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SELECTION OF A CHICKEN LACTOBACILLUS STRAIN WITH PROBIOTIC PROPERTIES AND ITS APPLICATION IN POULTRY PRODUCTION

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Ph.D.

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A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

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Selection of a chicken *Lactobacillus* strain with probiotic properties and its application in poultry production

One hundred and eleven lactic acid bacteria, of chicken origin, were tested for possible probiotic properties. One organism, *Lactobacillus salivarius* Salivarius NCIMB 41606 (*Lact.* Salivarius) showed: rapid autoaggregation ability, maximum co-aggregation ability with enteropathogens, tolerance to acidic pH and bile salts of the chicken GI-tract, very good antagonistic activity against several enteropathogens and good adhesive capacity to chicken epithelial cells. It was also found to survive in water for 24h and produce fermented feed, of low pH (<0.4) and containing >150 mmol L^{-1} lactic acid after 24h fermentation at 30°C. This organism was selected as a candidate that could be used *in vivo* as a chicken probiotic.

Lact. Salivarius NCIMB 41606 was assessed for its efficacy in reducing the shedding of *Salmonella enterica* Typhimurium Sal 1344 nal^r in one day old chicks and during their 40 day development, *in vivo*. The overall percentage of *Salmonella* shedding chickens was significantly greater in the probiotic and acid unsupplemented control birds (CON) at 93% than in the other three supplemented treatments. The overall percentage of *Salmonella* shedding chickens for the group fed moist feed, fermented with *Lact.* Salivarius NCIMB 41606 (FMF) was significantly (P<0.05) lower (48%) than for the group provided with *Lact.* Salivarius via water (WAT) (81%) and the probiotic free group, fed acidified moist feed (AMF) containing the same amount of lactic acid as FMF birds (75%). The overall mean faecal lactobacilli counts from chickens fed FMF were also significantly higher (P<0.05) at 8.35 log10 cfu/g than the three other groups which were 7.78, 7.99 and 7.67 log_{10} cfu g^{-1} for WAT, AMF and CON respectively. The overall mean *Salmonella* counts were significantly higher in the CON chicks at 5.35 log_{10} CFU gr^{-1} than WAT, FMF and AMF which had respective *Salmonella* counts of 4.95, 4.66 and 4.39 log_{10} CFU gr^{-1}. At 26 day *post mortem* the FMF group had significantly (P<0.05) lower ileal *Salmonella* counts (1.08 log_{10} CFU ml^{-1}) compared to the CON (6.61 log_{10} CFU ml^{-1}) and AMF groups (6.10 log_{10} CFU ml^{-1}) and FMF and WAT group had significantly (P<0.05) lower caecal *Salmonella* counts than the CON group (1.05, 1.20 and 5.03 log_{10} CFU ml^{-1}, respectively). There were no significant differences between the treatments, for 40 days *post mortem*. Determination of microflora of the caecum by cultivation on selective media showed significantly higher *Lactobacillus* counts for FMF group than for the CON and the WAT group (8.87, 8.28 and 8.12 CFU ml^{-1}). Molecular-based analysis of ileal microbial species diversity demonstrated that treatments with *Lact.* Salivarius (WAT and FMF) showed high similarity between them (53.9%).

FMF produced using *Lact.* Salivarius NCIMB 41606, is suggested as an effective means of controlling *S. typhimurium* infection to poultry.

FREQUENTLY USED ABBREVIATIONS

AMF	Acidified Moist Feed
CFU ml^{-1} or CFU g^{-1}	Colony forming unit per millilitre or per gram, a measure of viable bacterial numbers
CON	Control group
DNA	Deoxyribonucleic acid
FLF, FMF	Fermented liquid feed, Fermented moist Feed
GI	Gastrointestinal
HPLC	High Performance Liquid Chromatography
LAB	Lactic Acid Bacteria
Lact.	*Lactobacillus*
mmol L^{-1}	Milimole per litre
MRS	Mann Rogosa Sharpe (broth/agar)
NCIMB	The National Collection of Industrial, Marine and Food Bacteria
OD	Optical Density
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
RNA	Ribonucleic acid
SCFA	Short-chain fatty acids
Salm. Typhimurium	*Salmonella* enterica Typhimurium Sal 1344 nal^r
WAT	Water group

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AUTHOR'S DECLARATION

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Chapter 1

Literature review

1.1 Problems of modern poultry production

Modern poultry husbandry requires a system of highly intensive production, in which birds are hatched artificially in incubators, which are kept clean and disinfected. Thus, newly chicks, which normally acquire their intestinal microbiota from the fowl and surface of the eggshells, hatch in a sterile environment and may delay or fail to develop microbial ecosystem of the gastrointestinal tract with desirable microorganisms (Sterzo et al. 2005). Gut colonisation in the beginning of life with desirable microbes could act as a natural barrier against colonisation and multiplication of pathogenic bacteria (Oliveira et al. 2000) . Therefore, the poor or undeveloped intestinal microbiota may be a reason of the colonisation of the gastrointestinal tract by pathogenic bacteria, which may cause gastrointestinal disturbances and enteric diseases (Fowler and Mead 1989). Such conditions are a major concern for the poultry industry as they can have a negative effect on production, increase mortality and subsequently reduce the quality of poultry products for human consumption. Conversely, any improvement in the development of the microbial environment may lead to the improvement in the productivity of the birds.

Bacterial enteritidis is a widespread problem in chicken husbandry and causes serious economic problems in the broiler industry. Enteritidis can result either

from the invasion of pathogenic organisms or from the continuing changes in the number and the composition of the intestinal microflora. The intestinal microflora is regarded as stable in the healthy bird, although factors such as changes in the bird itself, deprivation of food or water, transportation, administration of antibiotics and radiation may result in instability of the gut microflora.

In 2007 the European Food Safety Authority (EFSA) published a survey of the levels of *Salmonella* detected in broiler flocks (chickens reared for meat) across the European Union in 2005-6. It was reported that one in four broiler flocks surveyed between October 2005 and September 2006 were *Salmonella*-positive. *Salmonella enteritidis* (*Salm. enteritidis*) and *Salmonella* Typhimurium (*Salm.* Typhimurium), which are the two most commonly reported *Salmonella* types in human cases in the EU, were identified in about 40% of *Salmonella* positive flocks. *Salmonella enteritidis* has been related to human salmonellosis, a common and widespread zoonosis worldwide (Nava et al. 2005). Both the association of *Salmonella* infections with the consumption of poultry products and the fact that in the live bird *Salmonella* carriage is mainly asymptomatic have been led to a demand to find ways of preventing infection of commercially reared poultry and product contamination (Revolledo et al. 2006). *Clostridium perfringens* (*Cl. perfringens)* was first referred to by Parish (1961) who stated that it is one of the main causes of necrotic enteritis, with very important economic consequences for broiler chicken production (Ficken and Wages 1997). *Cl. perfringens* is reported to cause subclinical disease (Stutz et al.

1983) or a mild clinical infection (including colonisation) (Kaldhusdal and Hofshagen 1992), and liver disease, as well as the more commonly recognised fulminant infection that can result in disease outbreaks with a significant mortality rate. *Salmonella* poses a continuing threat to consumer health. As far as *Clostridium* is concerned, it is probable that the economic significance has been underestimated, as growth decreases, condemnation increases and liver pathology is rarely included in assessments (Lovland and Kaldhusdal 2001).

For many years the use of antibiotics provided a solution to diseases related with enteric infections with pathogenic bacteria. Antibiotics have been used in animals basically for three purposes: therapeutic use, for a short period of time, with high doses to inhibit pathogens, to treat diseases; prophylactic use, for a defined period of time with moderate to high doses, to prevent infection in animals; as growth promoters (AGP's), over extended periods at subtherapeutic levels, to improve feed utilisation and production (Barton 2000). However, the spread of antibiotic resistant bacteria, which is a direct response to the use of antibiotics, has concerned scientists and consumers. Attention started to focus on the issue, when antibiotic residues in products from treated animals and antibiotic resistance spread to humans from treated animals via the food were observed after long term and extensive use of antibiotics in animal production (Salminen et al. 1998; Van Immerseel et al. 2002; Hayes et al. 2004). For example, Hayes et al. (2004) reported an *Enterococcus* strain isolated from poultry with high frequency of multi-drug resistance. The development of undesirable antibiotic resistance led to a ban on the use of antibiotic growth

promoters throughout the European Union in 2000 and the potential of a ban in the United States. Despite the ban in the use on antibiotics as growth promoters, reports suggest that the total amount of antibiotics used increased as a result of increased therapeutic use of antibiotics (Kobashi et al. 2008). In order to reduce antibiotic use there has been an increased commercial interest in developing alternatives to antibiotics for poultry production. As a result there has been increased interest in, and use of, dietary acids and probiotics.

1.2 Definition of Probiotics

Metchnikoff (1907) was the first to provide some evidence that intestinal bacteria have an important role in the maintenance of health when he noted the effect of lactic acid bacteria present in fermented milk products on longevity in humans. However, Lilly and Stillwell (1965) were the first to introduce the term 'probiotic' to describe growth promoting factors produced by microorganisms. 'Probiotic' is derived from the Greek and means pro: for and bios: life (hence 'for life'). This is the converse of 'antibiotic' which means 'against life' (Ghadban 2002). Parker (1974) defined probiotics as microorganisms or substances that contribute to intestinal microbial balance. Crawford (1979) defined a probiotic as a culture of specific living microorganisms, mainly *Lactobacillus* spp., which is established in the host animal and ensures the rapid and effective establishment of a population of beneficial organisms in the intestines. Having considered the definition given by Parker (1974), Fuller (1989) narrowed the definition of 'probiotics', wanting to exclude antibiotic preparations and he refined 'probiotic' as a live microbial feed supplement, which has a beneficial

effect on the host animal by improving its intestinal microbial balance. Havenaar and Huis In't Veld (1992) expanded Fuller's (1989) definition, which was restricted to feed supplements, animals and their intestinal tract, and redefined 'probiotic' as a mono or mixed culture of living microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora.

During 1989, the United States Department of Agriculture (USDA) proposed that manufacturers use the term 'direct-fed microbial' (DFM) instead of probiotic (Miles and Bootwalla 1991). According to Miles and Bootwalla (1991) the USFDA characterise a DFM as a source of live, naturally occurring microorganisms, including bacteria, fungi and yeasts. Vanbelle et al. (1990) indicated that the majority of researchers considered probiotics as selected and concentrated viable counts of lactic acid bacteria. In 1995 Gunther broadened the term even more and defined probiotics as organisms, live or dead, or as products of microbial fermentation, nucleotides and their metabolisable products, metabolites of the proteins and derived substances, organic acids, in addition to enzymes of hydrolytic type that beneficially affect the host. However, and according to Curbelo et al. (2005), the majority of authors agree in defining probiotics as feed additives, formed by live microbes that beneficially affect the health of the host (Schrezenmeir and de Vrese 2001).

Direct-fed microbials, initially described as probiotics, may be composed of a single specific strain, or several strains, or even several species of bacteria,

which do not have to be defined by identification of each organism in the mixture. This definition incorporates the Nurmi concept of 'competitive exclusion' (CE). Nurmi and Rantala's technique (1973) is considered as the basis of the competitive exclusion mechanism of probiotics in poultry (Jin et al. 1997; Mahajan and Sahoo 1998; Ghadban 2002). When 1-2-day old chicks orally inoculated with adult gut microflora of healthy birds, showed significant resistance to *Salm. infanti*, supplied by feeding one day later (Nurmi and Rantala 1973). This study set the basis for further development of the competitive exclusion and the introduction of oral administration of an undefined mixed culture from adult birds to newly hatched chicks. The CE bacteria preferentially establish in the gastrointestinal tract and become antagonistic to opportunistic pathogens. Several commercial CE products, with defined or undefined composition, have been developed such as *AviFree*, *Aviguard*, *Broilact*, *MSC* and *Preempt*, or *CF-3* (Edens 2003; Doyle and Erickson 2006). The Scientific Committee on Animal Nutrition (EUROPEAN COMMISSION, 2003) stated that the microbial products, presented in Table 1, are safe for use as feed additives in poultry production, when used according to the manufacturer's instructions.

Table 1. Safe microbial products for use as feed additives in poultry production (Scientific Committee on Animal Nutrition, 2003)

Name	Active Constituent(s)	Culture collection and accession number	Target animal categories
Bactocell®	*Pediococcus acidilactici*	CNCM MA 18/5M	Broilers
Bioplus 2B®	*Bacillus licheniformis* *Bacillus subtilis* [in a 1/1 ratio]	DSM 5749 DSM 5750	Broilers and turkeys
Cylactin LBC®	*Enterococcus faecium*	NCIMB 10415	Broilers
Lactobacillus acidophilus D2/CSL®	*Lactobacillus acidophilus*	CECT 4529	Laying hens
Microferm®	*Enterococcus faecium*	DSM 5464	Broilers
Oralin®	*Enterococcus faecium*	NCIMB 10415	Broilers
Probios PDFM Granular®	*Enterococcus faecium* *Enterococcus faecium*	DSM 4788 / ATCC 53519 DSM 4789 / ATCC 55593	Broilers

1.3 Mechanism of action of probiotics

1.3.1 Host intestinal anatomy

An understanding of the mechanism of action of probiotics in the chicken requires knowledge of the intestinal ecology and the anatomy of the host gastrointestinal tract. The intestinal anatomy of the chicken has been reviewed by Koutsos and Arias (2006). In summary, the gastrointestinal tract (GI) consists of several anatomical sections such as beak, oesophagus, crop, proventiculus, gizzard, small and large intestine, and caeca. The small intestine which is the main site for digestion of feed components and the absorption of nutrients, and the large intestine colonised by microorganisms, are of major importance.

The GI tract can be subdivided according to the cell type and function into the epithelial cell layer, lamina propria, muscularis, components of the immune system and the mucus layer. The first epithelial layer comprises enterocytes (primary absorptive cells of the intestine), goblet cells (secrete mucin and glycoproteins), endocrine cells (secrete hormones and neuropeptides), M cells and intraepithelial leukocytes ($CD8^+$ T lymphocytes and natural killer cells). All these cells (except M cells) are arranged into finger-like shapes known as villi, which comprise the basic absorptive structure of the gut. At the villus base the crypts of Lieberkuhn are formed and constitute the major site for production and differentiation of new enterocytes. The structure that lies beneath and stabilises the epithelium, the lamina propria, consists of nerve fibre, and several immune cells such as plasma cells (IgA secreting), T lymphocytes (generally $CD4^+$), macrophages, eosinophils, mast cells and dedritic cells. Immune system components, such as aggregated lymphoid tissues and Peyer's patches containing B and T lymphocytes ($CD8^+$ and $CD4^+$), are also found within the lamina propria and in the regions underlying the mesentery. Finally, the mucus layer, which coats the epithelium surface of the GI tract, provides lubrication for the epithelium, prevents enzymatic degradation of host tissues and functions as a barrier, permeable to nutrients but not to macromolecules. It affects indirectly the gut associated lymphoid tissue (GALT), by allowing the fixation of bacteria and providing a substrate for bacterial fermentation. The GALT is made up of several types of lymphoid tissue that store immune cells, such as T and B lymphocytes (Bar-Shira and Friedman 2006). The host intestinal anatomy interacts constantly and complexly with the intestinal microbial population affecting the health and the performance of the host.

1.3.2 Maintaining beneficial microbial population in the gastrointestinal tract and suppression of viable numbers of specific bacteria

1.3.2.1 Development and microbial ecology of the chicken

The avian digestive tract includes microbial populations that are variable in size and complexity (Barnes 1979; Bjerrum et al. 2006; Gabriel et al. 2006; Koutsos and Arias 2006; Fortun-Lamothe and Boullier 2007). Broadly, microflora of the digestive tract can be grouped into harmful bacteria, which may be involved in the induction of infection, intestinal putrefaction and toxin production and the commensal populations, which are involved in vitamin production, stimulation of the immune system and inhibition of harmful bacteria (Koutsos and Arias 2006).

Normally, newly hatched birds receive their first inoculum of bacteria from a combination of sources i.e. from the surface of their eggshells, through contamination with faecal material from mature birds, from their environment, and through the organisms populating their feed and water and through ingestion of litter. Chicks establish a protective microflora within the first couple of days after hatching. The digestive flora develops with age (Gabriel et al. 2006). Within one day, the previously sterile ileum and caecum contain 10^8 and 10^9 bacteria per gram of content respectively (Apajalahti et al. 2004) and 3 days later this increases to 10^9 and 10^{11} per gram of content respectively. Thereafter, the numbers remain relatively steble until 30 days of age (Gabriel et al. 2006). Coliforms and enteroccoci dominate the gut of the hatchlings initially. Lactobacilli 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 et al. 2004).

Bacterial activity takes place mainly to the crop and caecum and to a lesser extent in the small intestine. The bacterial population is diverse, especially in the caecum, where the slow turnover of the contents (1 to 2 times a day) results in the development of more and different types of bacteria (Apajalahti et al. 2004). The ileum contains 10^9 bacteria per gram of contents and this consists mainly of facultative anaerobes, predominantly lactobacilli, but also enteroccoci and coliforms. The caecum has 10^{11} bacteria per gram of contents and has less facultative than strict anaerobes (Apajalahti et al. 2004). Recent molecular studies for the characterisation of the composition of the chicken microbial ecology (using 16S ribosomal DNA for phylogenetic analysis) have shown that there are a large variety of unculturable bacteria, or bacteria for which the culture conditions are not known. Hence, these cannot be identified by traditional culture depended methods (Bjerrum et al. 2006). In addition to using species specific primers to identify bacteria population precisely, terminal restriction fragment length polymorphism (TRFLP) has been used to examine the diversity of the intestinal microflora. A better understanding of the microbial intestinal population will allow researchers to provide tools by which animal health and performance can be maximised (Koutsos and Arias 2006).

It is generally considered that a balanced intestinal microbial population is characteristic of a healthy and well functioning gastrointestinal tract. This in turn

results in more efficient feed conversion (Jin et al. 1997). The usual practise in modern, intensive, poultry production is to keep poultry incubators and hatching rooms as sterile as possible. However, this affects negatively the development of the chicken intestinal microflora (Apajalahti and Kettunen 2006). 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 of the environment may pose a continual challenge to the chicken. The protective mechanisms of the host, such as the low gastric pH (2.6 for gizzard), that will result in a 10 to 100 fold reduction of bacteria numbers in digested food or water, and the production of volatile fatty acids, that are known to suppress the population of enteropathogens, can partly protect the host (Jin et al. 1997; Edens 2003). Also, immune protection of poultry starts in the egg as yolk, which releases the maternal antibodies straight into the gut lumen of the developing embryo, guaranteeing protection against pathogens for the first four days of the hatched chick's life (Shat and Myers, 1991;cited by (Mahajan and Sahoo 1998). However, Spencer (1992;cited by Mahajan and Sahoo (1998)) claims that this maternal antibody IgG cannot guarantee any significant protection against *Salmonella*, so chickens must develop a protective gut microflora. In addition, supplementation with probiotics and competitive exclusion products can be very beneficial (Jin et al. 1997; Mahajan and Sahoo 1998; Ghadban 2002; Edens 2003; Nava et al. 2005). Administration of probiotics as soon as possible to hatchlings guarantees inoculation and colonisation of their GI tract with selected, safe and beneficial strains for the establishment of a healthy microflora, which can compete against pathogens. Early colonisation by

beneficial bacteria enables them to attach to the available receptor sites on the epithelium and exclude pathogens.

