnight was taken in each of four test tubes. For the *Lactobacillus* re-suspended in skim milk, 1ml of fresh culture was centrifuged and the pellet was re-suspended in 1ml of skim milk and taken in each of the four test tubes. For the freeze dried powder 1gm was taken into each of the four test tubes. All the tubes were kept in water bath at 80°C and at the interval of 0.5 min one test tube of each criterion was removed from the water bath this followed by time intervals of 1 min, 1.5 min and at 2 min. Then each of them was diluted in ringer's solution and 100µl was plated onto MRS laid agar plates and the cell count was taken.

2.3.8 Viability of Freeze Dried Probiotic

The viability of the freeze dried probiotic was determined by taking the cell count of the powder at regular intervals of time over a period of four months. A specific amount of powder was measured and diluted in ringer's solution, it was then serially diluted from 10^{-1} till 10^{-12} and then they were spread plated onto MRS laid plates and incubated for 24h at 37°C in anaerobic conditions. After incubation, the cell count was then taken to determine the viability of the freeze dried powder during the period of storage.

2.4 Field Trial

The field trial was carried out at BLRI (Bangladesh Livestock Research Institute) in Savar. 48 broiler chicks of 1 day age were divided into four groups with 12 chicks and each group was further sub-divided into three groups with 4 chicks so that each treatment was done in triplicate. The first group served as the control which was fed with poultry feed without any supplements. The second group were fed with poultry feed supplemented with antibiotic tetracycline. The third treatment group was fed with poultry feed with isolate 10 probiotic

additive and the fourth treatment group was fed with poultry feed with isolate 14 probiotic additive. At the beginning of the trial, each of the chicks was randomly assigned to a group and was weighed individually in the beginning. The feeding trial lasted 5 weeks after which the weight gain and growth of all the different treatment groups were determined.

2.4.1 Faecal Sampling

Samples of faeces were collected from each treatment group. The faeces was then homogenised in ringer's solution and then serially diluted from 10^{-1} to 10^{-5} and then 100µl of ringer was spread plated onto MRS laid plates and incubated under anaerobic conditions at 37°C.

Chapter 3

Results

3.1 Acid Tolerance

Most bacteria do not survive in harsh or extreme conditions. Hence the bacterial strains being used as probiotics must be tested whether it can survive in extreme conditions such as low pH. The severe acidic conditions of the crop and gizzard could have an adverse effect on the bacteria Thus, it has been suggested that microbial cultures to be used as probiotics should be screened for their resistance to acidity (Boonkumklao *et al.*, 2006). In this test, the tolerance to acid was determined of the isolates.

Table 3.1: The tolerance to different pH values of the isolates. The symbol of +/- denotes growth of 18 hours culture.

Isolates	pH 3	pH 4	pH 5	pH 6
2	***-**	++	++	++
8	***-**	+	++	++
10	***-**	++	++	++
14	***-**	++	+	++

* The pH 3 is not bactericidal, Isolates were surviving or slow in growth at those pH

The positive test of this result was indicated by the turbidity of the media indicating the growth of the isolates. At pH 6 the media was very turbid indicating maximum growths for all of the isolates. At pH 5 isolate 14 and at pH 4 isolate 8 the media was less cloudy or turbid compared to rest indicating lesser growth compared to others. At pH 3 the media was a little hazy indicating the least or no growth but it is considered that this pH is not bactericidal but growth is very slow compared to the other pH values.

3.2 Resistance to Bile

Once the living microorganisms in the probiotic reach the intestinal tract, their ability to survive depends on their resistance to bile (Gilliland *et al.*, 1984). Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria (Ding & Shah, 2007) Hence, it can be said that the success of a probiotic also depends on the bile-resistant qualities of the selected strains. Hence the isolates were tested for resistance to different concentrations of bile.

Table 3.2: The resistance of isolates to different bile concentrations. The symbol of +/- denotes growth of 18 hours culture.

Isolates	0.5 % Bile	1.0 % Bile	1.5 % Bile	2.0 % Bile
2	++	++	++	++
8	++	++	++	++
10	++	++	++	++
14	+	++	+	+

The positive result of the test was indicated by the turbidity of the media which meant isolates were growing in the media. From the results shown it can be concluded that the all the isolates grew in all the concentration of bile. Isolate 14 grew least in concentration of 0.5%, 1.5% and at 2 % when comparing the turbidity of the media to others. All the other growth of the isolates had similar turbidity and was taken as a positive result in their ability in resisting bile.

3.3 Antimicrobial Activity

One of the criteria of good probiotic is that it should exhibit antimicrobial activity against pathogens and maintain the homoeostasis of the intestinal flora. Antimicrobial is the ability of one species to inhibit the growth of another species (Fijan, 2016). Hence before selecting a strain for probiotic its antimicrobial activity should be checked in-vitro to determine its probiotic efficacy. In this test, the antimicrobial activity of the isolates was tested against four microorganisms, *Salmonella typhi, E. coli, Shigella and S. aureus.*

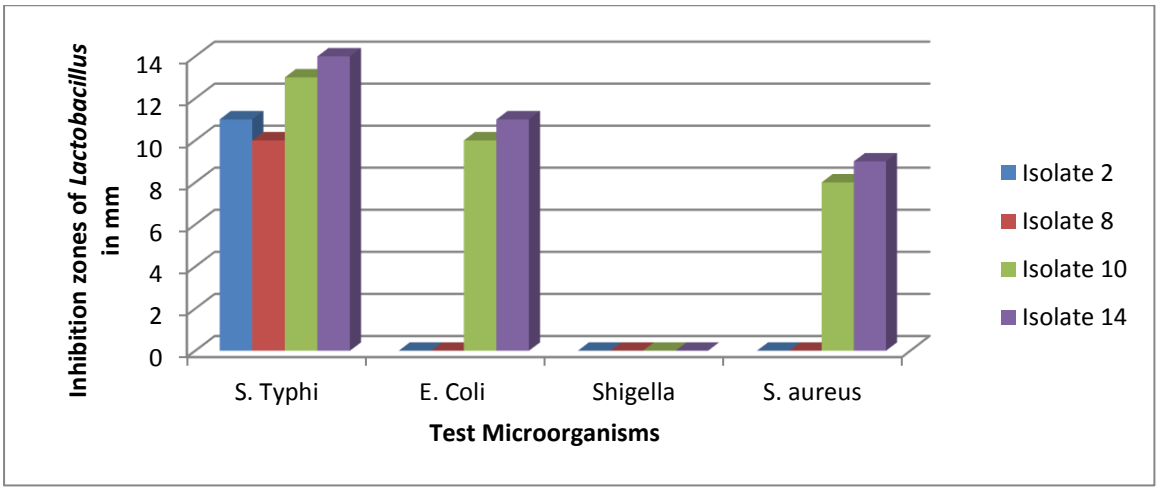

Figure 3.1: Antimicrobial activity of probiotic with the inhibition zones

As it can be seen in figure 3.1; all the four isolates showed no antimicrobial activity towards *Shigella*. Isolate 2 and 8 showed no activity against *E. coli* and S. *aureus* and had antimicrobial activity against *S. typhi* with an inhibition zone of 11mm and 10mm respectively. Isolate 10 and 14 had antimicrobial activity against *S. typhi* with 13mm and 14mm inhibition zones respectively. They showed activity against *E. coli* with 10mm and 11mm zones and against *S. aureus* with 8mm and 9mm inhibition zones respectively.