Even though a number of studies have been carried out to establish the effects of probiotics, their mechanism(s) of action have not been clearly defined. Most experimental work aimed at defining the different modes of action of probiotics, has been based upon work conducted in mammals, but the same principles may not apply to the avian species (Edens 2003). Researchers have proposed that the maintenance of a beneficial microbial population, which guarantees a well-functioning gastrointestinal tract and subsequently healthy animals, is based on the mechanism of competitive exclusion and the antagonistic activity against pathogenic bacteria (Jin et al. 1996a; Edens 2003).

1.3.2.2 Antagonistic activity

In general, most bacteria produce and secrete 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). For lactobacilli of chicken origin their antagonistic activity has not been extensively studied. However, Jin et al. (1996a) has summarized the possible mechanisms by which LAB may inhibit several Gram positive and Gram negative bacteria. Probiotic bacteria such as lactic acid bacteria are able to produce antibacterial substances, which have been shown to be inhibitory to poultry pathogens. Oyarzabal and Corner (1996) reported on two commercial *Lactobacillus* strains, *Lact. casei and Lact. lactis* with good inhibitory activity against six *Salmonella* serotypes. A year later, Jin et

al. (1996a) reported on twelve *Lactobacillus* strains isolated from chicken intestine, having inhibitory activity against five strains of *Salmonella* and three serotypes of *Esch. coli*. Such antimicrobial substances include bacteriocins (acidolin, bulgaricin, nisin, reuterin), bacteriocin-like substances (Mahajan and Sahoo 1998), sort chain volatile fatty acids (lactic, propionic, butyric, and acetic) and hydrogen peroxides (Fuller 1989; Jin et al. 1997; Mahajan and Sahoo 1998; Ghadban 2002; Curbelo et al. 2005). In particular, lactobacilli are known to produce bacteriocins, which are defined as peptides, proteins, or proteinaceous compounds and most of them have an inhibitory action against Gram positive or/and Gram negative bacteria. For example, *Lact. acidophilus* has been reported to produce a large number of bacteriocins with an inhibitory effect on pathogenic bacteria such as *Salmonella*, Coliforms and *Campylobacter* (Tahara and Kanatani 1997; Curbelo et al. 2005). Jin et al. (1997) reviewed a study by Vincent et al., (1959), claiming that a bacteriocin-like substance produced by *Lactobacillus spp* and called lactocidin plays an important role against numerous pathogenic bacteria including *Salmonella spp* and *Esch. coli*. However, according to the EU Council Regulations 429/2008 (Paragaraph 2.2), micro-organisms used as additives or as production strain shall not be capable of producing antibiotic substances that are relevant as antibiotics in humans and animal, such as reuterin (European Patent EP0357673).

Some strains of lactic acid bacteria, that have shown an antagonistic action against other bacteria, are reported to produce large amounts of hydrogen peroxide as a protective mechanism to oxidation of biomolecules (Stanier et al.

1992; cited by Curbelo et al. 2005). In other words 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. 1996a)

The bactericidal effect of organic acids (lactic, acetic, butyric and propionic) produced by *Lactobacillus*, *Enterococcus* and *Bifidobacterium*, has been attributed to their capacity to reduce intestinal pH (Jin et al. 1997). Organic acids, which are major end products of the metabolism of lactic acid bacteria such as *Lactobacillus*, *Enterococcus* and *Bifidobacterium* strains, have antagonistic activity against pathogens. The acidic environment of the gastrointestinal tract increases the level of acids in an undisociated form and consequently increases their inhibition ability against gram–negative organisms (Jin et al. 1997). Another mechanism of the acids against *Salmonella* is related 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* spp. at low doses (Van Immerseel et al. 2006a; Van Immerseel et al. 2006b).

1.3.2.3 Competitive exclusion

The Nurmi concept of 'competitive exclusion' (CE) suggests that the colonisation of the intestinal tract with normal gut flora from adult birds to newly hatched chicks discouraged colonisation with *Salmonella* (Ghadban 2002). In recent years, several studies, reviewed by Ghadban (2002) demonstrated the

efficiency of competitive exclusion for the control of pathogens such as *Salmonella*, *Esch. coli* and *Campylobacter*. Undefined cultures were found to be effective against pathogens *in vitro*, however, *in vivo* studies were more variable (Stavric and D'aoust 1993). The same study concluded that defined cultures are less effective than undefined cultures *in vitro*. Fuller (1989) suggests that competition between existing microflora and pathogens for sites of adherence on the intestinal surface is another mechanism by which beneficial organisms prevent colonisation by pathogens.

1.3.2.4 Adhesion to epithelial cells

A number of bacteria stay in constant contact with the mobile viscous layer of the mucosa (Savage 1983), which is considered to be a very complex structure because of the complicated interactions between the microflora and the different cell types. Savage (1983) describes the gastrointestinal surfaces as dynamic three-dimensional sections that consist of mucinous glycoproteins, flowing on glycocalix overlying the epithelial cell membrane that is convoluted into microvilli. The membranes are apparently in an active liquid condition with permanent movement and transposition of their macromolecular components. In particular, the chicken gastrointestinal epithelium consists of a multilayered and keratinised epithelium in the crop and gradually developing and migrating tall columnar enterocytes within the lower intestinal epithelia (Hodges and Muir 1974). Mucus consists of glycoproteins and is synthesised and secreted by the goblet cells, and, as mentioned above, covers the GIT epithelium and acts as a protective layer (Deplancke and Gaskins 2001; Edelman et al. 2003). There is

evidence that the growth of enteropathogens in mucus contributes to their pathogenesis (Wadolkowski et al. 1988). The pathogens adhere to ileal mucus through their type 1 fimbriae. Bacterial adhesion and rapid growth on mucus glyconjugates contribute to bacterial colonisation of the mucus covering epithelial surfaces (Edelman et al. 2003). Paradoxically, adhesion to mucus also contributes to the antimicrobial and the protective function of the mucus as it enables removal of bacteria from sites due to the rapid movement of mucus (Wadolkowski et al. 1988). In addition, adherence is essential for the proliferation of lactobacilli and for decreasing their exclusion from specific receptors on the gut epithelium, caused by the passage of digesta due to peristalsis (Ghadban 2002). Gusils et al. (2003) suggested that two particular strains *Lact. anomalis* CRL1014 and *Lact. fermentum* CRL1015 can grow in mucus preparations and have the ability to adhere to poultry intestinal mucus. A glycoprotein from the mucus probably acts as a receptor, at least for *Lact. fermentum* CRL1015. These two LAB could not reduce pathogen adhesion ($P>0.05$) suggesting that they do not interfere with the binding sites used by *Salmonella* to attach to the chicken small intestinal mucus. This probably means that they use different mechanisms for adherence to mucus from those used by lactobacilli. It is desirable that the probiotic strains have the ability to compete with the pathogens for the same receptors and to engage their potential binding sites in the gut including collagen and fibronectine (Neeser et al. 2000; Lorca et al. 2002). Edelman et al. (2003) describes an avian pathogenic *Esch. coli* 078 strain 789 (Babai et al. 1997) that shares similar, or identical, adhesion sites within the chicken intestinal tract as *Lact. crispatus*. This organism was isolated

from chicken and is an adhesive *Lactobacillus* strain that efficiently prevents pathogen adherence *in vitro*.

Adhesion is considered to be a complex but essential mechanism for bacterial colonisation. In the case of probiotics, adhesion is a multistep process involving the close association of the potential probiotic lactic acid bacteria with the intestinal mucosa and the epithelial cells. Gusils et al. (2002b) suggest that adhesion might be a prerequisite of competitive exclusion; as the LAB grow, attach to the gut wall and further colonise and inoculate the luminal contents especially in the crop and caecum of chickens. 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. Finlay and Falkow (1997) suggest that since the LAB adhere to the epithelial cells and thereby have a prolonged residence in the GIT, they could act as antibacterial agents by excluding the pathogens found on the mucosal surface.

Busscher and Weerkamp (1987) suggest that bacterial, and particularly LAB adhesion (Servin and Coconnier 2003) involves two to five steps, starting with the passive van der Waals attractive forces; electrostatic interactions; hydrophobic; steric forces; lipoteichoic acids; and lastly frequently active adhesion through the production of specific structures by the bacteria, such as external appendages covered by lectins (Neeser et al. 2000) and (or) extracellular polymers (polysaccharides). Not all the bacteria use the same attachment mechanism, for example *Esch. coli* uses particular structures called

pili which can be described as proteinaceous projections. On the other hand, lactobacilli use extracellular substances, containing mucopolysaccharides, proteins (Conway and Kjelleberg 1989), lipids and lipoteichoic acids (Sherman and Savage 1986) to attach to the epithelium cells. Granato et al. (1999) reported that *Lact. johnsonii Lal* uses lipoteichoic acid as an adhesion mechanism. Several researchers such as Jakava-Viljanen et al. (2002), Lorca et al. (2002) and Rojas et al. (2002) have managed to isolate and characterise adhesion-promoting proteins on the surface of *Lact. fermentum* and *Lact. brevis*. Gusils (2002a) reported the presence of lectinlike proteic structures on the surface of several lactobacilli (*Lact. animalis*, *Lact. fermentum* and particularly *Lact. fermentum spp.. Cellobiosus* was found to have high hydrophobicity values. Lectinlike structures and (or) polysaccharides tend to link to specific receptors of epithelial cells (Conway and Kjelleberg 1989; Gusils et al. 2002b) and by occupying the adhesion receptors on the surface or by steric hindrance, they manage to block the adhesion of pathogens. In all their studies Gusils et al. (1999a; 1999c; 2002a; 2002b; 2003) report that all the lactobacilli studied, showed lectin-like structures, though with different sugar specifities. Polysaccharides also play a significant role in bacterial attachment (Marshall 1971; Henriksson et al. 1991). Except from Gusils (2003) who observed polymeric substances on a number of chicken derived lactobacilli, Wadstrom et al. (1987) also reported *Latobacillus* strains from the small intestine of the pigs having carbohydrate capsule polymers and high hydrophobicity. High cellular hydrophobicity probably indicates a greater ability to adhere to epithelial cells. Generally, it is believed that hydrophobic lactobacilli adhere better to GI epithelial cells than hydrophilic LAB (Wadstrom et al. 1987). Ehrmann et al.

(2002) obtained results from their work with potential probiotic lactic acid bacteria of duck origin, showing that the strains with high hydrophobicity also showed strong adhesion to crop cells and Hep2 cells.

It is well documented in several *in vitro* adhesion models, using Caco- cells, that lactobacilli are able to competitively exclude enteropathogens (Tuomola and Salminen 1998; Tuomola et al. 1999; Lee et al. 2003). Generally, lactobacilli obtained from chickens have the ability to adhere to several epithelial cells such as crop, small and large intestines, though adhesion to the cells of small intestine is weaker. Gusils et al. (2002b) and Jin et al. (1996b; 1996c) demonstrated that some lactobacilli were able to adhere strongly to chicken ileal epithelial cells. Sarra et al. (1992) commented that the bacterial adhesion to the ileum helps the control of the intestinal flora of this region and prevents colonisation with undesirable bacteria from the caecum.

Most of the studies support the hypothesis that competitive advantage of the beneficial against the pathogenic bacteria for receptor sites is essential for host protection against infection. Though, there are some recent studies suggesting that adhesion of the potential probiotic strains is not required for these bacteria to exert their probiotic effect in the GIT (Bezkorovainy 2001). In addition to that, Hautefort et al. (2000) reported that *in vitro* adhesion of *Lact. fermentum* strains did not guarantee their persistence in the digestive tract of mice. Several researchers (Kabir et al. 1997; Matsumoto et al. 2001) have examined the ability of probiotic strains, previously shown to adhere *in vitro,* to colonise the

GIT of mice *in vivo. Lact. casei rhamnosus GG, Lact. johnsonii Lal, Lact. salivarius and Lact. plantarum* strain 299v colonised all the segments of the gut of gnotobiotic mice.

It is generally believed that when potential probiotic bacteria have the ability to adhere to intestinal mucosal cells the maximum probiotic effect is achieved. However, Servin and Coconier (2003) stated that there some evidence that exogenously administered organisms can have a probiotic effect, even though the organisms pass through into the faeces without having adhered or multiplied. Following clinical pharmacokinetic studies Bezckorovainy (2001) suggested that in order to have a continuing probiotic effect the probiotic culture must be constantly ingested, especially in the case that the probiotic culture hase low adhesive capacity. Mead et al. (2000) supported the view that the probiotic treatment is mainly prophylactic rather than therapeutic, which is in agreement with Jin et al. (1996b). They concluded that once Salmonellae have attached to the IEC lactobacilli are unable to replace them. *Salmonella* invasion starts with them entering the cell, though they very soon become isolated with membrane-bound inclusions. Once a *Salmonella* cell internalises, other *Salmonella* cells follow the same route into the epithelial cell and attach thoroughly to intestinal cells, which makes the replacement of the *Salmonella* by lactobacilli unlikely.

1.3.2.5 Autoggregation and coaggregation with pathogens

The aggregation or 'clumping' of bacterial cells belonging to the same (autoaggregation) or to different (coaggregation) bacterial cells is an example of bacterial interactions and comprises a part of the general research on screening lactic acid bacteria of chicken origin (Roos et al. 1999; Kolenbrander 2000). Several researchers have reported the importance of aggregation ability of lactobacilli in enabling colonisation of the oral cavity, the urogenital tract and in genetic exchange via conjugation (Gasson et al. 1992; Bensing and Dunny 1993; Mastromarino et al. 2002). Mojgani et al. (2007) have studied the mechanism of autoaggregation of *Lactobacillus* strains as potential probiotics and suggested that cell aggregation is probably due to the proteins present in the culture supernatant and proteins or lipoproteins, located on the cell surface. Cesena et al. (2001) reported that the aggregating phenotype of *Lact. Crispatus* has enhanced adhesion ability *in vitro* and improved GI colonisation *in vivo*. Autoaggregation of lactic acid bacteria may also be necessary for adhesion to intestinal epithelial cells and with the addition of their potential coaggegation ability they may form a barrier that prevents colonization of pathogenic microorganisms (Kos et al. 2003) by providing a competitive advantage in the intestinal microbiota (Ghadban 2002). Spencer and Chesson (1994) suggested that coaggregation between lactic acid bacteria and enteropathogens has a direct effect in excluding the pathogenic bacteria from the gastrointestinal tract. Boris et al. (1998) claims that the autoaggregation ability of lactobacilli can increase their adhesion to intestinal epithelial cells in environments with short residence time and even more, may speed up the passage of pathogenic *Esch. coli* through the piglet's gut by the formation of *Lactobacillus spp. – Esch. coli*

aggregates (Kmet and Lucchini 1999). 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 (Kmet and Lucchini 1999; Styriak et al. 2001)

The antagonistic activity of lactobacilli could also be associated with their ability to coaggragate. As the lactobacilli coaggregate with the pathogens, they create large contact areas between them, which enable the so called 'inhibitory microenvironment', which is based actually on the inhibitory activity of certain metabolites that are concentrated in these areas. Additionally, a continuity zone is formed between the two bacterial cytoplasms of the bacterial membrane surfaces, which probably leads to the transfer of intracellular metabolites (Drago et al. 1997)

1.2.3.6 Competition for essential nutrients

Mahajan and Sahoo (1998) suggest that lactic acid bacteria in the gut might utilise nutrients (like glucose and amino acids) that would be otherwise be available for pathogens. The hypothetical competition between commensal gut bacteria and pathogens for accessible nutrients in the gut as a competitive exclusion defence mechanism requires much more evidence to be confirmed (Mahajan and Sahoo 1998).

The mechanism of the 'competition for essential nutrients' is the basis of prebiotics, which are defined as non-digestible carbohydrate fractions fed in diets, that are beneficial to the host by stimulating the growth of one or more bacteria in the GI tract (Dunkley et al. 2009). Fructooligosaccharides have been shown to impact bacterial populations by promoting the growth of *Lactobacillus* and *Bifidobacterium* spp. (Dunkley et al. 2009). Oyarzabal and Conner (1995) suggested that fructooligosaccharides are not digested by intestinal enzymes and can be used by lactobacilli and bifidobacteria to satisfy their growth requirements for energy. At the same time the pH levels decreased because of the production of acids. Consequently, the growth of Gram negative bacteria, such as *Salmonella* and *Esch. coli,* is restrained as they are not able to use fructooligosaccharides and they are sensitive to low pH.

1.3.3 Increasing feed intake and digestion

Several researchers have reported improved feed intake and feed conversion of chickens provided probiotics (Kabir et al. 2004; Gil de los santos et al. 2005; Takahashi et al. 2005; Khaksefidi and Ghoorchi 2006; O'Dea et al. 2006; Timmerman et al. 2006; Apata 2008).

Gut microflora affects the digestion, absorption and generally the metabolism of dietary carbohydrates, protein, lipids and minerals and the synthesis of vitamins (Jin et al. 1997). According to Apajalahti et al. (2004) intestinal bacteria produce metabolites that provide energy for the host. Most of the VFA formed

by intestinal bacteria are absorbed and metabolised by the host, contributing to host energy requirements.

There is some evidence that microflora may affect the digestion and absorption of nutrients through effects on villus architecture and consequently the absorption of the nutrients. The enterocytes that comprise the villus epithelium are capable of digestive and absorptive activity (Moran 1985). Lactic acid bacteria, *Lact. rhamnosus* GG for example were found to increased enterocyte production in gnotobiotic rats (Banasaz et al. 2002). This suggests that lactic acid bacteria may increase digestion.

1.3.4 Alteration of metabolism by increasing digestive enzyme activity, decreasing bacterial enzyme activity and ammonia production

1.3.4.1 Digestive and bacterial enzyme activity

Ghadban (2002) reviewed several scientific papers suggesting that lactobacilli produce digestive enzymes *in vitro* that may enhance the concentration of intestinal and bacterial digestive enzymes. Enzymes of the gastrointestinal tract increase the digestion of nutrients, especially in the lower intestine (Jin et al. 1997).