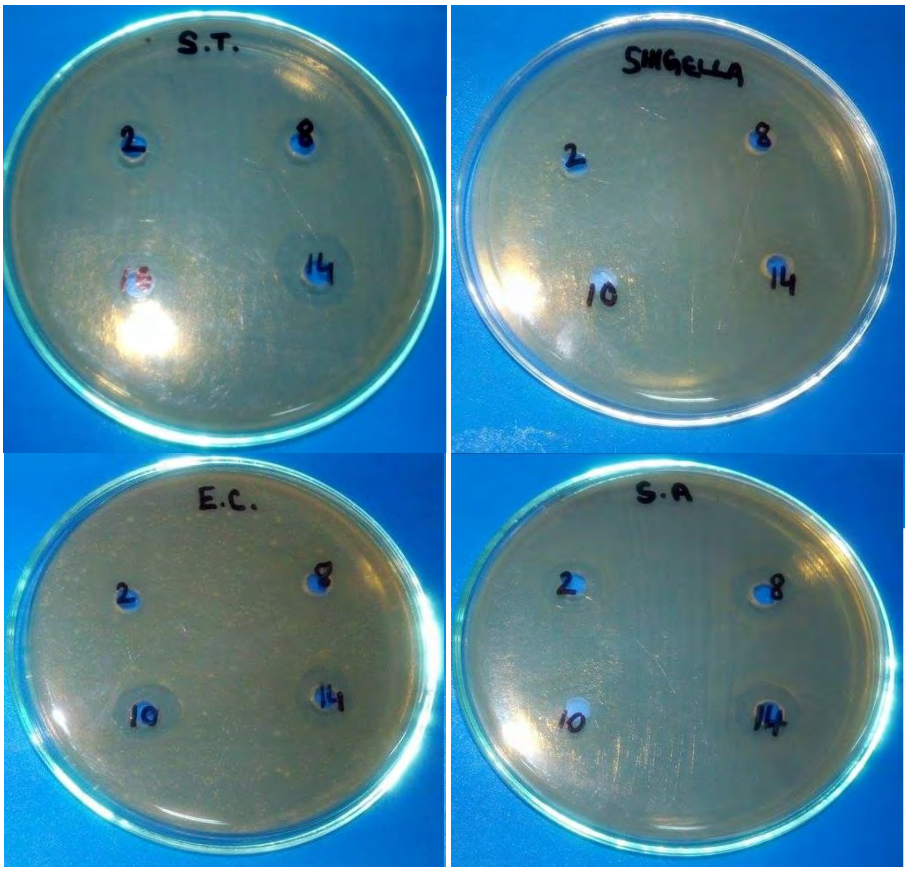

Figure 3.2: The antimicrobial activity of the isolates against the test microorganisms

3.4 Cell Surface Hydrophobicity

The role of cell surface hydrophobicity in mediating bacterial adherence to mammalian cells was conceived of over half a century ago in the studies of Mudd and Mudd (Rosenberg *et al.*, 1983). Adhesion to host tissues, in particular to epithelial cell line, is a well established probiotic criterion as it provides prolonged persistence in the gut and more efficient host-microbial interaction. Cell surface hydrophobicity is often considered as the primary factor influencing the strength of bacterial adhesion (Georgieva *et al.,* 2016). Strains are considered

to be hydrophobic if they have a hydrophobicity of more than 40% (Abdulla *et al*., 2013). The results of this test have shown that the isolates had a range of 23% to 48% hydrophobicity. The greatest affinity for xylene was shown by isolate 10 with 48% hydrophobicity followed by isolate 14 with 46%. The least affinity was shown by isolates 2 and 8 with both less than 40% i.e. 37.75% and 23.98% respectively and is considered to have least adhesion ability compared to isolate 10 and 14. Results are shown in table 3.3 and figure 3.3.

Table 3.3: The absorbance measured of the four *Lactobacillus* strains suspended in PBS and the aqueous phase after separating it from xylene.

Isolates	A_{540} PBS-suspended cells	A_{540} of Aqueous Phase
2	**0.821**	**0.711**
8	**0.813**	**0.718**
10	**0.750**	**0.551**
14	**0.829**	**0.631**

Using the data in the above table the percentage hydrophobicity is calculated using the following formula

$(A_{540}$ initial – A_{540} aqueous phase)/$A_{540}] \times 100$ = %hydrophobicity.

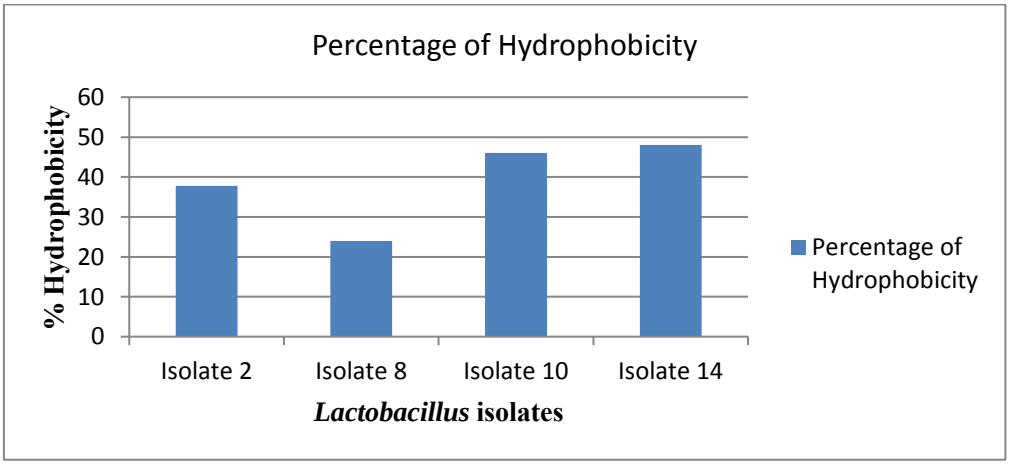

Figure 3.3: Percentage hydrophobicity of the *Lactobacillus* isolates.

3.5 Survival of Probiotic after Freeze Drying

Freeze drying is a common method to incorporate probiotics into foods however during its processing it affects the viability of cells and also during storage. Hence freeze drying probiotic organisms are protected by adding cryoprotectants (Savini, *et al.*, 2010). In this test skim milk was used as the cryoprotectant and the loss of cells after freeze drying was determined. The results show that no significant amount of cells was lost. The maximum loss was seen in isolate 8 where 3 log reductions was seen followed by isolate 10 and 14 where reduction was of 2 logs after freeze drying and the least reduction was seen in isolate 2 where only 1 log of cells was lost. The results are depicted below.

Table 3.4: The survivability of *Lactobacillus* in cell count (CFU/ml) after it was freeze dried.

Isolate	Cell count before Freeze Drying (CFU/ml)	Cell count after Freeze Drying (CFU/ml)
2	**5.2×10^{17}**	**3×10^{16}**
8	**3×10^{16}**	**4.2×10^{13}**
10	**2×10^{18}**	**6.7×10^{16}**
14	**1.3×10^{18}**	**2.5×10^{16}**

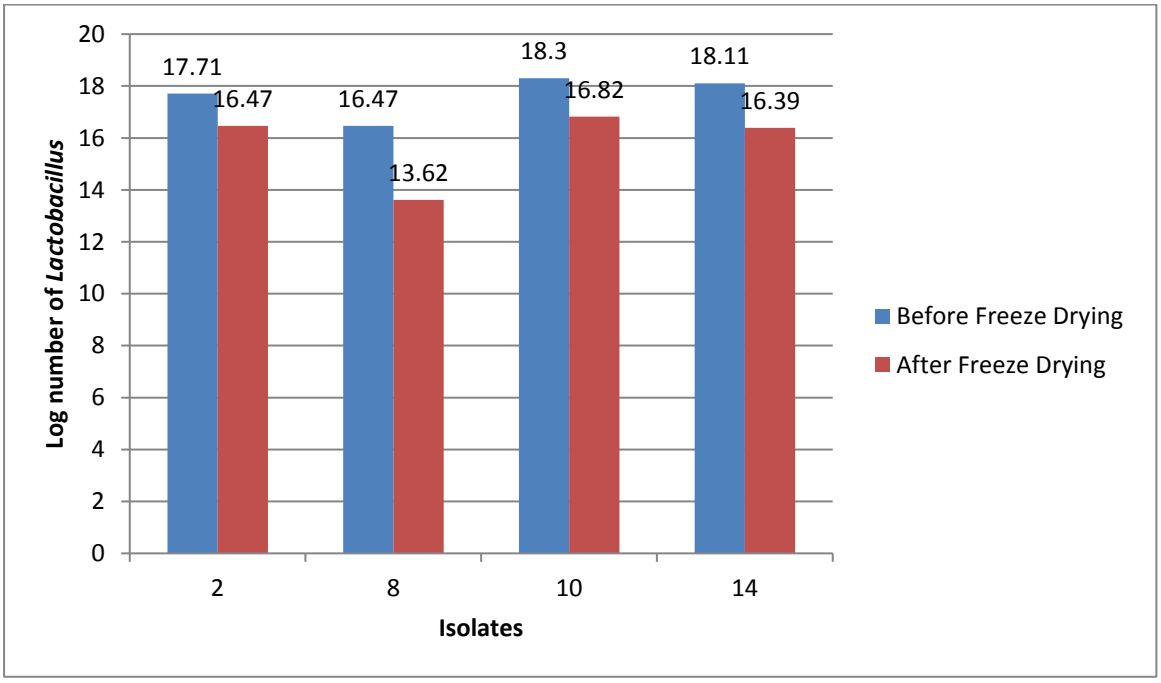

Figure 3.4: the log number of *Lactobacillus* before and after freeze drying.

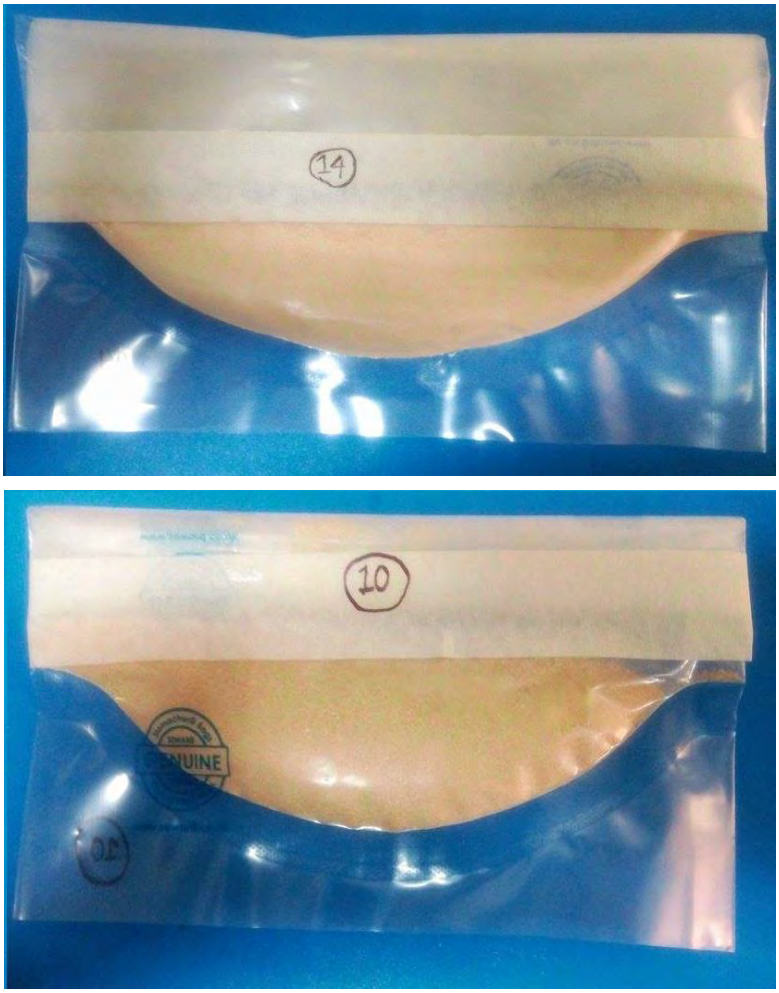

Figure 3.5: The probiotic additive of isolate 10 and 14 after freeze drying

3.6 Heat stability of *Lactobacillus*

Heat is used in the processing of poultry feeds such as in mixing, grinding and also in the process of pellet formation. Since probiotics are living microorganisms and must be consumed in enough amounts for it confer a beneficial effect, the thermal resistance of the microorganism becomes crucial. It is recommended that these bacteria must be active and abundant in foods in an amount of at least 10^7 CFU/g on the consumption (Mansouripour *et al.*, 2013). Hence the thermal stability of one of the isolate (isolate 10) was determined. The heat stability was determined at 80°C with three different culture media. The results show freeze dried probiotic has the least loss of cell count and the most loss of count was seen in MRS broth. The loss of cells in freeze dried probiotic and of skim milk was linear; with a loss of 1 log in cell count each 30 seconds in 80°C whereas in MRS broth there was a loss of 2 logs of cells each 30 seconds and at the end of two minutes only 4 logs of cells survived

whereas in skim milk 5 logs of cells survived and the best survivability was seen in freeze-dried probiotic where 7 logs of cells survived.