When chickens were provided with adherent *Lactobacillus* cultures, either as a single dried culture of *Lact. acidophilus,* or as a mixture of 12 *Lactobacillus* strains, the levels of amylase in the small intestine were found to be significantly

(P<0.05) higher than those of the control group fed just a basal diet. However, no differences were observed on the proteolytic and lipolytic activities in the small intestine. Supplementation of *Lact. acidophilus,* or a mixture of 12 *Lactobacillus* strains, significantly reduced (P<0.05) the intestinal and faecal β-glucuromidase and faecal β-glucosidase, but not intestinal β-glucosidase after 40 days of feeding (Jin et al. 2000). Mountzouris et al. (2007) evaluated the efficacy of a probiotic product (*Biomin Poultry5Star, BIOMIN GmbH*) comprising probiotic bacteria isolated from the crop (*Lact. reuteri*), jejunum (*Enterococcus faecium*), ileum (*Bifidobacterium animalis*), and cecum (*Pediococcus acidilactici* and *Lact. salivarius*) of healthy adult chicken, in promoting metabolic activities of broilers. Birds fed probiotic in feed and water had significantly (P<0.05) lower specific activities of α-galactosidase and β-galactosidase compared with birds on control and antibiotic supplemented diets.

1.3.4.2 Ammonia production

Ammonia produced by ureolysis and urea's activity in the intestinal mucosa can be toxic and harmful to the cell wall. So, reducing ammonia production and urease activity improves animal health and enhances growth (Jin et al. 1997). Yeo and Kim (1997) found that during the first three weeks, feeding a diet containing *Lact. casei* significantly (P < 0.05) decreased urease activity (per gram of collected contents) in small intestinal contents but not in large intestinal contents, compared with the control. More recently, *Aspergillus oryzae* was found to decrease significantly (P<0.05) ammonia gas production in broilers (Lee et al. 2006).

1.3.5 Other modes of action of probiotics

1.3.5.1 Enterotoxin neutralisation

Probiotics have been found to have an anti-enterotoxin activity (Jin et al. 1997), but the evidence for that is still limited. A commercially available lactobacilli preparation (*Lact. bulgaricus*) was shown to neutralise *Esch. coli* enterotoxin both *in vitro* (Mitchell and Kenworth 1976) and *in vivo* (Foster et al. 1980).

1.3.5.2 Stimulation of immune response in the digestive tract of the chick

1.3.5.2.1 Avian intestinal immunity

Th GI tract prevents enteric infections by non-immunological and immunological mechanisms. Intestinal peristalsis, that reduces the interactions of pathogens with epithelial cells, and mucus coating the surfaces of enterocytes, that reduces the attachment of pathogens on enterocytes, and the resident microflora that acts antagonistically against pathogens comprise the non-immunological intestinal defence mechanism against pathogens (Fortun-Lamothe and Boullier 2007).

At the same time, the mucosa associated lymphoid tissue (MALT) provides the first defence barrier against enteric microorganisms (Bar-Shira and Friedman 2006). An important part of MALT is the gut-associated lymphoid tissue (GALT). In chickens, GALT includes unique immune effector cells residing in the epithelial lining and distributed in the underlying lamina propria of specialised lymphoid structures, such as the bursa of Fabricius, the caecal tonsils, Meckel's

diverticulum, Peyer's patches and lymphocytes (Bar-Shira and Friedman 2006). The defence system in the digestive tract of the chick is immature at hatch and the posthach period is of major immunological importance (Revolledo et al. 2006). Immediately after hatch the chicks are exposed to environmental antigens. The intestinal lymphoides tissues develop in the first 4 posthatch days, though the GALT that contains T lymphocytes, which provide the major players in cell-mediated immune responses, and B lymphocytes, which synthesise and secrete antibodies (Davison 2003) and on antibodies, is observed only in the second posthatch week and maturation occurs later. Therefore, the hatching chicks are susceptible to pathogens immediately posthatch (Sklan 2005). Subsequently, GALT plays an important role on the host defence against pathogens and on the protection of the mucous membrane by controlling the inflammatory response.

Additionally, the gut and its resident microbiota also affect the immune system. Commensal bacteria are recognised by Toll-like receptors, of the dendritic cells. These are found in the lamina propria of the gut, and activation of these cells results in the secretion of cytokines, some of which are important for antibody production (Haghighi et al. 2005).

1.3.5.2.2. Stimulation of immune system

The immune system can be influenced by the probiotic by the activation of the lymphoid cells of the gut-associated lymphoid tissue (GALT). Lymphoid cells are diffusely distributed among epithelial cells and populate the lamina propria

and sub-mucosa (Madara 1997). Shin et al. (2002) report that one of the *Lactobacillus* strains they studied, *Lact. Fermentum* YL-3, managed to attach to caecal epithelial cells adjacent to intracrypts and the pericryptal region and suggested that this may be the cause of a transient translocation of small numbers of bacteria via M cells of the Peyer's patches and other GALT surfaces.

Evidence is accumulating that suggests that probiotics exert an essential role in stimulating the immune system in avian species (Jin et al. 1997). Nahashon et al. (1996) found that *Lactobacillus* supplementation of layer diets increased cellularity of Peyer' s patches in the ileum indicating a stimulation of the mucosal immune system that responded to the secretion of intestinal IgA. Haghighi et al. (2005) suggest that probiotic bacteria including *Lact. Acidophilus* and *Bifidobacterium Bifidum,* enhance the antibody-mediated immune response in chickens. Another probiotic mixture of *Lact. Acidophilus*, *Bifidobacterium Bifidum* and *Streptococus Faecalis* stimulated the production of natural antibodies in chickens (Haghighi et al. 2005). Dunham et al. (1993) reported that chickens provided *Lact. Reuteri* exhibited longer villi and a deeper crypt, which is a response associated with enhanced T lymphocyte function and increased production of anti–*Salmonella* IgM antibodies. Antibody production against Newcastle disease was significantly higher ($P<0.05$) in chickens provided *Bacilus Subtilis* (Khaksefidi and Ghoorchi 2006) or *Lact. Bulgaricus* (Apata 2008) than in control chickens. Also, Dalloul et al. (2005) observed an immunoregulatory effect of a commercial *Lactobacillus* – based probiotic

(*Primalac*, Star – Labs/Forage Research, Inc., Clarksdale, MO) on cytokine levels and on the local cell-mediated immunity in poultry when challenged with *Eimeria acervulina*. Another commercial probiotic (*Protexin/Boost*) provided to broilers, promoted significant antibody production (Kabir et al. 2004).

1.3.5.3 Histological alterations of the gastrointestinal tract

The intestine plays an important role in the digestion and absorption of the ingested feed ingredients (Yamauchi 2007). The avian crypt area has few cells at hatch that are formed completely within 48 hours posthatch and matures within 5 days posthatch (Koutsos and Arias 2006). Intestinal villi are the extension of the lamina propria into the intestinal lumen (Yamauchi 2007). Increased villus height indicates a greater surface area increasing absorption of available nutrients (Caspary 1992). It has been postulated by some people (Shamoto and Yamauchi 2000) that longer intestines, greater villus height and numerous cell mitoses in the intestine indicate that the function of the intestinal villi is stimulated. An evaluation of the effects of probiotics can made using histological methods in addition to nutritional-physiological (such as feed intake, bodyweight gain, feed efficiency), immunological, microbiological and pathological methods. The effects of probiotics on histological alterations to intestinal villi are still unclear. The permeability of the intestinal epithelium, which functions as a natural barrier against pathogens, may be altered by pathogens such as *Salmonella*, resulting in decrease of number, height and length of villi, increase of cell turnover and decrease in the digestive and absorptive activity. These changes have consequences for the health and

performance of the animal. Though, there is some evidence that probiotics improve the intestinal mucosa development. Pelicano et al. (2005) found that birds fed *Bacillus subtilis*-based probiotic showed significantly ($P<0.01$) greater villus height in jejunum and ileum and greater crypt depths in the duodenum, jejunum and ileum, compared with control. Dunham's team (1993) showed that chicks and turkeys treated *with Lact. reuteri* had longer villi, compared to birds provided a basal diet. Samanya and Yamauchi (2002) reported that birds fed dried *Bacillus subtilis* var. *natto* had a tendency to display improved intestinal histology compared with controls, such as increased villus height, cell area and cell mitosis.

1.4 Application of probiotics and competitive exclusion bacteria to poultry

1.4.1 Effect of probiotics on poultry performance

There are several reviews prior to 2003 discussing the effect of probiotics on poultry performance (Jin et al. 1997; Mahajan and Sahoo 1998; Ghadban 2002; Edens 2003; Patterson and Burkholder 2003). Some more recent studies, published since 2004, are reviewed here (Table 2).

Table 2. Performance of probiotic organisms used in chicken production

Reference	Organism used	Effects in performance (difference from control)
Kabir et al. (2004)	*Protexin®/Boost* 2mg/ $10L^{-1}$ drinking	15% significantly ($p< 0.01$) higher live weight gains
Gil de los santos et al. (2005)	*Saccharomyces boulardii* or *Bacillus cereus* var. toyoii supplemented in feed	60 and 75% significantly ($p< 0.01$) higher live weight gains and improved feed efficiency by 10 and 12% for *Sac. Boulardii* and *Bac.cereus*, respecteively
Khaksefidi and Ghoorchi (2006)	50 mg kg^{-1} *Bacillus subtilis* supplemented in feed	4 and 6% significantly ($p< 0.01$) higher live weight gains and improved feed conversion by 6% for 1-21 and 22-42 days, respectively
Timmerman et al. (2006)	A multispecies (MSPB- different probiotic species of human origin) and a chicken-specific (CSPB- 7 *Lactobacillus* species isolated from chickens) probiotic preparation, 10^{7}-10^{10} CFU ml^{-1} in drinking water	MSPB treatment resulted in a slight increase (by 1.84%) in broiler productivity based on an index taking into account daily weight gain, feed efficiency, and mortality
O'Dea et al. (2006)	Pr1: *Lact. acidophilus*, *Lact.bifidus*, and *Streptococcus faecalis*, in drinking water or spray, Pr2: *Lact. acidophilus*, *E. faecalis*, and bifidobacteria in feed (0.5 g of probiotic $2kg^{-1}$ of feed)	6, 6.5 and 7% significantly ($p< 0.01$) higher live weight gains for Pr1 in water, Pr 1 in spray and Pr2 in feed, respectively, at day 36 to 42
Opalinski et al. (2007)	*Bacillus subtilis* (strain DSM 17299), 8×10^{5} CFU g^{-1} feed or 3×10^{5} CFU g^{-1} feed	From 1 to 42 days of age: no significant difference in weight gain, feed intake, decreased feed conversion ratio by 1.4-1.8%
Apata (2008)	*Lact. bulgaricus* -based probiotic, 20, 40, 60 or 80 mg kg^{-1} in feed	Significantly increased feed intake and body weight, Feed/gain ratio improved significantly ($P < 0.05$) with the 20, 40 and 60 mg kg^{-1} *Lact. bulgaricus* diets

A study by Faria Filho et al. (2006) evaluated the efficacy of probiotics as growth promoters in broiler feeding, using a systematic review and meta-analysis of 27 Brazilian studies published between 1995 and 2005 found in the literature. Meta-analysis showed that probiotics increased the weight gain and the feed conversion in relation to the negative control (no antimicrobial) in the initial phase (1 to 20-28 days) and in the total period (1 to 35-48 days). Weight

gain and feed conversion were similar between the probiotics and the positive control (with antimicrobial) both in the initial and the total periods.

The effects on performance of chickens provided probiotics, reported in the literature, are not conclusive. Six of the seven papers reviewed, as well as the systematic review and meta-analysis of 27 Brazilian studies (Faria Filho et al. 2006) have reported increased body weight gain and feed intake of chickens provided with probiotics either by feed (Gil de los santos et al. 2005; Khaksefidi and Ghoorchi 2006; O'Dea et al. 2006; Opalinski et al. 2007; Apata 2008) or drinking water (Kabir et al. 2004; Timmerman et al. 2004), during either the whole period of study or a part of it. However, Opalinski et al. (2007) reported no significant difference in weight gain or feed intake and decreased feed conversion ratio by 1.4-1.8% of chickens fed *Bacillus subtilis*. The low dose level (10^5 CFU ml^{-1}) of the microorganism may explain the negative results of this study. However, when (Khaksefidi and Ghoorchi (2006) provided chickens with 50 mg kg^{-1} *Bacillus subtilis* reported significantly ($p< 0.01$) higher live weight gains and improved feed conversion for 1-21 and 22-42 days.

1.4.2 The effect of probiotics against *Salmonella* contamination

1.4.2.1 Salmonella: pathogenesis in poultry

Consumption of poultry products has been implicated in the incidence of human salmonellosis, a common and widespread zoonosis worldwide (Nava et al. 2005). As mentioned in the introduction, one in four broiler flocks surveyed in

Europe was *Salmonella*-positive (European Food Safety Authority 2007). However, *Salm. enteritidis* and *Salm.* Typhimurium, which are the two most commonly reported Salmonella types in human cases in the EU, were identified in only 40% of *Salmonella* positive flocks.

Salmonella serotypes that colonise a particular host species, such as *S. gallinarium* in poultry, are called host-restricted. *Salmonella* serotypes, such as *Salm. enteritidis* and *Salm.* Typhimurium that are associated with a broader range of unrelated host species and can induce systemic disease in them, are referred to as unrestricted or broad-range serotypes (Uzzau et al. 2000).

Chickens are usually infected by the faecal-oral route (Barrow et al. 1994), though other routes such as the respiratory route have also been reported (Cox et al. 1996). The upper intestinal tract of chickens is regarded as an acidic barrier to pathogens (pH 2.6 for the gizzard). Passage through the intestinal tract is a dynamic process, and although some bacteria stay in contact with antimicrobial substances in the crop and gizzard for a long time, others pass directly to the duodenum (Heres et al. 2003c). In poultry, the caeca are favoured sites for *Salmonella* colonization. Even the presence of low numbers of *Salmonella* allow fresh caecal contents to become inoculated after periodic emptying and refilling of the caecum (Revolledo et al. 2006). Once *Salmonella* bacteria enter the intestinal tract of the chicken they attach to the specific adhesion-receptor sites on the epithelium by bacterial fimbriae, and afterwards penetrate the mucous layer (Revolledo et al. 2006). When *Salmonella* crosses

the intestinal epithelium, contacts the lamina propria, where it can multiply, or enter into deeper tissues, and after reaching the blood stream, it infects internal organs such as the liver and spleen (Van Hemert et al. 2007).

Invasion of epithelial cells by *Salmonella* plays a critical role in *Salmonella* pathogenesis. According to Zhou and Galan (2001), upon contact with intestinal epithelial cells, *Salmonella enterica serovar spp.* injects a set of bacterial proteins into host cells via the bacterial pathogenicity Island 1 (SPI-1) type III secretion system. *Salmonella* uses the type III secretion system, which is a needle-like structure of the bacterial membrane that injects bacterial proteins into eukaryotic cells, as a basic virulence mechanism (Hueck 1998). *Salmonella* pathogenicity Island 1 (SPI-1) is a genetic element found on the chromosome which encodes various proteins that are essential to assemble the complex type III secretion system, regulatory proteins, plus some effector proteins injected by the needle complex into intestinal epithelial cell (Phoebe Lostroh and Lee 2001). Injected proteins mainly reorganize the cytoskeleton of the intestinal epithelial cells in such a manner that bacteria are surrounded by ruffles on the host cell membrane, resulting in uptake by the epithelial cell. This process is called invasion (Van Immerseel et al. 2006a). Injected proteins interact with host proteins of the intestinal epithelial cells and may also attract the immune cells to the gut wall. Macrophages may occupy bacteria entering the caecal mucosa, which is the beginning of the systemic phase of infection (Van Immerseel et al. 2006a).

1.4.2.2 Effectiveness of probiotics against Salmonella

Although several mechanisms by which probiotic bacteria could inhibit the colonisation of invaded organisms have been proposed, the exact mechanism involved in the exclusion of *Salmonella* in chicks has to be elucidated. Competition for receptor sites of the caecal epithelium, antagonistic activity through production of bacteriocins, production of volatile fatty acids by obligate anaerobes in the caecum have been suggested (Vanbelle et al. 1990; Jin et al. 1997; Ghadban 2002; Nava et al. 2005; Revolledo et al. 2006). Van Immerseel et al. (2006b) reviewed the use of organic acids to combat *Salmonella* in poultry. The antibacterial capacity of medium-chain fatty acids (C6 to C12, caproic, caprylic, capric and lauric acid) against *Salmonella* is greater than short-chain fatty acids (formic, acetic, propionic and butyric acid).

The aim of controlling carriage of *Salmonella* has met with varying degrees of success. Generally, the use of a single isolated lactic acid bacterium has been less successful than using simpler defined competitive exclusion mixtures and this approach is less successful than using undefined competitive exclusion complex mixtures. The effectiveness and the protective capacity against *Salmonella* of undefined mixtures of competitive exclusion products, based on the Nurmi concept have been demonstrated and discussed in several reviews (Ghadban 2002; Van Immerseel et al. 2002; Nava et al. 2005; Revolledo et al. 2006). *Broilact*® and *Aviguard* are two commercial products that consist of undefined intestinal bacteria obtained from healthy chickens that are available and used in *Salmonella* protection in poultry production. Schneitz and Renney

(2003) studied the effect of a commercial competitive exclusion product Broilact[r] on the colonization of *S. Infantis* in day-old pheasant chicks. Broilact[r] efficiently reduced colonization of *Salm. infantis*. *Salm. infantis* concentration per gram of caecal contents for the treated groups was 2.9 and in the controls groups 8.4 CFU g^{-1}. Mortality during the 1 week rearing period was 5.0% in the *Broilact[r]* treated groups and 8.5% in the *Salmonella* control groups.

In their study, Nakamura et al. (2002) evaluated the competitive exclusion action of *Aviguard* and its effects on the antibody response of chicks. Fourteen days after infection, fewer *Aviguard* pretreated than nonpretreated chicks shed salmonellae from their cloaca in both infected groups (*Salm. enteritidis* and *Salm.* Typhimurium), although much less from *Salm. enteritidis* infected chicks. In contrast, Stavric and Daoust (1993) reviewed a series of field trials, suggesting that the use of undefined competitive exclusion cultures, combined with good hygienic control measures on poultry farms did not consistently reduce *Salmonella* infection in commercial poultry flocks.