Table 3.5: The cell count of *Lactobacillus* placed in broth, skim milk and freeze dried at 80°C for different time periods.

Time (minutes) at 80°C	Cell count in Broth (Cfu/ml)	Cell count in Skim milk (Cfu/ml)	Cell count of Freeze Dried Powder (Cfu/ml)
0	**5.92×10^{10}**	**4.49×10^{9}**	**4.05×10^{11}**
0.5	**3.38×10^{8}**	**1.6×10^{8}**	**1.10×10^{10}**
1.0	**3.0×10^{6}**	**6.0×10^{7}**	**1.05×10^{9}**
1.5	**2.0×10^{5}**	**2.3×10^{6}**	**3.0×10^{8}**
2.0	**3.0×10^{4}**	**1.3×10^{5}**	**2.5×10^{7}**

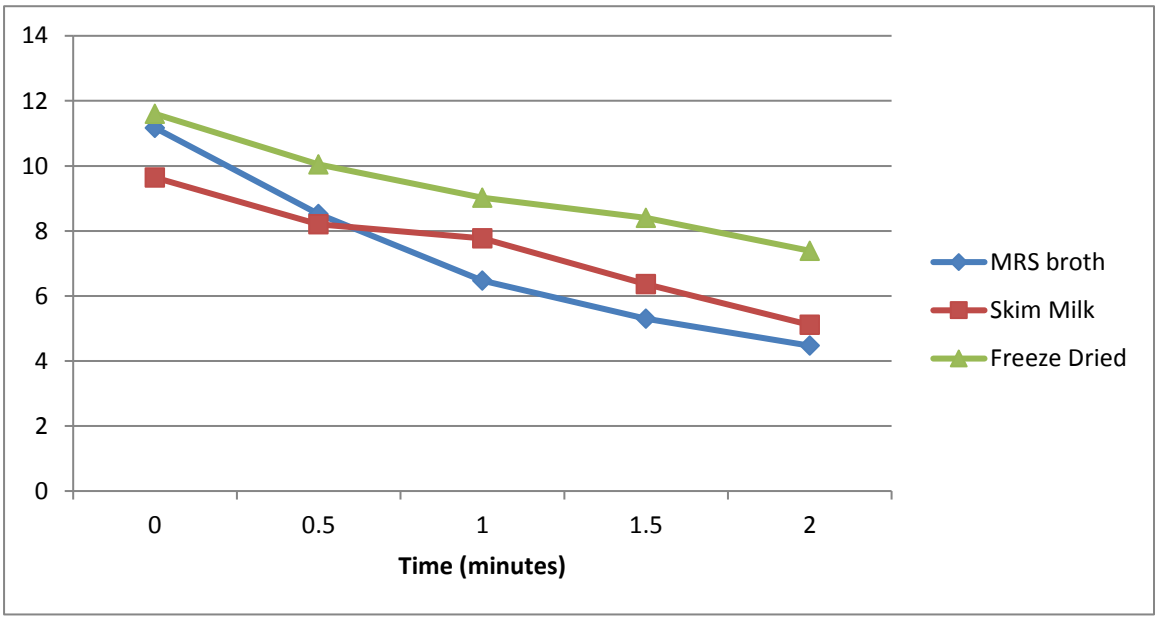

Figure 3.6: Comparison of survivability at 80°C of *Lactobacillus* placed in MRS broth, Skim milk and in freeze dried form.

Hence from the above results, it can be said that freeze dried probiotic had the highest heat stability compared to the *Lactobacillus* in MRS broth and skim milk.

3.7 Viability of freeze dried Probiotic

A beneficial probiotic should maintain high viability and stability during the process of freeze drying followed by long periods of storage (Carvalho *et al*., 2002). In this test, the viability of freeze dried isolate 10 and isolate 14 was determined for four months period of storage at 4°C. The result shows some amount of losses occurred for both the isolates. For isolate 10 the initial count was log 17.5 after freeze drying. After a month of storage, it decreased to log 15.8 followed by log 14.77 and log 12.7 in the next two months. At the end of the four months storage period the count was log 10.89, hence a total of log 6.61 loss of cells over the entire period. For isolate 14 the initial count was log 16.3 after freeze drying. After a month of storage the cell count reduced to log 14.92 and in the following two months it reduced further to log 13.37 and log 11 respectively. At the end the cell count was log 10.21, hence a total loss of log 6.09 cells during the period of storage. Based on the graph and total loss it can be said that the loss in both the isolates were linear

Table 3.6: Cell count of freeze dried isolate 14 over the period of 4 months

Date	Cell counts (CFU/ml)	Log number of cells
14.03.16	**2.5×10^{16}**	**16.3**
17.04.16	**8.31×10^{14}**	**14.92**
17.05.16	**2.35×10^{13}**	**13.37**
26.06.16	**1×10^{11}**	**11**
24.07.16	**1.62×10^{10}**	**10.21**

Table 3.7: Cell count of freeze dried isolate 10 over the period of 4 months

Date	Cell counts (CFU/ml)	Log number of cells
14.03.16	**3.7×10^{17}**	**17.5**
17.04.16	**6.30×10^{15}**	**15.8**
17.05.16	**5.9×10^{14}**	**14.77**
26.06.16	**6.0×10^{12}**	**12.7**
24.07.16	**7.9×10^{10}**	**10.89**