Studies with commercial products of defined probiotic mixtures such as *Avian Pac Plus*, *Pre-empt* and a lactobacillus based probiotic culture FM-B11TM proved successful and supported the view that characterised competitive exclusion cultures can reduce *Salmonella* incidence in poultry. Tellez et al. (2001) determined the effect of *Avian Pac Plus*, which contains *Lact. acidophilus*, *Streptococcus faecium*, and *Salm.* Typhimurium-specific antibodies on the colonization of *Salm.* Typhimurium in broilers. Chicks were spray-

vaccinated at the hatchery and given Avian Pac Plus for the first 3 days after placement, as well as at 10 and 14 days, 2 days prior to vaccination and 2 days postvaccination. Six hours after placement, the chicks were challenged with 10^7 CFU ml^{-1} *Salm. enteritidis.* The probiotic-treated group had a significantly lower concentration of *Salm. enteritidis* caecal colonization at days 3, 7, 10, 17, 24, 31, 38, and 41 when compared with the non-treated, control group ($P < 0.05$). Similarly, there was a significant difference ($P < 0.05$) in the frequency of isolation of *Salm. enteritidis* from the internal organs (liver and spleen) when probiotic-treated and nonprobiotic-treated groups were compared. The results obtained in Tellez and co-worker's study are in agreement with those from Promsopone et al. (1998) study. Chickens were administered *Avian Pac Plus* by spray at the hatchery, in the water for two days and were challenged, six hours postplacement, with 10^7 CFU $ml^{-1}$of *Salm.* Typhimurium. The mean caecal and colonic concentration of *Salm.* Typhimurium from the *Avian Pac Plus*-treated group was significantly lower at days 31, 38 and 43 than the nontreated control group. A 4 h posttreatment inoculation on the day of hatch with oral gavage of the *Pre-empt* bacteria, followed by challenges with 10^2 and 10^4 *Salmonella* CFU ml^{-1}, resulted in 3% and 3%, respectively, of the caeca testing *Salmonella*-culture-positive, compared with 28% and 95%, respectively, culture-positive caeca in untreated chicks (Hume et al. 1998). Another commercial product, FM-B11, made by 11 LAB isolates, has been shown to reduce significantly ($P<0.05$) the incidence of *Salm. enteritidis* (60 to 70% reduction) or *Salm.* Typhimurium (89 to 95% reduction) recovered from the caecal tonsils of day-old broiler chicks 24 h following treatment as compared with controls ($P < 0.05$) (Higgins et al. 2007; Higgins et al. 2008; Vicente et al. 2008).

Several researchers were unable to demonstrate the ability of monogeneric preparations to protect poultry against *Salmonella* infection (Stavric and D'aoust 1993). La Ragione et al. (2004) showed that a single oral dose of 10^9 CFU ml^{-1} *Lact. johnsonii* F19758 was not sufficient to suppress *Salm. enteritidis*. However, there are some older studies, and some very promising recent ones, in which competitive isolates for reduction of *Salmonella* carriage in chickens have been selected. Soerjadi et al. (1981) showed that native avian lactobacilli reduced the number of salmonellae adhering to the crop mucosa of chickens by 1 to 2 log units. However, treatments with lactobacilli did not lower the number of chickens shedding salmonellae, or reduce the number of salmonellae adhering to the mucosa of the caecum. Watkins and Miller (1983) treated a total of 205 chicks with *Lact. acidophilus* and found a significant ($P<0.05$) reduction of *Salm.* Typhimurium in crop contents but no, significant reduction in caecal or rectal contents at post-mortem. More recently, Zhang et al. (2007) observed a reduction of *Salm.* Typhimurium prevalence from 33 to 54% compared with controls and decrease of the average *Salm.* Typhimurium counts in positive chickens by 1.5 to 2.5 log CFU g^{-1}, when each of 6 strains typed as *Lact. salivarius* were administered by gavage (10^7 to 10^8 CFU g^{-1}/ chicken) on day of hatch and the following day. Mixtures containing 2 to 3 isolates were not superior to single isolates.

1.5 Methods of administration

Delivery of probiotic strains of lactic acid bacteria (LAB) to poultry may be achieved by application directly into the crop of the chick, by gavage, in

laboratory studies. However, in commercial production it is not practical and probiotics are usually administered either in the drinking water, by spraying hatching eggs or chicks in the hatching trays or shipping boxes, through feed slurries or in the dry feed (Doyle and Erickson 2006). Added benefit may be obtained if the feed is allowed to ferment to produce a feed containing at least 150 mmol lactic acid and a low pH <4.5, as this has been shown to reduce contamination of feed by enteropathogens such as Salmonellae (Heres et al. 2003a). *In ovo* methods such as applying the probiotics prior to hatching, were ineffective up to now due to low hatchability or killing the embryo (Doyle and Erickson 2006).

1.5.1 Administration of LAB through water

Administration of LAB through drinking water is a usual practice in poultry production. However, osmosis, chlorine and calcium salts in the water could affect the survival of the LAB delivered in water. The survival of the LAB delivered in water is considered a prerequisite for obtaining a positive effect on production through treatment of animals with probiotic bacteria. However, there is some evidence that the protective effect of probiotics is partially mediated by viable but also by non-viable 'dead' probiotic bacteria. Their cell wall structure, rather than their metabolites or their ability to colonise the colon, might be partially responsible for their therapeutic benefit on the host (Rachmilewitz et al. 2004; Mottet and Michetti 2005).

When LAB are suspended in water the phenomenon of osmosis is observed (Garbutt 1997; Tortora et al. 1998; Adams and Moss 2003). The difference in the mineral concentration inside and outside the cell results in a net movement of water through cell wall, which is semi-permeable, and causes the disruption of the cell.

Chlorination of water is a common practice to avoid transmission of harmful bacteria through water consumption. When chlorine is used as a disinfectant in piped distribution system, a free chlorine residual of 0.2-0.5mg L^{-1} throughout is desirable to reduce the risk of microbial growth (World Health Organization 1997). Chlorination using an in-line proportioner (a device for accurately injecting the correct proportion of chlorine into the water line) was successful in poultry production when the residual chlorine level in the waterers was at least 1 mg L^{-1} (Carter and Sneed 1996). Previous studies at the University of Plymouth (Azhar 2005) showed that LAB survived at chlorine concentrations of 0.05, 0.23 and 0.42-0.46 mg l^{-1}; at 20°C, in hard water. *Lact. plantarum* (Alltech, Kentacky, USA) counts decreased numerically from 7.36 to 7.31 $\log_{10}$ CFU ml^{-1} after 24 hours, but the differences were not statistically significant (Azhar 2005).

1.5.2 Administration of LAB through fermented feed

In pig production, liquid feeding has been practiced since 1814 (Russell et al. 1996) and its usage has increased over the last decades. Liquid feed (LF) involves the use of a complete, nutritionally balanced diet, prepared either from a dry, moist, or liquid feed ingredients mixed with water. A typical liquid diet

contains 200-300 g/Kg dry matter per litre (Pedersen et al. 2005). Several million tones of fermented liquid co-products are used annually. These liquid co-products derived from human food industries, such as liquid wheat starch from the starch industry, potato steam peel from potato processing, cheese whey from the dairy industry and other residues from brewing and sugar processing industries. In addition fermented diets, made by mixing dry compound feed and water and stored for at least 8h (Russell et al. 1996) to permit fermentation under controlled conditions are currently used for pig feeding in Europe and around the world (Pedersen et al. 2005).

In fermented diets both bacteria and their fermentation products are present , whereas when probiotics are included to dry diets, only the bacteria are present and the products of their metabolism appear in the GI tract after being consumed. The numbers of bacteria also differ and moreover in the fermented diets bacteria grow quickly, whereas in dry diets they have to be reaccelerated (Scholten et al. 1999).

Brooks (2008) discriminates non-fermented liquid feed (NFLF), described as liquid feed that has been mixed and fed to pigs immediately, with limited opportunity to ferment, with spontaneously-fermented liquid feed (SFLF), which is feed that unintentionally or intentionally has been allowed to ferment through the action of the indigenous microflora for a period of time before delivering and inoculated fermented liquid feed (IFLF), which is liquid feed fermented following inoculation with selected lactic acid bacteria. As Canibe and Jensen (2003;

2007) have described, this is stored in a tank at a controlled temperature and for a specific period of time before being provided to animals.

A general definition of fermentation is the chemical conversion of carbohydrates (starch, sugar) into alcohols or acids (lactic acid, organic acids) by microbes (Scholten et al. 1999). Fermentation starts as soon as feed and water are mixed and several changes as far as the microbial characteristics that occur with time affect the value of the feed (Niven et al. 2006). Brooks (2008) describes the three phases through which the fermentation of cereal grains and complete pig feeds progress. As soon as feed materials are mixed with water coliforms start proliferating rapidly because of the high, around 6, pH of the feed (Phase 1). Just after that LAB start converting the carbohydrates producing mainly lactic acid and other organic acids and lowering the pH, which results in the inhibition of the pathogenic bacteria. As more and more acid is produced and subsequently the pH drops, Enterobacteriaceae are inhibited and eventually excluded (Phase 2). Phase 3 starts with LAB numbers and pH stabilising in the feed, while the yeast population continues to grow. There is a risk that yeast will eventually dominate the feed.

The procedure followed to prepare FLF affects the quality of the product obtained (Canibe et al. 2008). A good quality fermented feed is characterised by: high numbers of lactobacilli, high concentration of lactic acid and a low pH.

FLF has been demonstrated to be effective against several food-borne pathogens (Brooks 2008). In order to reduce *Salmonella* and Enterobacteriacae numbers in FLF, a concentration of around 10^9 CFU g^{-1} LAB and more than 150 mmol of lactic acid and a pH below 4.5 is necessary. As far as pig nutrition is concerned a low concentration of acetic acid (<30mM) is desirable to maintain palatability. Care should be taken to avoid fermentation of FLF by heterofermentative LAB, as this can result in the production of undesirable SCFA such as acetic, propionic and butyric acids, that affect negatively the palatability and the nutritional value of the feed and consequently the feed intake (Niven et al. 2004). Scholten et al. (1999) reviewed the mechanisms by which FLF might act and beneficially affect the pigs. High lactic acid concentration in the stomach is connected with the inhibition of the growth of many undesirable microorganisms, coliforms and *Salmonella spp.* (Mikkelsen and Jensen 1997). An additional benefit of reduced gastric pH is the stimulation of the activity of pepsin and the reduction of gastric emptying, so there is more opportunity for acid and/or enzymic hydrolysis of protein. A proportion of ingested lactic acid and short chain fatty acids (SCFA), produced in the fermented liquid feed, manages to reach the small intestine and stimulates the secretion of pancreatic juice, particularly bicarbonate. SCFA are generally considered to have a positive effect on the intestinal epithelium and in particular, it has been reported that villus height in pigs is correlated positively with the luminal level of butyric acid. This suggests that fermented liquid feeding might be beneficial for villus and mucosal structure and subsequently digestion and nutrient absorption (Brooks et al. 2003b).

Fermented feed has high numbers of lactic acid bacteria, some of which might have potential probiotic properties. Spontaneous fermentation of LF has been considered as unreliable and failed to give consistent good results. Backslopping is usually practiced in piggeries. This is the practice of maintaining a continuous fermentation by retaining a proportion of a previous successful fermentation and using this as an inoculum for the next batch. However, this has proved to be risky, as yeasts eventually dominate the resident microflora and negatively affect palatability and the nutritional value of the feed. In addition, SFLP often fails to generate sufficient lactic acid. In conclusion, production of IFLF using carefully selected inoculum organisms that rapidly produce high concentration of lactic acid is desirable. It is advantageous if the selected strain also has probiotic properties such as being tolerant of the high acid concentration in the stomach and bile salts in the duodenum; has antimicrobial activity, either by competition for attachment sites, co-aggregation with pathogens or production of antimicrobial substances such as bacteriocins and lastly exerts immunomodulatory ability on the host.

For a good quality LF preparation, parameters such as liquid to feed ratio, steeping time and other feeding strategies like addition of exogenous enzymes in LF should be considered too. As the liquid to feed ratio increases, the feed conversion, the dry matter and energy digestibility of the feed improve (Geary et al. 1996). Other authors such as Pedersen et al. (2005) reported no improvement in digestibility of organic matter and energy in comparison to dry feed. Choct et al. (2004) who examined the effect of water to feed ratio,

steeping time and enzyme supplementation on the performance of weaner pigs found that a liquid : feed ratio of 2 : 1 and 3: 1 had similar digestible energy (DE) content to the control diet, though the 4 : 1 diet had significantly lower DE. They concluded that grain processing had a greater effect on nutrient availability than the water to feed ratio. Brooks (2008) reviewed the effect of steeping in liquid feeding systems and suggested that steeping feed for 8-16h activates the phytase that occurs naturally in the pericarp of some grains and also increases the bioavailability of nutrients such as phosphorus, calcium, magnesium and copper in the grain, and subsequently allows the dietary inclusion of exogenous minerals to be lowered. Choct (2004) reported that in his experiment steeping may have activated the naturally occurring β-glucanase and xylanase, causing partial polymerisation of the NSPs, and consequently removing their antinutritive effects on nutrient digestion and absorption. Care should be taken with duration of steeping, though, as there is a possibility that more than 24h may cause excessive losses of organic matter, or increase the risk of harmful organisms (such as yeasts) in the feed (Brooks 2008).

The benefits of fermented liquid feed have been documented in many studies, especially for pig production and to a lesser extent in poultry production, and fermenting liquid feeding is considered a promising strategy for various reasons.

Feeding liquid feed to piglets might be a more appropriate way of their adaptation from sow's milk suckling than dry pellets in the initial post weaning stages (Choct et al. 2004). Van den Brink and van Rhee (2007) reported that

early access to semi-moist diets for day old chicks can stimulate the development of their gastrointestinal tract and prevents them from dehydration during transport. Chicks are often left without any feed or water for an extended period of time during transportation from the hatchery to the farm. The early provision of energy and water in the first 48h after hatching resulted to chicks with significantly ($P<0.05$) longer intestines (104.6% of length at hatching) compared with both the non fed chicks (103. 7%) and the chicks fed dry feed (104.0%).

There are a number of studies indicating that FLF improves gastrointestinal health of the animal. FLF was found to reduce the number of Enterobacteriacae along the gastrointestinal tract and especially in the lower small intestine, caecum and colon of pigs (Van Winsen et al. 2001b; Canibe and Jensen 2003; Canibe et al. 2008). Kobashi et al. (2008) reported that feeding FLF (produced with *Lact. fermentum* LQ80) to weaner pigs for 28 days resulted in reduction of chlortetracycline-resistant *E.coli* from 88.9% to 22%. Brooks (2008) reviewed a number of studies that have shown a reduction of *Salmonella* incidence in pigs fed liquid diets in comparison with those fed dry pelleted diets. He also suggested that undissociated acids, produced by fermentation rather than bacteriocins produced by LAB, are responsible for the antimicrobial properties of FLF. FLF has been reported to impair colonisation of other enteropathogens too, i.e *Brachyspira hyodysenteriae* (Lindecrona et al. 2003) and *Lawsonia intracellularis* (Boesen et al. 2004).

Feed:water ratio of FLF provided to feed ranges between 1:2 and 1:4 (Plumed-Ferrer and Von Wright 2009). Heres et al. (2002; 2003a; 2003b; 2003c; 2004; 2004b) suggest a feed:water ratio of 1:1.4 of the fermented feed provided to chickens, which could be considered as moist rather than liquid.

Feeding FLF has also been correlated with a lower prevalence of *Salmonella* in chicken husbandry. A reduced probability of *Salmonella* colonisation in chickens fed FLF. was reported by Heres (2004). In his study, 14 out of 18 pens of chickens fed dry feed, but only 4 out of 18 pens of chickens fed FLF, were found to be *Salmonella* positive following inoculation with a dose of 10^3 CFU ml^{-1} *Salm. enteritidis*. The protective effect of FLF against *Salmonella* was also demonstrated by the low incidence of transmission between chickens, because chickens fed FLF were not as easily contaminated with the faeces or infected litter particles they consumed. Heres et al. (2003b) have also shown 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, Heres et al. (2003a) found that chickens fed FLF required a longer time after inoculation of *Salm. enteritidis,* or a higher inoculation dose, to get the same number of infected chickens compared with chickens fed dry feed. They also reported that there was a 1 to 10 times difference in *Salm. enteritidis* susceptibility between chickens fed FLF and chickens fed dry feed. In particular, FLF had a positive effect on the upper intestinal barrier against *Salmonella* in broiler chickens. A decrease in *Salmonella* has been observed in the crop and gizzard of chickens fed FLF, compared with chickens fed

conventional dry feed. However, there was no significant reduction of *Salmonella* in the lower intestinal tract (Heres et al. 2003c). This may have been because the lactobacilli present in the specific FLF did not have probiotic properties against *Salmonella*.

In some studies, LF, NFLF or FLF, have been reported to improve feed intake and protein digestibility (Brooks 2008). Chah et al. (1975) reported significantly ($P<0.05$) improved weight gain and feed efficiency in broilers that were fed soybeans moisture conditioned to *ca.* 31% moisture and fermented in a chamber for 2-3 days at 23-30 °C with several strains of Aspergilli. They concluded that this was probably due to a greater supply of the essential amino acids. More recently, Dung et al. (2005) found that organic matter and protein digestibility were significantly greater ($P<0.05$) in a FLF diet made with broken rice and fed to growing finishing pigs, than at dry feed, or a non–fermented feed, acidified with lactic acid to pH 4. Reduced viscosity of feed and the dry matter content of digesta have been also mentioned as positive effects of liquid feed (Brooks et al. 2003b; Brooks 2008). Recently, Canibe et al. (2008) supported the view of several other researchers that FLF fermented with *Lact. plantarum* VTT E – 78076 improved the growth performance of piglets. Feng and co workers (2007) provided fermented soybean meal to one day old broilers in an 6 week experiment and observed an increase in average daily gain ($P<0.05$) by 3.8 and 17% and average daily feed intake ($P<0.05$) by 2.3 and 10.3%, for 0-3 weeks and 4-6 weeks, respectively, and increased feed

conversion by 6.3% ($P<0.05$) only in growing period, compared with chicks that were provided unfermented soybean meal.

Probiotic and immunomodulatory properties of LAB present in FLF have also been reported. In 3 week old chickens, the IgA and IgM content in the serum of birds fed soybean meal fermented with *Aspergillus oryzae* from hatching and for a 6-week period after hatch, were significantly ($P<0.05$) improved by 50 and 25% for IgA and IgM, respectively, compared with chickens fed soybean meal (Feng et al. 2007).

A potential advantage of using fermented liquid feeding for pig production, and generally all liquid feeding systems, is the opportunity to recycle liquid residues and co-product from the human food industry as animal feed which otherwise would have been additional loading for the environment. However, the opportunity for recycling co-products for chickens is limited because the use of liquid feeding is limited. It has been reported that accurate diet formulation and careful management of liquid feeding systems have the potential of reducing the nitrogen output and phosphorus loading through activation of endogenous phytase in cereal grains or the addition of exogenous enzymes to diets (Brooks et al. 2003a; Brooks 2008). Santoso et al. (2001) suggested that feeding broiler chicks a fermented product from *Bacillus subtilis* at a level of 1% or 2% of the basal commercial diet reduced ammonia gas release significantly ($P<0.05$), compared with the basal commercial diet.

FLF increases the flexibility in raw material use, with the possibility of using co-products of lower cost. Modifying raw materials prior to feeding through simple steeping in water for a sufficient period of time can activate naturally occurring enzymes, which produces a possibility of improving the feed utilisation efficiency. For example Yamomoto et al. (2007) used a traditional Japanese feed, called Koji-feed that is produced by wheat bran and Shochu distillery by-product, fermented with *Aspergillus awamori*, to examine its effect on the growth performance and nutrient availability in broilers. Results showed that broilers fed the FLF had an improved performance (0.05%, $P<0.05$) and a lower abdominal fat content (1.0%, $P<0.05$), compared with chickens fed a basal feed.

In Europe ca. 30% of finishing pigs are fed using automated liquid feeding systems with LF delivered to the pens through a pipe line. These systems can act both as feed mixing and distribution systems. Moreover using a computer allows control of feed distribution to each pen, adjusting it more accurately to the individual nutrient requirements of the pigs (Choct et al. 2004).

A reduction of the physical activity of pigs fed FLF as well as the reduction of fighting for feed at the feeding trough has been reported by Scholten et al. (1999) and Jordan (1997; cited by Choct et al. 2004). Brooks et al. (2001) observed that pigs consuming FLF spent more time sleeping/resting than pigs fed a dry diet. This might be attributed to the correlation of physical activity with

the absorption of SCFA and alcohol, produced by fermentation, from the gut wall and to the altered microbial population.

1.6 Selection criteria for probiotics

Many researchers have been working on selecting probiotic strains from animals (O'Sullivan 2001; Mojgani et al. 2007) and plants (Chiu et al. 2008) for animal use, or humans use (Jacobsen et al. 1999). Selection criteria used, for potential probiotics for animal use in order to achieve well established and positive probiotic effects vary in the literature. Edens (2003) suggested a number of desirable probiotic characteristics and functions for potential probiotics (Table 3), considering safety for the host and the capacity of the strains to be viable as well as metabolical active within the gastrointestinal tract (GI) as preresquisite for a good probiotic.

However, the most commonly reported desirable characteristiques of probiotic strains include 1) resistance to gastric acidity and bile salt resistance which ensures their viability and capacity of being biological active within the acidic chicken GI tract (Jacobsen et al. 1999; O'Sullivan 2001; Barbosa et al. 2005) 2) adhesion capacity to mucus (Jin et al. 1996c; Gusils et al. 2003) and epithelial cells (Jin et al. 1996c), which ensures the bacterial maintenance in the GIT and thereby prevents their rapid removal by contraction of the gut (Jacobsen et al. 1999), 3) ability to remove pathogen adhesion to surfaces, which may be facilitated by the aggregation properties of the probiotics (Kos et al. 2003;

Collado et al. 2007) and 4) antimicrobial activity against potentially pathogenic bacteria (Tsai et al. 2005; Olnood et al. 2007).

European regulation about feed additives, regards Nurmis and Rantala (1973) proposed the practice of dosing chicks with gut contents of adult birds to protect them from *Salmonella* infections, as too risky mainly due to the high possibility of transmission of undesirable pathogenic bacteria. So, in recent years, research has focused on developing probiotics consisting of pure probiotic bacteria or mixtures of defined probiotic bacteria that have been demonstrated to contribute to the mixture, to conform with the authorization procedure for feed additives in the EU (Regulation 1831/2003EC, Articles 7, 8 and 9) (Plumed-Ferrer and Von Wright 2009). However, La Ragione et al. (2004), suggest that attempts to use simpler defined CE mixtures have been less successful than using undefined CE complex mixtures.

Selection and screening of *Lactobacillus* strains is widespread, as they are commonly known for their probiotic properties (Nemcova 1997; Garriga et al. 1998; Gusils et al. 1999a; Gusils et al. 1999b; Ehrmann et al. 2002; Gusils et al. 2002a; Gusils et al. 2002b; Van Coillie et al. 2007). Lactobacilli are gram-positive, non- spore forming bacilli. They grow better under anaerobic conditions and their major product of glucose fermentation is lactic acid (Tannock 1997). Lactobacilli can be found in all sites of the chicken digestive tract, whether shed from epithelial surfaces or multiplying in the digestive tract (Fuller 1978; Tannock et al. 1982). Fuller and Turvey (1971) were the first to

show that lactobacilli adhere to the surface of the crop wall. Jin et al. (1996c) stated that the ileum contains a much larger population of bacteria than other parts of the small intestine.

Table 3. Desirable Probiotic Characteristics and Functions

Desirable Probiotic Characteristics:

• Normal inhabitant of the host or host adapted
• Non-toxic and non pathogenic
• Accurate taxonomic identification
• Tolerate processing, storage and delivery
• Capable of survival, proliferation and metabolic activity in the target site, which implies:
  o Resistance to gastric acids and bile salts
  o Readily bind to epithelium and mucus
  o Persistent viability in the gastrointestinal tract
  o Ability to compete with the resident flora
• Produce inhibitory substances against other bacteria
• Alter microbial activity
• Modulate immune responses
• Actively competes for receptor sites
• Be able to exert at least one clinically documented health benefit
• Genetically stable

Desirable Probiotic Functions:
• Exclude (prevent colonisation) or kill pathogenic bacteria • Stimulate the immune system • Reduce inflammatory reactions • Enhance animal performance • Decrease carcass contamination • Increase production of volatile fatty acids • Increase vitamin B synthesis • Improve nutrient absorption • Decrease diarrhoea • Competition for essential nutrients for bacterial growth • Creates a restrictive physiological environment • Stimulates peristalsis

Adapted from (Edens 2003)

1.6.1 Aggregation experiments

Autoaggregation of lactic acid bacteria may be necessary for adhesion to intestinal epithelial cells and with the addition of their potential coaggegation ability they may form a barrier that prevents colonization of pathogenic microorganisms (Kos et al. 2003) by providing a competitive advantage in the intestinal microbiota (Ghadban 2002). Spencer and Chesson (1994) suggested that coaggregation between lactic acid bacteria and enteropathogens has a direct effect in excluding the pathogenic bacteria from the gastrointestinal tract.

There are few studies analysing the aggregation ability of lactic acid bacteria with pathogenic bacteria and comparing different methodologies to measure aggregation. Collado (2007) compared different methods to measure the aggregation of bacteria based on radiolabelling, fluorescent stains and spectrophotometry after mixing suspensions of pathogens as *Escherichia coli* K2 strain and *Salmonella enterica* serovar Typhimurium ATCC 12028 with selected commercial probiotic strains, *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12. The aggregation ratio (%) was calculated by comparing the absorbance (A_{600}) of the pathogen suspension to the A_{600} of the pathogen–probiotic mix at different incubation times. The author suggests that radioactive labels in bacterial aggregation assays offers the best reproducibility and sensitivity, and also, a potential method to analyse aggregation between cells instead traditional methods based on absorbance measurements. However radiolabelling safety issues are considered the main drawback of this method.

A simpler, safer and more cost effective method of measuring bacterial aggregation suggested by several authors (Kmet and Lucchini 1997; Kmet and Lucchini 1999), previously used successfully in the laboratories of the University of Plymouth (Demečková 2003) measures the bacterial aggregation intensity in relation to the time needed for the bacteria to form aggregates that can be observed to the bottom of the tubes. Scanning electron microscopy (Drago et al. 1997) can be also used to confirm the bacterial aggregation.

1.6.2 Antagonistic activity

There are many *in vitro* methods for measuring the antagonistic action of probiotic microorganisms against pathogenic bacteria. For example, critical dilution assays flip-streak method, well diffusion assay, disc diffusion assay, spot-on-lawn method (Çadirci and Çitak 2005). Some of the above methods are based on dilution of the antimicrobial agent in broth, but most of the techniques are based on the diffusion through solid or semi-solid culture media to inhibit the growth of sensitive organism. Different inhibitory compounds, such as bacteriocins, lactic acid or hydrogen peroxide, produced by LAB bacteria exhibit different effect on susceptible microorganisms (Jin et al. 1996a).

The agar spot test, described by Jin et al. (1996a) method is a practical and suitable technique. However, the disadvantage of this method is the potential for false results due to the possible effect of the media and the chemicals used for cultivation on the bacterial antagonistic activity.

1.6.3 Acidic pH and bile salt resistance

The viability of the potential probiotic strains under the physical and chemical stressing conditions of acid and bile salt in the GI tract was considered by Gibson et al (2000) as one of the desirable characteristics for probiotics to provide health benefits. The fact that the avian gastrointestinal tract is shorter than the mammalian one and that the feed requires only 2.5 hours to pass through the GI tract, suggests that the use of resistance to low pH as a selection criterion for potential probiotics for avian species is not as crucial as for mammalian species. On the other hand, the pH of the chicken GI tract could be as acidic as 2, which may be an obstacle for the survival of lactobacilli.

Studies have demonstrated that the pH tolerance of bacteria is strain specific and some strains are better able to tolerate, or even thrive in, the low pH levels found in the gizzard (Adamberg et al. 2003; G-Alegria et al. 2004). Nevertheless, it could be mentioned, that exposure to the extreme acidic environment of the GI tract may be eliminated, or reduced, as the food particles and/or other carrier matrix molecules that are consumed by the chicken act as a buffer and protect the lactobacilli.

In addition to acid tolerance, successful probiotics also need to be bile tolerant to grow and exert their action to GI tract. Chicken bile contains bile salts and lipids (cholesterol and phospholipids). Two thirds of the bile salts, which are different from those found in mammals, consist of tauro-chenodeoxycholate. Also found are taurocholate and tauro-allochates, but not deoxycholates

(Larbier and Leclercq 1994). Bile secreted into the duodenal section of the small intestine has been found to reduce the survival of bacteria. This is believed to be due to the cell walls of the bacteria consisting of lipids and fatty acids, which are broken down by the bile salts (Jin et al. 1998a). Several researchers such as Floch et al. (1972), Gilliland and Speck (1977), Tannock et al. (1989) considered resistance to low pH and bile salts as criteria for selecting potential probiotics and linked it to the ability of lactobacilli particularly to de-conjugate bile acids.

Recently, several researchers (Liong and Shah 2005; Mojgani et al. 2007) suggest that taurocholic acid, which is conjugated bile, is more inhibitory than oxgall and the reason for that might be the fact that conjugated bile salts are possibly more toxic than their de-conjugated counterparts, because of their higher solubility and detergent activity.

1.6.4 Adhesion of LAB to chicken intestinal epithelial cells

Adherence of bacteria to the gut epithelium is essential for their proliferation and prevents their removal from specific receptors on the gut epithelium, caused by the passage of digesta (Ghadban 2002). A study by Bengmark (1998) confirmed that a *Lactobacillus* strain (*Lactobacillus Plantarum)* is able to adhere and colonise the intestinal mucosa. When lactic acid bacteria bind to the sites of adhesion on the epithelium, the pathogens colonising them may be reduced. The essential condition for this principle is the "parallel attack mechanism" with both the pathogenic and lactic acid bacteria attempting to colonise the site at

the same time. Fuller (1973; 1989) and Jin et al. (1997) suggested that lactobacilli colonising the crop can efficiently control the *Esch. coli* population. Light and electron microscopy revealed that the adherent bacteria of the intestinal microflora are connected by fibres to the mucosal surface (Jin et al. 1996b).

Although adhesion is often considered as a selection criterion for potential probiotics, there is no clear evidence of the importance of adherence of lactic acid bacteria as far as the colonisation to the GI tract is concerned. Chan et al. (1985), Bruce et al. (1988) and Reid et al. (1985; 1987) have been the pioneers in demonstrating by *in vitro* and *in vivo* studies that several selected *Lactobacillus* strains of urovaginal origin have adhesive properties that facilitate them to restrain and/or prevent the colonisation of uroepithelial cells by uropathogens. Consequently, the same mechanism of action has been anticipated for *Lactobacillus* strains of intestinal origin. According to Nielsen (1994), adherence and colonisation of the probiotic bacteria, ensures that the bacteria are maintenaned in the GIT and their rapid removal by contraction of the gut is prevented. Potential probiotic strains should have the ability to confer a competitive advantage against pathogens i.e. to compete with pathogens for the same receptor and to occupy their potential binding sites in the gut including binding to collagen and fibronectine (Neeser et al. 2000; Lorca et al. 2002; Bouzaine et al. 2005). So, the capacity of potential probiotics to adhere to intestinal mucosa and subsequently to prohibit and dislocate potential pathogens is of great significance for bacterial maintenance in the GIT and it is

generally accepted that adhesion properties also contribute to the modulation of the immune system (Schiffrin et al. 1997; Salminen et al. 1998) and the therapeutic management of the enteric microbiota in particular (Collado et al. 2005a), and the efficacy of probiotic strains in general (Servin and Coconnier 2003).

There are several ways of studying the adhesion of LAB experimentally. *In vivo* studies are generally considered to be the most reliable. However, the complexity of the gastrointestinal tract and its microflora makes *in vivo* studies of adhesion and colonisation problematic (Saremdamerdji et al. 1995). Tannock et al. (1982) suggested that species specificity of microorganisms is an obstacle for *in vivo* adhesion experiments, as bacterial strains that have been isolated from the indigenous microflora of one animal species will not necessarily colonise the same site in another animal species. Fuller (1975) had negative results when he studied the potential adhesion of lactobacilli isolated from birds to chicken crop cells *in vitro*. Suegara et al. (1975) found that lactobacilli isolated from rats could only adhere to epithelial cells derived from the non-secretory region of the rat intestine. *Lactobacillus* strains isolated from domestic pigs and the closely–related wild boar had the ability to colonise only pig squamous epithelial cells *in vitro* (Barrow et al. 1980), likewise. Surprisingly, Collado et al. (2005b) reported that some LAB strains of animal origin that adhered better to human mucus than did human fecal isolates. Rada and Rychly (1995) in their *in vivo* experiments reported colonisation of newly

hatched chickens with *Lact. Salivarius* 51R, although the *in vitro* experiments showed negligible adhesion of this particular strain to IEC.

Another disadvantage of *in vivo* studies is that they are time consuming and expensive. The complications involved in studying bacterial adhesion *in vivo*, have led to the development of *in vitro* model systems for the initial selection of potentially adherent strains (Collado et al. 2005a). Savage (1983) examined bacterial adhesion using isolated epithelial surface intact in living animals and with mucosal cells or tissue removed from animals and maintained *in vitro*. Recently, various permanent intestinal cell lines like Caco-2 and HT-29, isolated extracellular matrix (ECM) proteins, Basement Membrane Matrigel (BMM) (Bouzaine et al. 2005) and the intestinal mucus have been used to study bacterial interaction with the epithelial cells. These methods have the drawback that the cell lines are of human origin (for example, the Caco-2 cell lines have been isolated from human colonic adenocarcinoma) and their use in animal studies is controversial. Also the level of adhesion can be enumerated either by microscopic visualisation, bacterial culture counts or by using metabolically radio labelled bacteria (Bouzaine et al. 2005). These methods cannot be considered of the highest accuracy. Vesterlund et al. (2005) compared different adhesion methods that have been commonly used: radioactive labelling fluorescence tagging and staining bacteria. Their results showed that radioactive labelling had the best reproducibility and sensitivity. Though, radiolabels are regarded as undesirable due to safety and cost concerns.

1.6.5 Hydrophobicity

The small surface area of bacteria makes short range interactions possible. The approach of two hydrophobic surfaces leads to the dislocation of water from the system, benefiting the interactions between the surfaces (Derjaguin, Landau, averwey, Overbeek (DLVO) Theory). So, when a potential probiotic is selected, hydrophobicity should be taken into consideration. The mutual attraction between the probiotic bacterium and the epithelial cell prevents its removal by the normal intestinal flow.

The higher affinities of lactobacilli for nonpolar solvents, such as xylene, suggest that these bacteria possess a hydrophobic rather than hydrophilic cellular surface (Chichlowski et al. 2007). Higher percentage of hydrophobic bacteria adhere to intestinal epitheilal cells than do hydrophilic strains (Chichlowski et al. 2007). Hydrophilic cell surface properties and strong affinities to chloroform shows the strains tested are strong electron donors. The presence of (glyco-) proteinaceous material at the surface results in higher hydrophobicity (Kos et al. 2003), whereas hydrophilic surfaces are associated with the presence of polysaccharides (Pelletier et al. 1997). Cell hydrophobicity in bacteria is attributed to pronase- and papsin-sensitive surface molecules.

There are several bacterial surface hydrophobicity assays, such as bacterial adherence to hydrocarbons, salt aggregation, hydrophobic interaction chromatography, adhesion to polystyrene and latex particle agglutination. The most commonly used "MATH" (Microbial adhesion to hydrocarbons) assay

(Rosenberg et al. 1980) is considerd as a simple, reliable, economical and well established assay used for studying the hydrophobic interactions of cells (Gusils et al. 2002a).

1.6.6 Homo/heterofermentation test

Lactic acid bacteria are separated into two groups: the homofermentative LAB, which convert sugars entirely, or almost entirely, to lactic acid, and heterofermentative LAB, which produce more than one major end product such as lactic acid, acetic acid, ethanol, mannitol and CO_2 from hexoses (Müller 1990). Homofermentation is important if LAB are to be used to ferment feed. It has been suggested that homofermentation rather than heterofermentation establishes a good pig feed (Mayrhuber et al. 1999). The production of metabolites such as acetic acid and carbon dioxide during the heterofermentation leads to malfermentation and as far as the pig is concerned the feed becomes unpalatable because of the pungent smell of acetic acid. Chickens have their olfactory organs located in the base of the upper jaw and not highly developed, so smell does not seem to affect the choice of the food. So, there is no evidence that homofermentation affects the palatability of the chicken feed and consequently the feed intake.

1.7 Problems of probiotic development and usage

There are some drawbacks of undefined competitive exclusion cultures, such as the fact that their unknown bacterial composition raises worries on the potential transmission of human or/and avian pathogens that may be present in

the source material from donor birds, and that the bacterial composition and the efficacy could not be standardised, that made the development of probiotic products of known bacterial composition a demand (Stavric and D'aoust 1993).