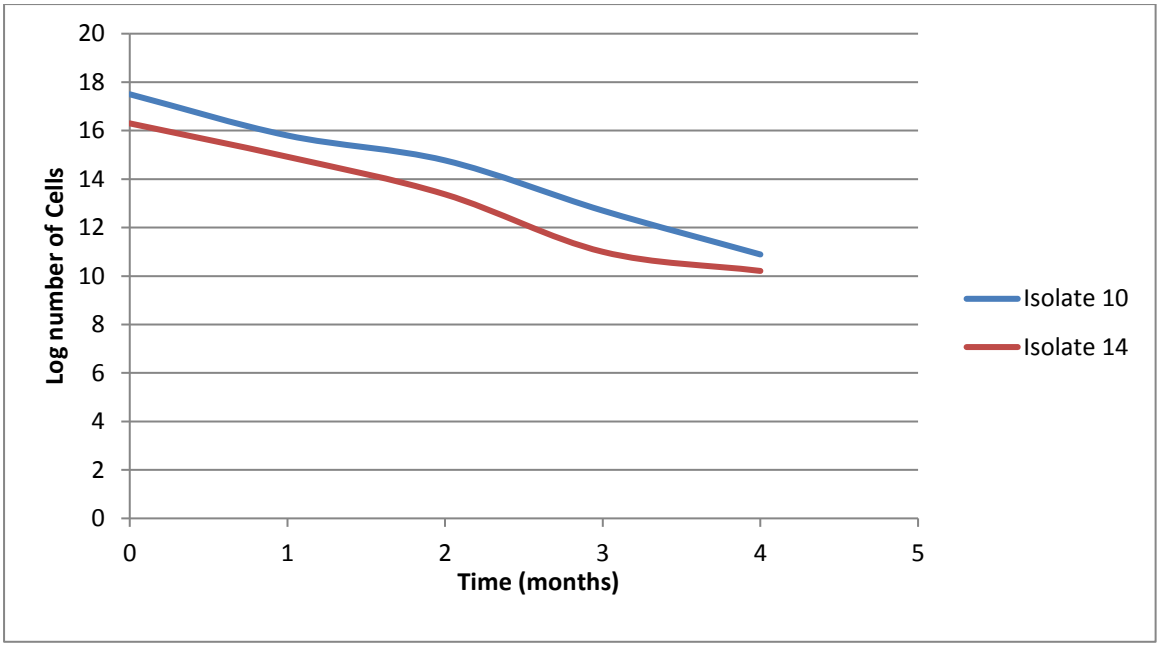

Figure 3.7: The viability of freeze dried isolates 10 & 14 after storage at 4°C for four months

3.8 Growth curve of *Lactobacillus*

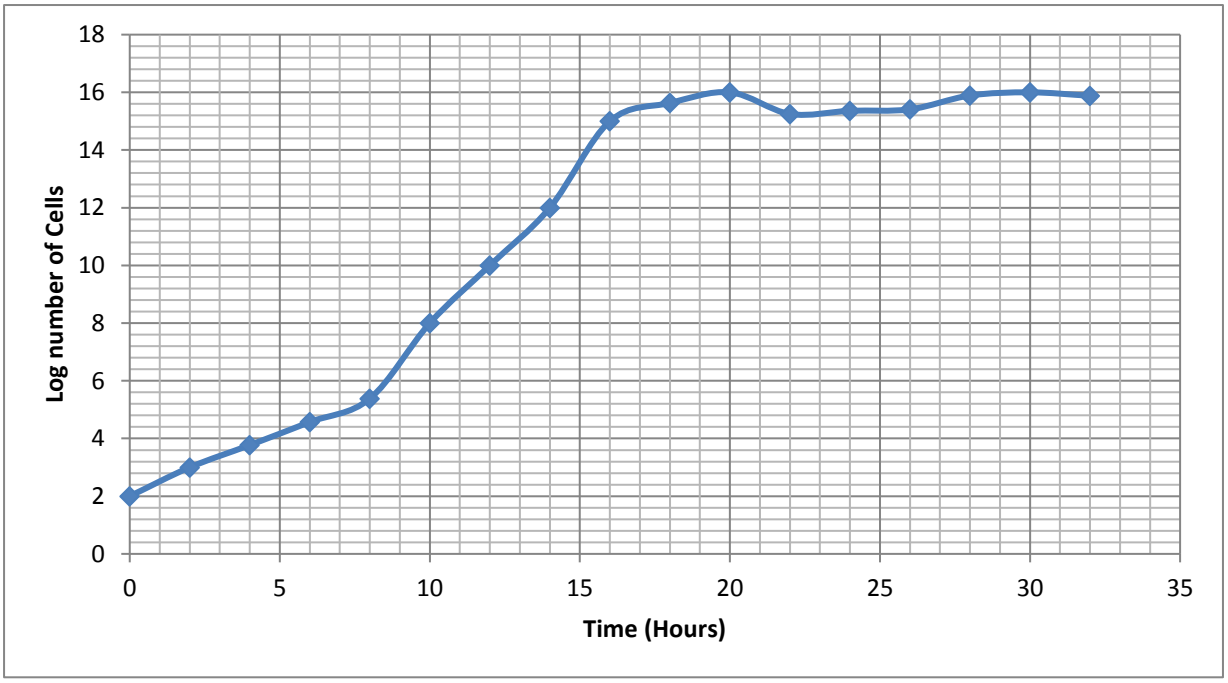

Figure 3.8: Growth curve in 32 hours of isolate 10.

The performed test evaluated the growth dynamics of the isolate over the period of 32 hours. A better understanding of the growth patterns gives the idea of optimum timing for the

maximum growth of cells when performing other tests on the isolate. From the results, it can be determined that between the hours of 0-8 was the lag phase. From hours 8-16 can be said to be the log phase where there is an exponential growth of the cells. From 20-32 hours the cells are in stationary phase there is no significant net increase or decrease in cell number.

3.9 Field Trial

At the end of 5 weeks feeding trial, the chickens were weighed

Table 3.8: The average weight of each treatment groups at the end of feeding trial

Treatment Group	Weight (gms)	Percentage Increase
Control	**1818.54**	**-**
Antibiotic feed	**1853.81**	**1.93**
Probiotic 10	**1836.08**	**0.96**
Probiotic 14	**1849.67**	**1.76**

The total body weight (BW) was determined by the body weights of all the chickens in a particular treatment and then calculating the average of all the chickens in that treatment group. It can be seen from the above table that the maximum weight gain was seen in the chickens fed with poultry feed supplemented with antibiotic tetracycline and least seen in the control group. The second maximum weight gain was seen in chickens fed with poultry feed added with probiotic 14 followed by probiotic 10. The antibiotic feed had 1.93% increase in total BW than the control group. The probiotic feed 14 had 1.76% increase in weight gain and probiotic feed 10 had an increase of only 0.96%.