On the other hand developing probiotics based on defined cultures is problematic too. First of all, there is still lack of a sound scientific basis for the selection process of potential probiotic strains. Wet microbiology methods have proved to be inadequate and unreliable and on the other hand molecular methods are costly. Additionally, there is evidence that probiotic mixed cultures potency decrease during cold storage and repeated laboratory manipulation (Stavric and D'aoust 1993). Reliable diagnostic schemes for identification of strains for broad knowledge of avian microflora and its interaction with gastrointestinal tract function as well as the standardisation of the probiotic selection criteria could be useful for the development of probiotics.

Many consumers, animal producers, even scientists are sceptical about the positive effects of probiotics due to the differences in the results obtained in different studies. This variation in the probiotic effects may be attributed to the variation in the strains and forms of bacteria used and in their concentrations of dietary supplements.

The method of administration of probiotics raises concerns too. Oral gavage, although used in laboratory trials, is not convenient in field, while administration

of probiotics in the drinking water does not guarantee the intake of an adequate inoculum by all birds. The method of administration by fermented liquid feed to chickens is still developing and is faced with discredit. European Union legislation controls the technology of fermented liquid feed on commercial units, as organisms used for fermentation have to be registered. This might be a risk for consumers and producers to benefit from the potential positive effects of using lactic acid bacteria and fermented feed (Brooks et al. 2003b).

Liquid feeding raises one more problem, as the wet feed stays in the trough for a long time, spoilage bacteria and molds can accumulate, which if consumed might be risky for animal health. Addition of >300 ppm of chlorine dioxide, a strong oxidising and sanitising agent with broad antimicrobial spectrum, could help overcoming this problem (Demečková et al. 2001).

1.8 Rationale for the study

Increasing scientific awareness of the role of some intestinal bacteria in promoting health and improving production has increased the use of probiotic bacteria as active functional ingredients in animal and human nutrition. The probiotic properties of lactic acid bacteria have been widely studied, demonstrating that when delivered to poultry, they can colonise the gastrointestinal tract and exert their probiotic effect (Fuller 1989; Jin et al. 1997; Mahajan and Sahoo 1998; Ghadban 2002; Edens 2003; Patterson and Burkholder 2003; Apata 2008). Delivery of probiotic strains of lactic acid bacteria (LAB) to poultry may be mediated by addition to either the water or the

feed. Added benefit may be obtained if the feed is allowed to ferment to produce a feed containing at least 150 Mmol lactic acid and a low pH <4.5 and high numbers of LAB 10^9 CFU ml^{-1}, as this has been shown to reduce contamination of feed by enteropathogens such as Salmonellae (Heres et al. 2003a; Heres et al. 2003c; Heres 2004; Heres et al. 2004b).

Previous work at our laboratory screening porcine derived LAB has resulted in the identification of suitable LAB starter cultures for fermentation of liquid feed (Moran 2001; Demečková 2003). This study aimed to discover whether the same approach has benefits in chickens too.

The specific objectives of this study were

- to isolate, characterise and select beneficial *Lactobacillus* strains in an attempt to predict candidates that could be used *in vivo* as chicken probiotic adjuncts and additionally to control fermentation

- to investigate whether the selected *Lactobacillus* strain, supplied to chickens either in water or fermented liquid feed, has a beneficial effect *in vivo* against *Salmonella* contamination

- to examine whether the potential positive effect of fermenting moist feed with a selected *Lactobacillus* strain is due to the strain itself, or due to the lactic acid produced during fermentation

- to determine the *in vivo* effect of the selected *Lactobacillus* strain on microbial, immunological and histological parameters in the chicken.

Chapter 2

Selection, characterisation and screening of lactic acid bacteria of chicken origin that have probiotic properties

2.1 Introduction

Many researchers have attempted to select probiotic strains from animals (O'Sullivan 2001; Mojgani et al. 2007) and plants (Chiu et al. 2008) for use in animals, or humans (Jacobsen et al. 1999). Selection and screening of *Lactobacillus* strains is widespread, as they are commonly known for their probiotic properties (Nemcova 1997; Garriga et al. 1998; Gusils et al. 1999a; Gusils et al. 1999b; Ehrmann et al. 2002; Gusils et al. 2002a; Gusils et al. 2002b; Van Coillie et al. 2007).

Previous work in our laboratory, screening porcine derived LAB, has identified LAB suitable for use as starter cultures for fermentation of liquid feed (Moran 2001; Demečková 2003). The aim of the current study was to apply the same approach in chickens. The screening method described in this chapter was based on the screening programmes described by Moran (2001) and Demečková (2003), which was adjusted to take account of the gastrointestinal conditions in poultry.

The screening programme described in this chapter started with the aim of finding a potential bacterial strain that is non-toxic, non pathogenic and of chicken origin, to ensure colonisation of the chicken GI tract. Although several authors (Hautefort and Hinton 2000; Bezkorovainy 2001) did not consider species and location specificity an essential selection characteristic, viability and biological activity within the acidic chicken GI tract was included as a selection criteria in the present screening programme. Adhesion to mucus and epithelial cells was considered a prerequisite for the selection of a probiotic strain.

In vitro methods are commonly used for screening potential probiotic strains because they are inexpensive and more rapid than *in vivo* methods. However, given the complexity of the chicken GI tract, the proof of efficacy of the probiotic bacteria in broilers requires *in vivo* studies. The aim of this study was the selection, characterisation and screening of lactic acid bacteria of chicken origin that have probiotic properties. *In vitro* methods for screening potential probiotic strains were used in an attempt to reproduce a dynamic model that mimicked *in vivo* chicken GI conditions as closely as possible.

2.2 Procedure and discussion

The steps involved in the screening process that were followed, are presented schematically in Figure 1.

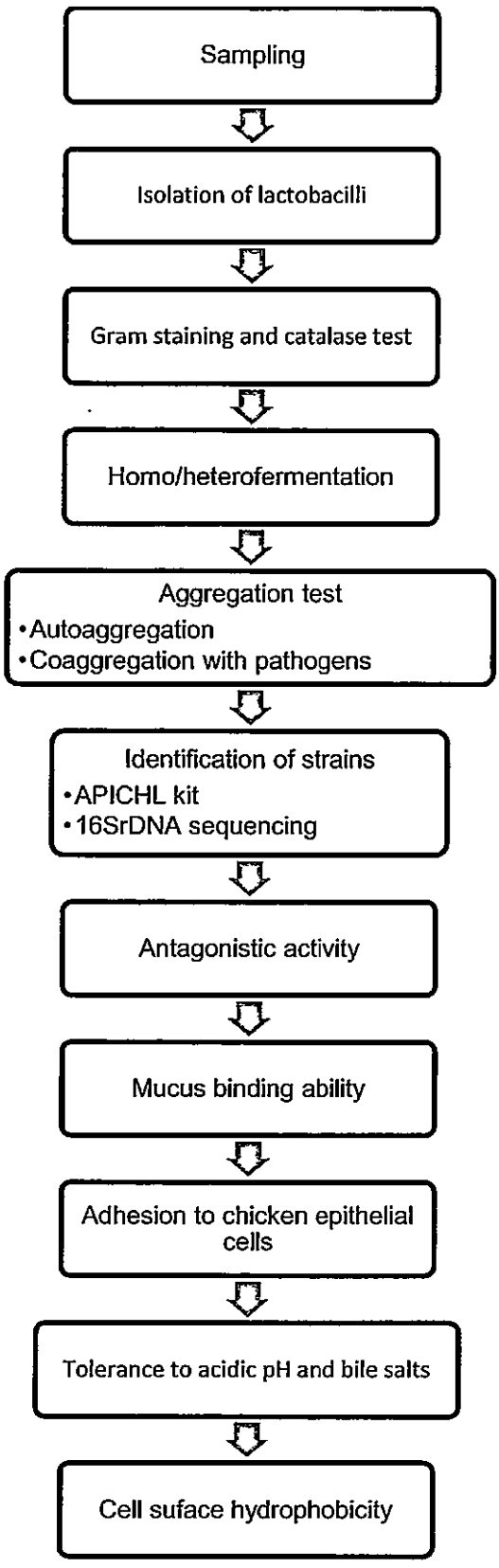


Figure 1. Flow chart of the screening and selection process

The number of organisms used and progressing from each stage during the screening process is shown in Table 4.

Table 4. The number of strains subjected to each test during the screening process and the number of isolates processed to the next stage.

Procedure	Strains	Organisms to the next stage
Isolation of lactobacilli	111	111
Gram staining	111	109 positive rods
Catalase test	111	111
		Fridge damage - 58 survived and had good growth
Autoaggregation test	58	25 good and normal activity
Coaggregation test with pathogens	25	19 good aggregation with pathogens
Identification (API CHL kit)	19	2 representative from each species, 8 in total
Antagonistic activity	8 (Nos 3, 6, 10,15,16,18, 22, 24)	All
Mucus binding assay	8 (Nos 3, 6, 10,15,16,18, 22, 24)	All
Adhesion to epithelial cells	8 (Nos 3, 6, 10,15,16,18, 22, 24)	5 representative from each species with best antagonistic activity
Tolerance to acidic pH	5 (Nos 3, 6, 10, 15, 16)	
		2 with the better aggregation, antagonistic ability against pathogens and adhesion to epithelial cells capacity
Cells surface hydophobicity	2 (Nos 3 and 16)	
		25 with good and normal and 16 with weak and non-aggregating ability
Fermentation test	41	
Identification (16S rDNA sequencing)	4 (Nos 3, 6, 16, 18)	

Chicken derived bacteria were obtained from three outdoor reared chickens, 9 weeks and 2 days old, of the Hubbard breed, obtained from a local organic farm. The chickens were fed *ad libitum* on an organic grower diet plus grass and clover. The chickens were humanly slaughtered and their entire GI tracts were removed and transported to the laboratory in ice, immediately after

slaughtering. Contents from the caecum, jejunum, ileum and crop were removed aseptically, and small intestine and crop epithelial cells were taken by scraping the epithelium with a slide. All samples were diluted in 10 ml phosphate-buffered saline (PBS) (Oxoid, England) and plated in de Man Rogosa and Sharpe (MRS, Oxoid, England) and Rogosa (Oxoid, England) agar. The streak plate method of isolation was used to obtain pure cultures from the mixed culture of LAB. After selection, the pure colonies were subcultured for a 2nd time on MRS agar plates and incubated in anaerobic jars (to prevent any oxygen from coming into contact with samples of cultures) at 37 °C, in 5% CO_2 atmosphere, for 72 h.

MRS and Rogosa agar have been used for the isolation and enumeration of the intestinal lactobacilli. According to the Oxoid technical information sheet, MRS agar and broth contain peptone and dextrose, which supply nitrogen, carbon and other elements necessary for growth. Other ingredients of the these media such as polysorbate 80, acetate, magnesium and manganese provide growth factors for culturing a variety of lactobacilli and may also inhibit the growth of some organisms other than lactobacilli. Rogosa agar has high acetate concentration and low pH value which suppresses the growth of other organisms than lactobacilli. It also contains low concentrations of manganese, magnesium and iron which ensure optimal growth of lactobacilli (Oxoid technical information sheet).

2.2.1 Isolation of lactobacilli, bacterial growth and acid and gas production of selected lactobacilli

One hundred and eleven lactic acid bacteria were isolated on MRS (isolation medium for *Lactobacillus* and *Pediococcus* strains) and Rogosa agar (lactobacilli) and maintained at -80 °C in 1ml aliquots of MRS broth (Oxoid, England) with 20% v/v glycerol. Stock cultures were reactivated by subculture in MRS broth and incubation at 37 °C, for 24 h, before their further experimental use. Lactobacilli cultures were maintained in a fridge (4 °C) and were subcultured on MRS agar plates once every week to retain viability, maintaining them in the fridge again. The gram stain and the catalase test were used to confirm that the organisms were lactobacilli (gram positive and catalase negative rods). The presence of the catalase enzyme was detected using hydrogen peroxide. When a small amount of of catalase positive bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed.

In total, 111 of the isolated lactic acid bacteria were found to be gram positive rods (106) and cocci (2 of them) and were kept for further screening. All the organisms showed negative results in the catalase test. Eight bacteria showed poor growth, seven in MRS agar and one in Rogosa agar (Oxoid, England) and they were rejected from further screening.

Some of the lactobacilli (48) died accidentally during storage and 8 showed poor growth and they were not used further.

2.2.2 Aggregation and coaggregation

The remaining fifty eight lactobacilli were tested for aggregating ability, and 25 that showed good and normal aggregating ability were tested for coaggregating ability with pathogens. *Salmonella enterica enteritidis* (NCTC 5188) obtained from the National Collection of Type Cultures, Central Public Health Laboratory, and *Clostridium perfringens* (NCIBM 8693) obtained from the University of Plymouth stock cultures, were used in the coaggregation tests.

Prior to use, the strains were subcultured twice in nutrient broth at 37 °C, for 24 hours and Clostridial broth (Oxoid, UK) at 37 °C for 24 hours, under anaerobic conditions, respectively.

Aggregation ability was assessed as follows. *Lactobacillus* strains were grown overnight in MRS broth at 37 °C, in 5% CO_2 atmosphere in glass tubes. The next day the cultures were centrifuged for 10 min at 10000xg and washed three times with sterile distilled water. They were then resuspended in the same initial volume of phosphate-buffered saline (PBS) at a concentration of 10^9 CFU ml^{-1} (pH 6.0) and incubated, at room temperature, in the presence of 10% (v/v) freshly prepared filter-sterilised culture of their own *Lactobacillus* supernatant fluid. The total volume of the aggregation mixture was 1ml. Autoaggregation was considered as positive when clearly visible, sand-like particles (formed by the aggregated cells), gravitated to the bottom of the tubes, leaving a clear supernatant fluid, within 2h. The reaction time was recorded for the measurement of aggregation intensity; and classified as rapid: 15min (+++),

particles, (formed by the aggregated cells) gravitated to the bottom of the tubes, leaving a clear supernatant fluid, within 2h. The reaction time was recorded for the measurement of aggregation intensity: and classified as maximum (4) <15min, marked (3): 15-30min, good (2): 30-60min, partial (1): 60-90min, almost or no (0): > 90min (Kmet and Lucchini 1997; Kmet and Lucchini 1999).

Coaggregation was also assessed using the method of Drago et. al. (1997). *Salmonella spp.,* and *Esch. coli* were grown at 37 °C in nutrient and clostridial broth respectively, for 24h, in 5% CO_2 atmosphere, and under anaerobic conditions, respectively, and *Lactobacilli* were grown at 37 °C, in MRS broth, for 24h, in 5% CO_2 atmosphere. 500µl of each *Lactobacillus* suspension was mixed with 500 µl of each pathogenic bacterium suspension for at least 10 sec on a vortex mixer and then incubated in 24-well microtrays (Corning, Italy) at 37 °C, under agitation (Drago et al. 1997). After 4 hours the suspensions were prepared for observation by scanning electron microscope.

Some lactobacilli were further tested for their ability to autoaggregate with *Salm. enteritidis* of chicken origin (PH Exeter, I.Ap. 29), *Salm.* Typhimurium (AFCC 14028) and *Esch. coli* (obtained from the University of Plymouth stock cultures).

After the coaggregation experiment a loop from each suspension was streak plated on nutrient agar to determine whether the pathogens were still alive.

Suspensions from the autoaggregation and coaggregation tests were prepared for observation by scanning electron microscope, as follows. Ten µl of each *Lactobacillus* suspension were placed onto a slide (Thermanox, Nunc, Inc), air dried, fixed with 2.5% glutaraldeyde for 2h at room temperature, washed three times in 0.1 % (w/v) sodium cacodylate buffer pH 7. Samples were dehydrated through a graded series of ethanol and acetone mixtures, critical point dried at 35 °C and 1250psi for 15min, coated with gold and observed by SEM, under high vacuum with a JEOL 5600 Low Vacuum SEM. Six randomized fields were evaluated in each sample.

Of 58 lactobacilli that were tested for their capacity to aggregate, 23 were found to be non-aggregative. Twelve bacteria showed rapid autoaggregation (within 15 minutes), thirteen lactobacilli had a normal reaction and the remaining strains showed weak autoaggregation activity. Scanning electron micrographs (Figure 3 and 4) show examples of autoaggregation of *Lactobacillus* strains and coaggregation with *Salm. enteritidis.*

The 25 lactobacilli that showed normal and rapid aggregative activity were further tested for their ability to aggregate with *Salm. enteritidis and Cl. perfringens*. One *Lactobacillus* strain showed maximum aggregation (scored as 4) with *Salm. enteritidis* , two showed marked aggregation (3), six showed good aggregation (2), nine partial aggregation (1) and three showed no, or almost no aggregation (0). Results were confirmed by scanning electron microscopy so that autoaggregates were excluded from coaggregation scores (Drago et al. 1997). Results of coaggregation of *S. enteritidis* of chicken origin with *S. typhhimurium, Esch. coli* and *C. perfringens* are presented in the Table 5. The microscopic appearance of the aggregates formed between aggregating lactobacilli alone (autoaggregation) and aggregating lactobacilli with some of the pathogens that were tested (coaggregation) are illustrated in Figure 4 and 5. No interaction was found between the origin of the LAB, the intensity of autoaggregation and the ability to coaggregate with pathogens. The pathogens survived after the coaaggregation test.

There was no evidence of an interaction between the origin of the organisms, the intensity of autoaggregation and coaggregation with *Salm. enteritidis*. However, it could be mentioned that the three LAB with maximum and marked aggregation with *Salm. enteritidis* bacteria were obtained from the contents of chicken small intestine. The *Lactobacillus* strain No 3 was found to have a rapid autoaggregation activity and a maximum ability to aggregate with *Salm. enteritidis* and, on this basis, it was selected for further screening for its potential use as a probiotic in chicken nutrition.

Table 5. Identification, autoaggregation of lactobacilli, and their coaggregation with several pathogens.

Lactobacilli	Origin	Identification		Autoaggregation	Coaggregation with				
		API 50 CHL (% identity)	Sequencing of 16S rDNA gene (% identity)		*Salmonella enteritidis (5188)*	*Salmonella enteritidis* (chicken)	*Salmonella* Typhimurium	*Escherichia coli*	*Clostridium perfringens*
No 7	caecum, contents	-		+++	1	-	-	-	1
No 22	caecum, contents	*Lactobacillus fermentum* (98.3)		++I	3	-	-	-	2
No 23	caecum, contents	-		++	0	-	-	-	-
No 4	caecum, contents	*Lactobacillus salivarius* (99.9)		++	0	-	-	-	1
No 11	small intestine, scrape	-		+++	1	-	-	-	2
No 9	small intestine, scrape	*Lactobacillus plantarum* (99.9)		++	1	-	-	-	3
No 6	small intestine, scrape	*Lactobacillus salivarius* (98.8)	*Lactobacillus salivarius* Salivarius *(99.96)*	+++	2	2	2	3	2
No 10	small intestine, scrape	*Leuconostoc lactis* (99.3)		+++	2	2	1	1	2
No 20	small intestine, scrape	*Lactobacillus salivarius* (99.2)		++	1	-	-	-	-
No 13	small intestine, scrape	*Lactobacillus salivarius* (83.0)		+++	1	-	-	-	1
No 16	small intestine, scape	*Lactobacillus salivarius* (99.9)	*Lactobacillus salivarius* Salivarius *(100.0)*	++	2	2	2	3	2
No 3	small intestine, scape	*Lactobacillus plantarum* (99.9)	*Lactobacillus salivarius* Salivarius *(9.97)*	+++	4	3	3	4	3

Lactobacilli	Origin	Identification		Autoaggregation	Coaggregation with				
		API 50 CHL (% identity)	Sequencing of 16S rDNA gene (% identity)		*Salmonella enteritidis (5188)*	*Salmonella enteritidis* (chicken)	*Salmonella* Typhimurium	*Escherichia coli*	*Clostridium perfringens*
No 17	ileum, contents	*Lactobacillus salivarius* (99.2)		+++	1	-	-	-	-
No 5	ileum, contents	*Lactobacillus salivarius* (99.2)		++	1	-	-	-	-
No 18	ileum, contents	*Lactobacillus plantarum* (99.9)	*Lactobacillus plantarum (100.0)*	+++	2	-	-	-	1
No 12	jejunum, contents	*Leuconostoc lactis* (99.5)		+++	1	-	-	-	2
No 1	jejunum, contents	*Lactobacillus salivarius* (98.8)		+++	2	-	-	-	2
No 8	jejunum, contents	*Lactobacillus salivarius* (99.2)		++	2	2	-	-	1
No 15	crop content	*Lactobacillus fermentum* (99.0)		++	2	1	2	1	1
No 21	crop, scrape	*Lactobacillus plantarum* (99.9)		++	0	-	-	-	3
No 24	crop, scrape	*Lactobacillus fermentum* (97.3)		++	3	-	-	-	2
No 14	crop, scrape	-		+++	2	-	-	-	-
No 2	crop, scrape	*Lactobacillus salivarius* (99.2)		++	1	-	-	-	-

[a] The score is based upon (Kmet and Lucchini 1997; Kmet and Lucchini 1999) methodology.
b The score is based upon (Drago et al. 1997) methodology

Aggregation of LAB was examined as part of the screening process because there is some evidence that it may be necessary for adhesion to intestinal epithelial cells and, with the addition of coaggegation ability, may provide them with a competitive advantage in the intestinal microbiota (Ghadban 2002) and can help them forming a barrier that prevents colonization of pathogenic microorganisms (Kos et al. 2003). Boris et al. (1998) claims that the autoaggregation ability of lactobacilli can increase their adhesion to intestinal epithelial cells in environments with short residence time and coaggregation may speed up the passage of pathogenic *Esch. coli* through the piglet's gut by the formation of *Lactobacillus spp.–Esch. coli* aggregates (Kmet and Lucchini 1999). The aggregation ability of the lactobacilli may be attributed to 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 (Kmet and Lucchini 1999; Styriak et al. 2001). However for this experiment the selected LAB were not assesd for production of the APF protein. Strong autoaggregation ability is not necessarily correlated with strong co-aggregation ability. For example, Ehrman et al. (2002) reported several lactobacilli (*Lact. agilis* TMW1.964 and two strains of *Lact. reuteri,* TMW1.966 and TMW1.967), which, despite having a strong autoaggregation ability, did not coaggregate with the pathogens tested. In the current study, only the strains that showed some or high autoaggregation capacity were tested for their potential coaggregation ability.

All the bacteria were cultured under the same conditions and using the same culture media because the method of culture may affect bacterial aggregation (Spencer and Chesson 1994). Resuspending each strain on their own culture supernatant fluid helped to prevent the possibility of removing extracellular components, which might influence autoaggregation.

2.2.3 Identification of the selected strains

Lactobacillus strains were identified using API CHL kit (BioMéreux, UK), which is based on the principle of differential carbohydrate metabolism of the microorganisms tested, following the manufacturer's instructions. The results were analysed with the appropriate identification software, apiweb® (BioMéreux, UK).

The 23 lactobacilli that were selected to be used for the coaggregation experiment were identified using the API identification kit. The results of the identification of the lactobacilli and the percentage reliability of the identification are presented in Table 5. The *Lactobacillus* strain that showed rapid autoaggregation and a maximum coaggregation with most of the pathogens was identified as *Lact. plantarum*. Of the 24 lactobacilli that were selected for identification, two were identified as *Leuconostoc lactis*, four as *Lact. plantarum*, three as *Lact. fermentum* and ten as *Lact. salivarius*. Two had an unacceptable profile and two had a doubtful profile.

The NCIMB identification report was in accordance with the API identification kit for three of the strains that have been subjected only for a basic identification using the first 500bp of the 16S rRNA gene (Table 6).

One strain No 3 that had the full 16S rRNA gene sequence identification performed, was identified as *Lactobacillus salivarius* Salivarius (99.97%), contradicting the results obtained from the API identification kit, which had identified the organism as *Lactobacillus plantarum* (99%).

Table 6. 16S rRNA gene sequence identification and National Collection Number of *Lactobacillus* Nos 3, 6, 16 and 18

Microorganism	16S rRNA gene sequence identification	National collection number (NCIMB)	% identification
No 3	*Lactobacillus salivarius* Salivarius	41606	99.97
No 6	*Lactobacillus salivarius* Salivarius	41609	99.96
No 16	*Lactobacillus salivarius* Salivarius	41610	100.00
No 18	*Lactobacillus plantarum*	41607	100.00

Commercial diagnostics have been widely used in bacteriology laboratories to identify common organisms, such as *Streptococcus*, to the species level, or to identify unusual gram-positive organisms in clinical specimens, e.g. *Aerococcus, Lactobacillus*, as they are considered to be a simple and inexpensive method of identifying bacterial species. However, studies have illustrated inaccuracies in the Analytical Profile Index system (API 20 STREP and API 50 CHL, Biomérieux, Inc., Lyon, France) and in bacterial identification (Kulwichit et al. 2007). Kulwichit et al. (2007) also reported inaccurate identification of *Leuconostoc* at the genus level using the API identification kits,

suggesting that for accuracy and reliability, conventional phenotypic assays, such as the API identification kits should be performed with the assistance of a genotypic confirmation where available.

2.2.4 Antagonistic activity

A representative number of each species, i.e Nos 3 and 18 identified as *Lact. plantarum*, Nos 6 and 16 identified as *Lact. salivarius*, Nos 15 and 24 identified as *Lact. fermentum* and Nos 10 and 22 identified as *Leuconostoc lactis*, using API CHL kit, that showed good aggregation and coaggregation ability, were tested for their antagonistic activity against several enteropathogens.

The eight lactobacillus isolates (Nos 3, 18, 6, 16, 24, 15, 22, 10) were tested for their antagonistic activity against several pathogens according to the method of Olnood et al. (2007). The *Lactobacillus* isolates were grown in MRS broth, at 39 °C, for 24h, under anaerobic conditions. After 24h incubation, each of the *Lactobacillus* cultures was streak plated on Wilkins-Chalgren anaerobic agar (Oxoid, CM0619), using a sterile cotton swab and incubated at 39 °C, for 24h, under anaerobic conditions. The same day, 5 pathogenic bacteria were grown in thioglycollate broth (Oxoid, CM0391) (Table 7). After 24h of incubation, pathogenic suspensions were streak plated, using a sterile swab, on the plates that *Lactobacillus* cultures were cultured, in a way to form a cross with the *Lactobacillus* streaks. The presence or not of an inhibition zone around the cross formatted by each *Lactobacillus* and pathogen streak was recorded after incubation.

All the strains were found to inhibit the pathogens, so, their antagonistic activity was quantified by the agar spot test described by Jin et al. (Figure 5). Cultures of the selected strains to be tested for their antagonistic activity against 5 pathogens were grown in MRS broth and incubated at 37 °C, under anaerobic conditions, for 24h. The overnight cultures were spotted onto the surface of MRS agar plates and incubated anaerobically, for 24h, at 37 °C, to allow colonies to develop. Approximately 10^7 CFU ml^{-1} of each pathogenic bacterium, in 15ml of nutrient agar, were poured on the same plate that each *Lactobacillus spp.* was grown on and incubated for 24h, at 37 °C. The plates containing *Cl. perfringens,* were grown under anaerobic conditions, at 39 °C, for 48h. After the incubation the plates were checked for inhibition zones around the lactobacilli spot and the radius of inhibition zone was recorded. The test of each *Lactobacillus* strain against each of the five pathogens was carried out three times with duplicates each time.

All the lactobacilli tested were able to inhibit the growth of *Salm. enteritidis* (5188), *Salm. enteritidis* of chicken origin, *Salm.* Typhimurium, *Esch. coli* and *Cl. perfringens* to varying extent. The results are shown in Table 8.

No 16 that have been isolated from small intestine had the best inhibitory ability against the Gram negative and Gram positive pathogenic bacteria that have been tested.

Table 8. Antagonistic activity of *Lactobacillus* spp. isolated form chickens against *Salmonella, Esch.coli* and *Cl. perfringens**

	Radius of inhibition zones (cm)						
Lactobacillus spp. isolates	*S. enteritidis* 5188	*S. enteritidis* (PH Exeter) E.Ap.29 (human)	*S. enteritidis* (PH Exeter) I.Ap.29 (chicken)	S. Typhimurium AFCC 14028	*Esch. coli*	*Cl. perfringens* NCIB 8693	Mean
No 3	1.67±0.06	1.57±0.12	1.57±0.12	1.53±0.15	1.37±0.06	1.93±0.15	1.61^{ab}
No 6	1.43±0.06	1.60±0.10	1.63±0.06	1.43±0.06	1.37±0.06	1.73±0.21	1.53^{cd}
No 10	1.60±0.00	1.50±0.00	1.53±0.12	1.43±0.12	1.27±0.06	1.70±0.00	1.51^{ef}
No 15	1.50±0.10	1.63±0.15	1.50±0.00	1.47±0.06	1.50±0.00	1.80±0.17	1.57^{gh}
No 16	1.60±0.00	1.53±0.12	1.57±0.12	1.47±0.06	1.33±0.06	1.93±0.25	1.57^{ij}
No 18	1.50±0.10	1.60±0.00	1.67±0.06	1.67±0.06	1.33±0.06	1.70±0.00	1.58^{kl}
No 22	0.97±0.50	1.00±0.00	0.83±0.12	0.93±0.06	0.77±0.06	1.37±0.12	0.98^{acegik}
No 24	0.93±0.12	1.10±0.10	0.93±0.12	0.90±0.10	0.87±0.12	1.27±0.21	1.00^{bdfhjl}
Mean	1.40^{AF}	1.40^{BG}	1.44^{CH}	1.35^{DI}	1.23^{EFGHI}	1.68^{ABCDE}	

*Each value is the mean of the triplicate of each sample. Same superscripts show significant ($P<0.05$) different values

Jin et al. (1996a) reported on twelve *Lactobacillus* strains isolated from chicken intestine that had inhibitory activity against five strains of *Salmonella* and three serotypes of *Esch. coli*. The nine lactobacilli tested in their experiment were found to inhibit the growth of 3 strains of *Salm. enteritidis*, one strain of *Salm.* Typhimurium, *Esch. coli* and *Cl. perfringens in vitro*. The mechanism of the antagonistic activity was not examined in this study. Lactic acid bacteria are generally known to produce antibacterial substances, which have been shown

to be inhibitory to poultry pathogens. Such antimicrobial substances include bacteriocins (acidolin, bulgaricin, nisin, reuterin), bacteriocin-like substances (Mahajan and Sahoo 1998), organic acids and hydrogen peroxide (Fuller 1989; Jin et al. 1997; Mahajan and Sahoo 1998; Ghadban 2002; Curbelo et al. 2005).

There are some consideration is regards to the validity of the method. It could be suggested that competition of nutrients rather than inhibition of pathogens is evaluated using the agar spot test. However, each of the lactobacilli and pathogens that used are grown in different culture media, i.e lactobacilli in MRS agar and pathogens in nutrient agar, so the competition of nutrients between them might be limited. Also, an indicator *Lactobacillus* strain that has been proved earlier that inhibits the growth of the pathogenic bacteria could be used as control for the comparison of the inhibitory effect of the lactobacilli tested. However, the aim this test was to compare the *Lactobacillus* strains between them and exclude those that performed worse than the others from further screening.

2.2.5 Mucus binding assay

Eight lactobacillus isolates (Nos 3, 18, 6, 16, 24, 15, 22, 10) that showed good aggregation and coaggregation ability were assessed for mucus binding ability. The method used for assessing mucus binding of LAB, was described by (Demečová 2003). In summary, Nunc-Immuno microtitre 96 – well plates with Maxi Sorp surface (Invitrogen, UK) were coated with 100µl mucin from porcine stomach Type II solution (Sigma, UK) at a concentration of 1000 µg mucin

proteins per ml and incubated at 4 °C, for 24h. Mucin solutions were removed and the plates were washed three times with PBS. Bacterial suspensions (100µl; 10^9 CFU ml^{-1}) of individual LAB strains were added and the plates were incubated on an orbital platform shaker for 2 h, at 37 °C. The unbound bacteria were removed by washing the wells three times with PBS. Bacteria in the wells were fixed at 60 °C, for 20 min and stained with crystal violet (95 ml per well) for 45 minutes. Then the wells were washed six times with PBS to remove excess stain. This was followed by addition the of 100µl of citrate buffer (pH 4.3) to each well and 45 min incubation at room temperature to release the stain bound to bacteria. The absorbance values (A550nm) were determined by an ELISA reader and the average of 10 absorbance values was calculated. Each batch of assays also included blank wells (only mucin and PBS without bacteria) and *Lact. reuteri* (NCIB 11951) as a positive control (Aleljung, 1994). Lactobacilli were classified as strongly adherent (A550nm> 0.3), weakly adherent (<0.1<A550nm> 0.3), or nonadherent <0.1<A550nm (Styriak et al. 1999)

Eight of eleven chicken-derived lactobacilli tested, were found to be non adherent to porcine mucus and two (Nos 18 and 10) were found to be weakly adherent. This might suggest that there is a species specificity of some of the chicken derived lactobacilli tested. *Lact. reuteri* (NCIB 11951) used as a positive control (Aleljung et al. 1994), but did not adhere to porcine mucin (Figure 6), which raises concerns on the validity of the test. Also, one more control well may be necessary, containing bacteria but no mucin, to check if the bacteria were attached to the plates without the need for mucin.

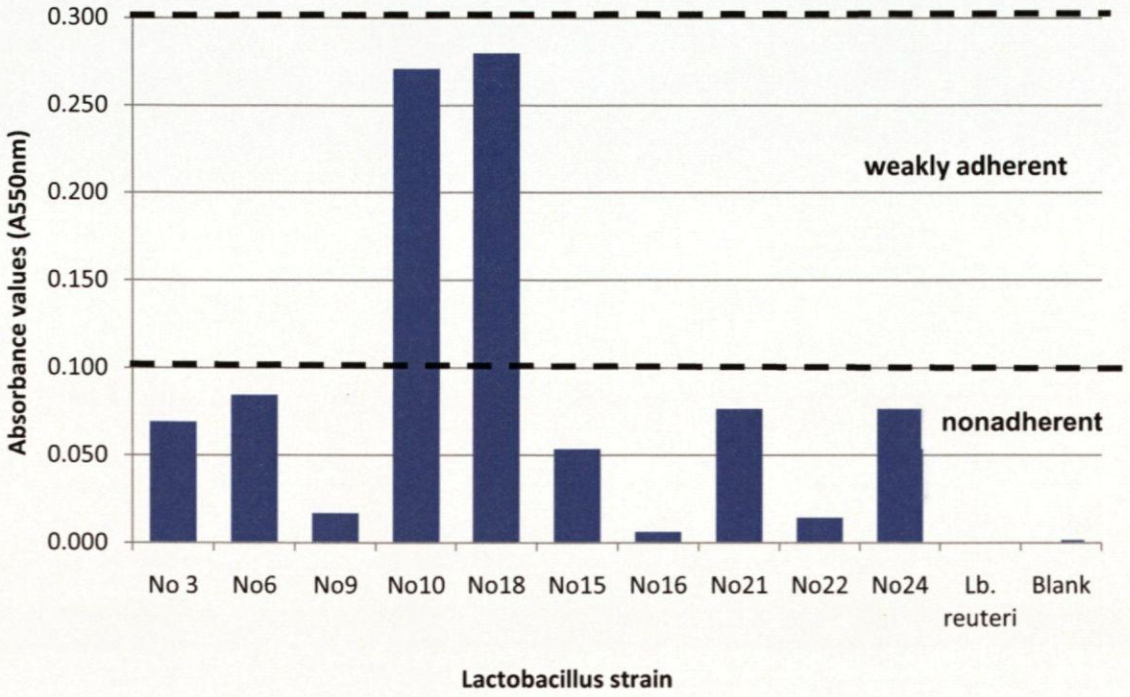


Figure 6. The level of mucus binding of 9 *Lactobacillus* strains.

2.2.6 Adhesion of LAB to chicken intestinal epithelial cells

Eight lactobacillus isolates (Nos 3, 18, 6, 16, 24, 15, 22, 10) that showed good aggregation and coaggregation ability were assessed for their ability to adhere to chicken gut epithelial cells *in vitro*, using the methodology first described by Fuller (1973) and used later by Garriga et al. (1998).

Organically farmed chickens were humanly slaughtered and the entire GI tract was removed and transferred to the laboratory in ice. Gut contents were removed aseptically and ileal segments were opened, washed with PBS and held in PBS (pH 7.2), at 4 °C, for half an hour, to loosen the surface mucus. The ileal epithelial cells were collected by gently scraping the epithelium with the edge of a microscope slide and scrapings were placed in sterile universal bottle

samplers and suspended in PBS. The suspended scrapings were left in ice for 15min to allow the large debris to settle. The debris that gravitated to the bottom was removed and the supernatant fluid centrifuged for 1min, at 500 rpm, to remove large tissue clusters and was further centrifuged, for 5mins, at 500 rpm, to spin down the suspended cells. The pellet containing ileal cells was washed twice with PBS and suspended in PBS. The cells were examined microscopically to ensure that they were free from any adherent bacteria. The number of cells present was determined using a hemocytometer. Cells were stained with Trypan Blue to identify dead cells. The number of live cells in the suspension was found to be 10^6 ml^{-1}.