3.10 Faecal Sampling

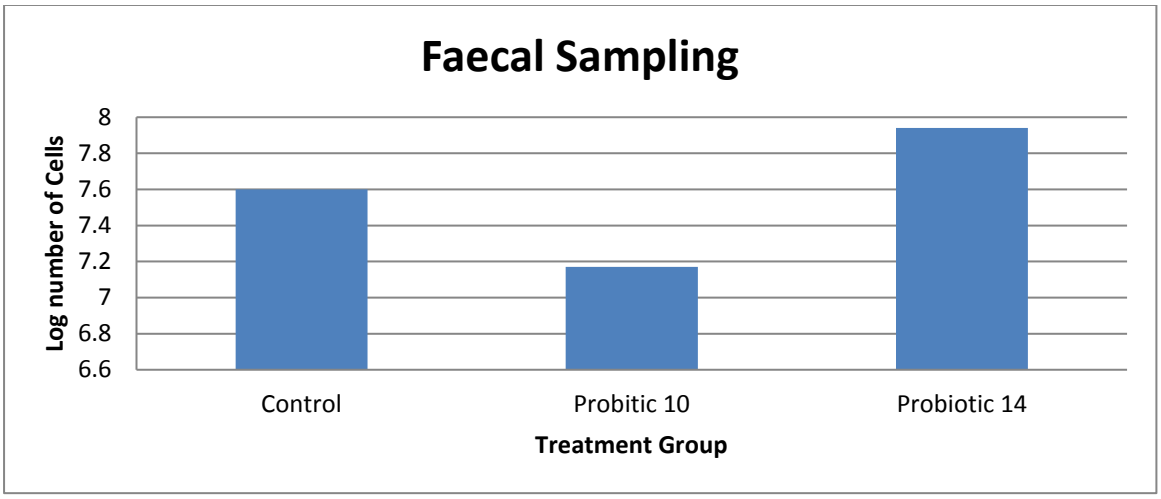

Figure 3.9: The log number of *Lactobacillus* cells found in faeces of each treatment group

The purpose of testing faeces was to determine the faecal count of *Lactobacillus* and to determine if the probiotic was effective. From the above results, it can be seen that highest number of cells was found in probiotic 14 with 7.94 logs of cells followed by control with 7.60 logs of cells and the least was seen in probiotic 10 with 7.17 logs of cells. The control treatment was fed with poultry feed without any supplements or probiotics, but it can be seen from the faecal sampling the growth of *Lactobacillus* this is because overtime the intestinal tract of chickens gets inhabited by the natural microflora from the environment.

Chapter 4

Discussion

4.1 Selection Criteria of an Effective Probiotic

The use of antibiotics in animals and poultry feed for growth promotion and for preventing diseases are causing concerns as this may lead to antibiotic resistance in humans and therefore, in many countries, the use of antibiotic growth promoters in animal feed are facing a ban (Salim *et al.*, 2013). As an alternative to antibiotic growth promoters in animal feed, probiotics are being used to prevent diseases by controlling the overgrowth of pathogenic bacteria and to also maintain the intestinal microflora balance in the animals (Raja *et al.*, 2009). Many studies have shown that probiotics enhance the growth and are beneficial for the animals while some studies show there are no beneficial effects on the animals, this could be due to the differences in strains or species of microorganisms used (Jin *et al.,* 1998). In order for the probiotic to be effective the bacterial strains should be individually tailored for each animal species since the probiotic properties are strain specific (Ehrmann *et al.,* 2002). The selection criteria of probiotics require a series of in-vitro and in-vivo analysis. According to FAO/WHO Working Group Report a guideline was published for the selection of probiotics, and as stated by the report emphasis has been placed on the safety of the microorganism used, its effectiveness, it also recommends in-vitro tests to evaluate the probiotic potential and in vivo tests in animals. There are certain criteria that should be met before a microorganism is selected as a probiotic. A probiotic should have the following ability: (i) it should be a strain that is capable of exerting a beneficial effect on the host consuming it, (ii) the strain should be non-pathogenic and non-toxic, it shouldn't be harmful to the host itself, (iii) it should be able to withstand in foodstuff at high cell counts and be viable throughout the shelf-life of the product, (iv) the strain should be able to adhere to the intestinal cell lining and be able to survive in gut conditions and colonize there, (v) produce antimicrobial substances towards pathogens (Fuller, 1989).

4.2 Acid and Bile Tolerance

This study focused on in vitro tests such as acid tolerance, salt tolerance, antimicrobial activity against pathogens and adhesion to epithelial cells by determining the cell surface hydrophobicity of the isolates. According to the study of Jin *et al.,* in 1988 the time required for the feed to pass through the entire alimentary canal for chickens is short of about 2.5 hours so therefore it can be said that the acid tolerance of the strains in the probiotic is not as crucial as it is for other animals whose feed passage time is longer. The acid tolerance test was determined at pH of 3, 4, 5 and 6. All the four isolates showed good tolerance to acid at

pH 4, 5, and 6. At pH 3 the isolates were very slow growing, compared to the rest of the pH values. Tolerance to bile salt showed similar results with all four isolates capable of surviving and growing at 0.5%, 1.0%, 1.5% and 2.0% of bile salt concentrations. These results are supported by the findings in study of Boonkumklao *et al.,* in 2006, where all five strains of thermotolerant lactic acid bacteria showed acid tolerance when incubated at pH 3.0 and was found to be tolerant to 0.3% bile concentration. Also the study of Pan *et al.,* in 2009 had similar findings. *Lactobacillus acidophilus* isolated from infant faeces were tested for resistance to pH 2-4 and 1-3% bile and results showed they had certain resistance to acid and bile and the strains were expected to survive acidic conditions that exist in stomach and intestinal juice. Hence based on the findings of this study it can be said that the isolates are tolerant towards acid and bile and could be able to pass through the crop and gizzard and be able to survive in the small intestine.

4.3 Cell Surface Hydrophobicity

The cell surface hydrophobicity of the four isolates was investigated to determine attachment to epithelial cells in-vitro. Cell surface hydrophobicity is often considered as the primary factor influencing the strength of bacterial adhesion. Adhesion to intestinal epithelial cells is an important prerequisite for colonisation of probiotic strains in the gastrointestinal tract (Abdulla *et al.,* 2014). The hydrophobic properties of bacterial surfaces are a major determinant in the adhesion of bacteria (Georgieva *et al.,* 2016). In a study carried out by Dhewa *et al.,* in 2009, four *Lactobacillus* species was examined for cell surface hydrophobicity by bacterial adherence to hydrocarbons assay. They reported that the general range of hydrophobicity in *Lactobacilli* is between the ranges of 6% to 73%. Their findings showed that *L. helveticus* and *L. fermentum* showed 73% hydrophobicity in xylene. According to Abdulla *et al.* in their study; they investigated the percentage hydrophobicity of six *Lactobacillus* species and found that the percentage hydrophobicity ranges from 29.5% to 77.4% and reported that microorganisms with cell surface hydrophobicity greater than 40% are hydrophobic and are capable of attaching to cell surfaces. Based on previous study findings the four isolates investigated in this study, only isolate 10 and 14 had hydrophobicity percentage greater than 40%. The rest of the isolates 2 and 8 had less than 40%. So, therefore, it can be said that the isolate 10 and 14 could be effective in attaching to epithelial cell surfaces better than the other two. A higher value of percentage hydrophobicity could indicate a better ability of *Lactobacilli* to adhere to epithelium cells. Although only xylene was used to determine cell surface hydrophobicity in this study other hydrocarbons such as

hexadecane, octane and etc. should have been used but due to lack of other hydrocarbons in the lab, only xylene was used. The usage of other hydrocarbons could have given more conclusive results about the cell surface hydrophobicity of each isolate.

4.4 Antimicrobial Activity

The antimicrobial ability of probiotics is a very important trait and it also involves the production of antimicrobial compounds, competitive exclusion of pathogens, enhancement of the intestinal barrier function (Fijan, 2016). The antimicrobial test was carried out against test pathogens of *Salmonella typhi, E .coli, Shigella* and *Staphylococcus aureus* as these are some of the bacterial species that can cause infections in chickens. It is important to test their antimicrobial activity to determine the efficacy of the probiotic in-vivo. A strain with good antimicrobial activity will help the host to prevent infections from other pathogenic microorganisms. Other investigations have reported that *Lactobacilli* are capable of producing antimicrobial substances. A study carried out by Silva and coworkers in 1987 reported that *Lactobacillus* strain GG produced a substance which inhibited a wide range of bacterial species. It inhibited the species of *Clostridium, Bacteroides, Bifidobacterium Staphylococcus*, and *Streptococcus.* Another study by Abdulla and coworkers in 2014 also reported the antimicrobial activity of *Lactobacillus* species. In their study, the antimicrobial activity of six *Lactobacillus* strains was tested against test pathogens and results showed that they were all effective against *Serratia marcescens,* five of the strains effective against *S. typhi* and four of the strains effective against *S. aureus.* Earlier studies back up the results of this study where all the isolates had antimicrobial activity against *Salmonella typhi.* Certain species of Salmonella are known pathogens of poultry and causes pullorum disease and fowl typhoid. Pullorum disease is highly fatal to young chicks and mortality may reach 90% if left untreated. *Shigella* was the test microorganism against which none of the isolates had any antimicrobial response. Shigellosis rarely occurs in animals, but in a study by Shi *et al.* in 2014 reported that chickens were directly injected with *shigella* and their results showed *Shigella* can cause death via intraperitoneal injection in chickens, but only induce depression via crop injection. The other test microorganisms were *E. coli* and *S. aureus* against which isolate 2 and 8 had no response, whereas isolate 10 and 14 has shown antimicrobial activity against them. *E. coli* infections are widely distributed among poultry of all ages for e.g. it causes omphalitis, a navel infection with reddening and tissue oedema in umbilical region and salpingitis, inflammation of the oviduct. Infections of chickens with *S. aureus* are more common where bones, tendon sheaths and joints are particularly affected.

Therefore based on the results of this study isolate 10 and 14 had better antimicrobial activity than the others so were selected for further in-vivo study.

4.5 Media Components and its Function

Based on these in-vitro results it was determined that isolate 10 and 14 could be more effective probiotic in-vivo since they showed good antimicrobial activity, had better cell surface hydrophobicity than the other isolates and showed good tolerance to different concentrations of acid and bile salts so therefore were used to produce the probiotic additive to the poultry feed. Both of the isolates were grown in large numbers and then freeze dried. Culturing of the cells in large number was carried out in a synthetic medium developed at the IFST, Industrial Microbiology lab of BCSIR. Although MRS broth is a medium suitable for *Lactobacillus* growth but since huge amount of this media was required and a single bottle of this media makes only 10 litres, another cost-effective growth medium was required. The components of media which are similar for both MRS and the composed synthetic media are: yeast extract, beef extract, proteose peptone, dextrose, magnesium sulphate and dipotassium hydrogen phosphate. Yeast extract is the water-soluble portion of autolysed yeast and it provides nitrogenous compounds, carbon, sulphur, trace nutrients, vitamin B complex and other important growth factors, which are essential for the growth of diverse microorganisms. It is often used as additive for culture media. Proteose Peptone is the widely used source of nitrogen in microbial media. It is prepared from peptic or enzymatic digest of animal tissue. It is rich in proteoses, peptones and free amino acids. It is a highly nutritious ingredient employed in media used for bulk production of bacterial cells, antibiotics, enzymes, and bacterial toxins (Eddleman, 1992). Beef Extract is not exposed to the harsh treatment used for protein hydrolysis, so it can provide some of the nutrients lost during peptone manufacture. It is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals and some vitamins. Therefore the function of beef extract can be said to complement peptone by contributing minerals, phosphates, energy sources and those essential factors missing from peptone (Bridson & Brecker, 1970). The dextrose is a monosaccharide in the media acts as an energy source and it is the primary source of carbohydrates. All bacteria utilize their energy sources to produce ATP. ATP is required for all of the biosynthetic processes that bacteria use for their maintenance and reproduction. Magnesium Sulphate was also used in this media. The function of magnesium is in the activation of cell division in gram-positive bacteria, and activation of bacterial growth (Webb, 1951). In Webb's paper in 1948, it was shown that

magnesium is essential for the growth and cell division of rod-shaped bacteria. Dipotassium hydrogen phosphate acts as a buffering agent in the media. The other components of synthetic media different from MRS were: sodium chloride and ammonium sulphate. The presence of NaCl maintains a salt concentration that is similar to the cytoplasm of the microorganisms. Ammonia in ammonium sulphate acts as a source of nitrogen. Whereas MRS broth has: polysorbate 80, ammonium citrate, sodium acetate and manganese sulphate. Polysorbate 80 acts as a surfactant and assists the uptake of nutrients by the *Lactobacillus*. Sodium acetate suppresses the growth of other bacteria and ammonium is a source of nitrogen and manganese provides essential cations. Breaking down the components of the synthetic media, it shows that the *Lactobacillus* isolates got all the nutritional requirements needed for growth, metabolism, reproduction and its maintenance. There are no significant functional differences between the composition of MRS media and the composed synthetic media. After growing a single isolate for 40 hrs in the synthetic media the bacterial cells pellet obtained after centrifuging was 8 gm in six litres of media. Hence the synthetic media also favoured the luxurious growth of the *Lactobacillus* species. Also using this media was less costly in comparison to using MRS broth as most of the components needed to make this media were already available in the laboratory. In the beginning stages of this study the freeze drying of the probiotic wasn't achieved after the first try, after many unsuccessful attempts the probiotic was freeze dried in perfect powdery form, so after each unsuccessful try the isolates had to be grown again in the media and due to this huge amounts of media was consumed in trials and errors, on certain times the media got contaminated and had to be discarded. So when carrying out this study using of MRS broth would have made it more costly as more of it needed to be ordered, hence increasing the cost.

4.6 Viability of the Probiotic

After freeze drying the survivability of the probiotic was determined. The cell count was taken before the starting of freeze drying and after freeze drying to determine the loss of cells. Also the viability of the probiotic was determined for a period of four months. Freeze drying is often used for long term storage of biological samples; it produces undesirable side effects that hamper viability of many species. Damage to biological systems resulting from freeze drying can be attributed primarily to changes in the physical state of membrane lipids or to changes in the structure of sensitive proteins (Carvalho *et al.,* 2002). Generally the cryoprotectants used are polyhydroxy compounds such as sugars (mono-, di-, and polysaccharides), polyalcohols, and their derivatives. In order to reduce the loss of cells many

studies have been carried out where several compounds have been investigated as protective agents for freeze drying. Such investigated compounds were sorbitol, inositol, fructose, trehalose, glycerine, dextrin, skim milk and etc. In this study skim milk has been used as the protective agent to minimize the loss of cells during freeze drying. As the results suggests that no significant losses in cells was seen after freeze drying in all of the isolates and also after the storage period the cells were viable for a period of four months with significant counts giving the probiotic product a shelf life of four to five months. These results were also backed up by the study of Savini and coworkers in 2010 where skim milk was used as the control cryoprotectant and the effectiveness of other cryoprotectants was compared with skim milk. Their results show that there are no significant differences in cell counts were observed between the tested cryoprotectants and the skim milk control over a period of five months. Leja and coworkers in 2009 also observed that the highest cell survival rate of 83-91% after storage was seen in freeze dried preparations with skim milk as a protective substance. They also concluded that the cells survival rate strongly depends on the protective agent in which bacteria is suspended before drying. The concentration of viable cells after storage also depends mainly on a protective medium utilized in the process of drying rather than the storage temperature and drying method and has low impact on cell viability. On the contrary Carvalho and co-workers in 2002 reported that survival was higher during storage when drying took place in the presence of a cryoprotectant and sorbitol had more significant effects than other compounds in maintaining the viability of freeze dried *L. plantarum* and *L. rhamnosus.*

Skim milk was also used as a cryoprotectant in this study with the aim that the freeze dried probiotic would be fed to chickens mixed with poultry feed. Skim Milk is a source of lactose and casein and other nutrients required for the growth of *Lactobacilli* (Bergey *et al*., 1974). Also, the skim milk is a reliable protein source, it may not fulfil the entire protein requirement of the chickens, this requirement has to come from the poultry feed but it does not have any great adverse effects on the health of the chickens.

4.7 Field Trial

The field trial was carried out to determine the efficacy of the probiotic in-vivo. Based on the results of this study the maximum weight gain was seen in antibiotic treatment group with a total body weight increase of 1.93% from the control group. The second increase in total body weight was seen in treatment group with probiotic 14 with 1.76%, followed by probiotic

10 treatment group with 0.96%. The effects of probiotics on animal productions have been widely studied and the results are often in agreement whereas in many cases they were contradictory (Jin *et al.,* 1997). Some of the study where probiotic had an increased weight gain compared to that of control was carried out by Lan and colleagues in 2003. In their study two probiotic strains of *Lactobacillus* isolated from chicken intestine was administered in chicken feed. Their results showed that the probioitc feed increased the count of intestinal *Lactobacilli* and was significantly higher compared to the control group and they also reported that the probiotic effect of the two strains resulted in significantly increased weight gain which was 10.7% than the control group. Another study carried out by Timmerman and co-workers investigated the growth performance of broilers giving drinking water supplemented with probiotics and they concluded that probiotics had beneficial effects on broiler performance. In their field trials, probiotic treatment significantly improved feed conversion. They also reported that in all of their field trials the final body weight increased by supplemental probiotics ranged from 0.74% to 1.64% and mortality was reduced by the addition of probiotics to the drinking water. Based on the results of other studies it can be said the probiotics administered in this study did have a beneficial effect in increasing the weight gain when compared to the control group.

Conclusion

In conclusion it can be said that the isolates 10 and 14 showed good probiotic characteristics in all of in-vitro analysis and showed moderately good efficacy in-vivo. The freeze dried probiotic had a shelf of four to five months at 4°C making it easier for storage and transport. Although further studies need to be carried out to completely replace antibiotic growth promoters. Further studies should strongly focus on more field trials with large a number of chickens and also with different concoction of probiotics rather than a single strain of probiotic and recording data of weight gain but also investigate further criteria such as meat and carcass quality. Further screening of probiotics is always necessary to discover strains with very good probiotic characteristics.

Chapter 5

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Appendices

APPENDIX- I

Media used in this study

MRS agar (HiMedia)	Gms/Litre
Peptone	10.00
Beef Extract	10.00
Yeast Extract	5.00
Dextrose	20.00
Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00
Agar	12.00
Final pH	6.5±0.2

Synthetic Media	Gms/liter
Yeast extract	5.00
Beef extract	7.50
Proteose peptone	5.00
Dextrose	10.00
NaCl	3.00
K_2HPO_4	1.00
$(NH_4)_2SO_4$	1.00
$MgSO_4.7H_2O$	5.00
Final pH	6.5±0.2

Mueller Hinton Agar (Micromaster)	Gms/liter
Beef, infusion from	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH	7.3±0.2

Brain Heart Infusion broth (TM Media)	Gms/ liter
Brain heart, infusion from	250.00
Calf brain, infusion from	200.00
Protease peptone	10.00
Sodium Chloride	5.00
Di-sodium phosphate	2.50
Dextrose	2.00
Final pH	7.4±0.2

Buffers used in this study

Phosphate Buffered Saline (PBS) (1X)	Gms/liter
NaCl	8.00
KCl	0.20
Na_2HPO_4	1.42
KH_2PO_4	0.24
pH adjusted	7.4

Ringer's Solution (1X)	Gms/Liter
NaCl	6.50
KCl	0.42
$CaCl_2$	0.25
$NaHCO_3$	0.20

APPENDIX-II

Laboratory Instruments used throughout the study

Incubator (37°C)	Memmert
Freeze (-20°C)	Siemens
Micro-centrifuge	Mikro 120
Microscope	Olympus BX41
Weighing Balance	A and D company ltd.
Large capacity Centrifuge	Kubota, Japan
pH meter	Hanna Instruments
Laminar Flow Cabinets	ESCO
Micropipette	Eppendorf
Laboratory glass bottles with screw caps	Schott Duran
Conical Flasks	Pyrex
Petri Dishes	Sterilin
Autoclave	Systec
Freeze Dryer	Christ (Alpha 1-2 LD plus)
CO_2 Incubator	Sanyo
Spectrophotometer	PG Instruments (T-60 UV-Visible)