Overnight cultures of the selected lactobacilli in MRS broth (10^9 CFU ml^{-1}) were resuspended in PBS to give a cell density of 10^8 CFU ml^{-1}. One hundred µl of each selected *Lactobacillus* suspension was added to 400 µl of the epithelial cell suspension and the mixture was further incubated for 30min, at 37 °C, in a shaking water bath (20rpm). The resuspended mixtures were stained with Gram stain and the number of bacteria adhering to an epithelial cell was determined by light phase contrast microscope. Duplicates of the samples were first fixed with methanol, which is supposed to be the superior method of fixation, even though flame fixing is used more frequently. When organisms are fixed with methanol, are more resistant to decolorisation. Ten epithelial cells were selected randomly and the mean number of bacteria attached per epithelial cell was calculated. Chains or pairs of bacterial cells were counted as one unit. The adhesion efficiency of the lactic acid bacteria to intestinal epithelial cells was

scored 1 to 3 which represent 1=no, 2=weak and 3=strong adherent efficiency, respectively, with a subjective assessment.

Scanning electron microscopy pictures of each *Lactobacillus*-epithelial cells suspension were taken to confirm the adhesion of the lactic acid bacteria to intestinal epithelial cells. A ten µl sample of each *Lactobacillus*-epithelial cell suspension was placed on a slide (Thermanox, Nunc, Inc), air dried, fixed with 2.5% glutaraldeyde for 2h at room temperature and washed three times in 0.1 % (w/v) sodium cacodylate buffer (pH 7). Samples were dehydrated through a graded series of ethanol and acetone mixtures, critical point dried, at 35 °C and 1250psi for 15min, coated with gold and observed by SEM, under high vacuum, using a JEOL 5600 Low Vacuum SEM.

The adhesion of 8 lactobacilli to chicken epithelial cells is summarised in Table 9. The adhesion of lactobacilli Nos 3 and 16 to chicken epithelial cells is illustrated in Figure 7 and 8. It was apparent from both the scanning electron micrographs and the light microscopy photographs that both lactic acid bacteria adhere to chicken epithelial cells.

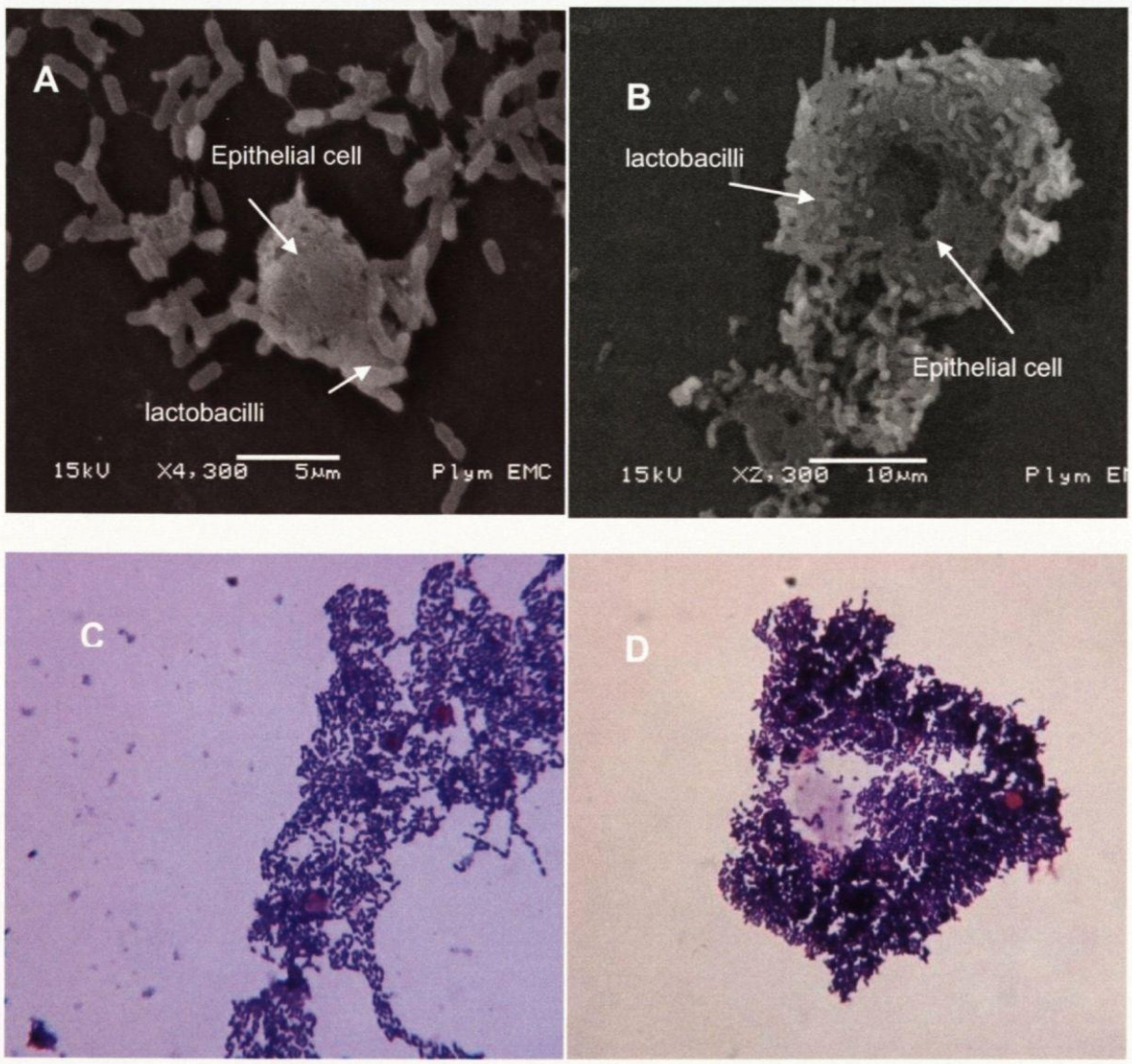


Figure 8. A. Adhesion of *Lact.* Salivarius (No16) and B. *Lact. plantarum* (No18) to chicken epithelial cells (Scanning electron microscopy -SEM). C. and D. Adhesion of *Lact. plantarum* (No18) (–blue colour) to chicken epithelial cell (–red colour) (Light microscopy –LM, 400x magnification)

In this study, no correlation was observed between the origin of the lactobacilli (i.e. from different parts of the intestine) and their ability to attach to the intestinal epithelial cells. Jin et al. (1996c) presented results of their study showing that lactobacilli isolated from the ileum had a higher affinity than those from the caecum, though those isolated from the jejunum were found to be unable to adhere to ileal epithelial cells. In addition to these results, one more

study of Barrow et al. (1980) found that *Lact. fermentum* isolated from the stomach of a pig adhered strongly to the squamus epithelial cells from the oesophagus and the stomach of the pig, but not to the columnar epithelial cells of the small and large intestine. This suggests that there is a bacterial specificity to certain intestinal locations. Moreover, Ehrmann (2002) observed that adhesion is based on strain-specific attributes rather than on taxonomic grouping.

The complications involved in studying bacterial adhesion *in vivo*, have led to the development of *in vitro* model systems for the initial selection of potentially adherent strains (Collado et al. 2005a). Therefore, for the present experiment, *in vitro* tests were used to examine which of the selection criteria were met and finally to reduce the number of strains and find the most efficient organism to be used *in vivo*. Epithelial cells of the gastrointestinal track of chickens were obtained and used according to (Jin et al. 1996c), as a simple and low cost method of assessing the adhesion ability of the isolated potential probiotic bacteria and also taking into consideration the fact that adherence of lactobacilli is affected by host specificity (Nemcova 1997). However, this method has all the disadvantages of conventional methods, since the enumeration of the adherent bacteria are based on microscopy counting. Also, it can be mentioned that enumeration by microscopy after staining of the sample is laborious and requires that bacteria remain detectable after fixation and Gram staining, which might reduce accuracy. It also requires counting of many fields, which may also be prone to observer error. On the other hand enumeration by plating is even

less accurate as it requires selective media, and requires that the bacteria are released first and after that also remain viable and culturable (Vesterlund et al. 2005).

For the present experiment, the temperature chosen for the incubation of the strains and the epithelial cells was 37 °C; according to the methodology of Jin et al. (1996c) who reported that this was the normal physiological human and experimental animal body temperature. Adhesion could be different if the experimental temperature was 41.1 °C, which is the avian body temperature. Fuller (1975), who tested the adhesion of the bacteria to chicken crop epithelial cells at 45, 37, 25 and 4 °C, reported that temperature did not affect adhesion. In his study, the lactobacilli managed to adhere even at temperature of 4 °C, suggesting that active metabolism is not a factor required for bacterial adhesion. These results are consistent with those obtained by Gusils et al. (1999a), though Morata de Ambrosini et al. (1998) suggested that the adhesion phenomenon is very sensitive to temperature changes.

The pH of the GI tract ranges from 6 to 8 (Gusils et al. 1999a; Gusils et al. 1999b). The pH of the suspending PBS for this experiment was around 7. Jin et al. (1998b) stated that pH values between 4 and 7 of the suspending buffer for the bacteria did not significantly affect the adhesion ability of bacteria to chicken crop epithelial cells, which is in agreement with Fuller's (1975) observations. However, both of them report that a pH more that 7 and 8 results in significantly ($P<0.05$) reduced attachment.

The time that the lactobacilli are in contact with the IEC may also affect adhesion. Jin et al. (1996b) reported that incubation for 30 minutes resulted in less bacteria attached to the IEC than for 1, 2 and 3 h; which had no significant differences between them. Although Fuller (1975) suggested that incubation beyond 10 minutes did not affect positively the adhesion of the bacteria in IEC, in a later study (Fuller 1978), he stated that incubation-contact of 30 mins was not enough. Conway et al. (Conway et al. 1987) reported a *Lactobacillus* strain that attached to human ileal cells within 2 minutes and on the other hand, Coconier et al. (1993) noticed that *Lact. acidophilus* needed 1h of incubation to adhere to human enterocyte-like cells HT-29 and Caco-2 and mucus-secreting HT 29-MTX cells.

Curbelo et al. (2005) reviewed a study of Bengmark (1998) that suggested that *Lact. Plantarum* was able to adhere to and colonise the intestinal mucosa. When lactic acid bacteria bind the sites of adhesion in the epithelium, the pathogens that are attached to them may be reduced. The essential condition for this principle is the parallel attack mechanism of both the pathogenic and lactic acid bacteria. Several researchers (Fuller 1978; Jin et al. 1997) suggested that when lactobacilli colonise strongly on the crop they can efficiently control the *Esch. coli* population. Light and electron microscopy revealed that the adherent bacteria of the intestinal microflora are interconnected by fibers to the mucosal surface.

2.2.7 Tolerance to acid and bile salts

The strains of each species that showed the greatest antagonistic activity, i.e No 3 (identified as *Lact. plantarum* using API CHL kit), Nos 6 and 16 (identified as *Lact. salivarius)*, No 15 (identified as *Lact. fermentum*) and No 10 (identified as *Leuconostoc lactis*) were tested for their ability to survive under the acidic environment of the chicken gut, using an *in vitro* simulation of passage through the chicken GI tract. In order to simulate the protective effects of the digesta matrix, the selected lactobacilli strains were sprayed onto pelleted poultry grower feed (Mole Valley Farmers, Devon, UK) that had been sterilized by irradiation with 25kGy from Co^{60} (Becton and Dickinson, Plymouth, UK).

The composition and proximate analysis of the poultry feed used is given in Table 10. The pH was chosen to correspond with the pH found at each stage of the digestive tract, namely: pH 4.4 - 4.5 in the crop and proventiculus, pH 2.6 in the gizzard and pH 6.2 in the small intestine. The digestion time for each segment corresponded with the time digesta takes to pass through the digestive tract of the chicken, namely: 45 min for the crop and proventiculus and 90 minutes for the gizzard (Chang and Chen 2000). A flow chart showing the overall process for assessing tolerance to acidic pH (and bile salts) is shown in Figure 9.

The simulation of the small intestine was not followed by a simulation of the large intestine, as it was hypothesised that since the cultures were able to survive through the small intestine they would also survive transit through the large intestine where the pH is 6.3 (Chang and Chen 2000).

Each of the cultures was added to MRS broth and incubated at 37 °C, for 24 h, prior to application. One ml of the suspension was then diluted with nine ml of distilled water, mixed and then 10 ml of the active culture was sprayed over 100g of chicken feed and incubated at room temperature, for either 30min, or 24h, before the start of the simulated digestion process. However, it was found that the sprayed culture did not survive for 24h, which may suggest that this practice is not practical on farm, so only the 30 min treatment was tested further.

Pilot studies had been conducted prior the main experiment to determine the time points and the quantity of concentrated HCl that needed to be added to prevent the increase of the pH due to the buffering capacity of the chicken feed. It was concluded that to maintain a pH of 4.4 - 4.5 for the simulated crop, 20-30µl of HCl needed to be added at the start (T=0) then an additional 20-30µl at 20 min (T=20). For the simulated gizzard, 120-140µl of HCl needed to be added at T=45 and subsequently 80-100µl of HCL at T=65, and T=95. At T=135, 0.5g of $NaHCO_3$ and 0.21g of bile salts, (oxgall, Sigma) were added to increase the pH to 6.2 in the simulated small intestine (0.3% bile is the quantity found in the intestines of poultry according to Jin et al. (1998b). However, due to

the buffering capacity of the chicken feed used, it was necessary to add 20-50 µl HCl at T=155 and T=185, to prevent the pH from increasing above the required level.

Three replicates for each of the five different *Lactobacillus* cultures at both application times, were filled with 70mls of distilled water and heated in a water bath at 41.4 °C, which is the body temperature of chickens (Chang and Chen 2000). Ten g of the feed sprayed with the *Lactobacillus* cultures was then added to each of the flasks and all of them were mixed well. One ml of the sample was taken from each flask at the start of the experiment to estimate the number of bacteria present before any acid was added. A flask containing only feed without addition of *Lactobacillus* culture was used as control to confirm that the feed was remaining sterile through the experiment.

One ml from each sample was taken at T=45, before the pH was reduced to pH 2.6, at T=135, before the pH was adjusted to pH 6.2 and lastly at T=225 at the end of the experiment, so the population of bacteria present could be counted. The one ml samples of digesta solution were diluted with 9 ml of sterile peptone water and 10 fold serial dilutions were prepared. 100 µl of each dilution were spread over MRS agar using aseptic techniques and the plates were then incubated at 37 °C for 24 hours before the plates were counted.

The data were statistically analysed by using the General Linear Model (GLM) of the Analysis of Variance (ANOVA), Minitab v, 15 Statistical Software.

The mean death/recovery rates of each culture over the period of the experiment are shown in Figure 10 and Table 11. All the cultures survived in the acid environments they were subjected to.

Survival rates of the cultures were significantly ($P<0.05$) affected by the strain, the simulated site in the GI tract and the interaction of these two factors. In the gizzard, where the pH was lowest (pH 2.6), both *Lact. salivarius* strains (Nos 6 and 16) and the *Lact. fermentum* strain (No 15) suffered from the acid environment they were subjected to. In the small intestine, where the pH increased to 6.2, both strains Nos 6 and 16, but not strain No 15 managed to recover.

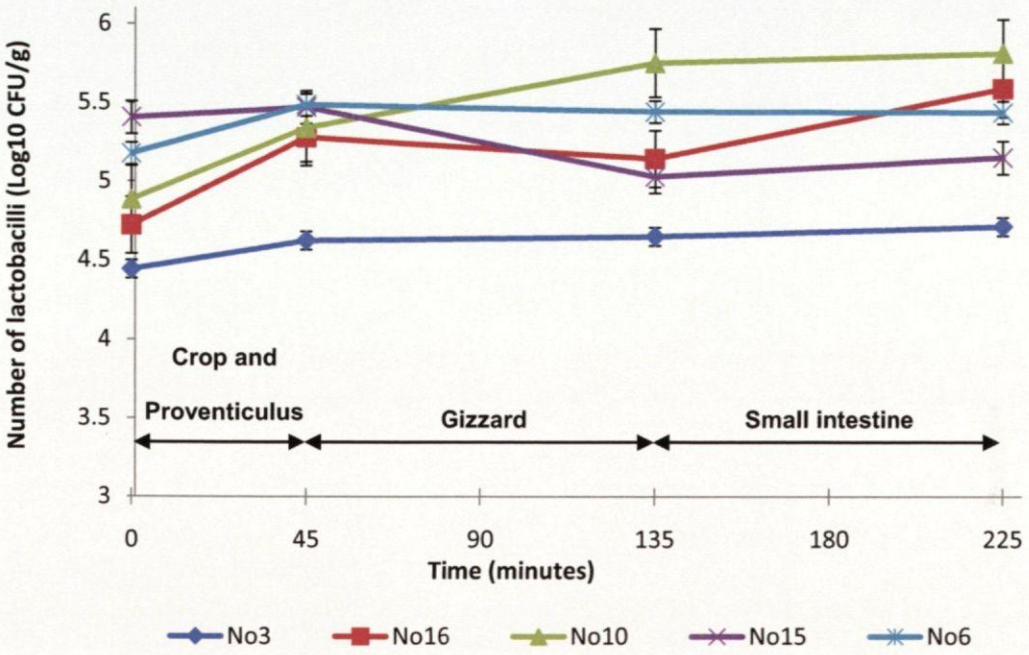


Figure 10. Number of *Lactobacillus* (log_{10} CFU ml^{-1}) Nos 3, 16, 6, 10 and 15 at each site of simulated GI tract*

Table 11. Number of *Lactobacillus* (log_{10} CFU ml-1) Nos 3, 16, 6, 10 and 15 at each site of simulated GI tract

Organism	Time				Mean
	0	45	135	225	
No 3	4.841	5.104	5.163	5.185	5.073^{a}
No 6	5.044	5.487	5.443	5.438	5.353
No 10	4.883^{ef}	5.339	5.754^{e}	5.813^{f}	5.447^{a}
No 15	5.406	5.471	5.029	5.151	5.264
No 16	4.720^{g}	5.278	5.144	5.590^{g}	5.183
Mean	4.979^{bcd}	5.336^{b}	5.307^{c}	5.336^{d}	

*Each value is the mean of the triplicate of each sample. Same superscripts show significant ($P<0.05$) different values

The pH of the chicken GI tract could be as acidic as pH 2, which makes the resistance to acidic pH of *Lactobacillus* strains uncertain. Chicken bile contains bile salts and lipids (cholesterol and phospholipids). Two thirds of the bile salts, which are different from those found in mammals, consist of tauro-chenodeoxycholate. Taurocholate and tauro-allochates, but not deoxycholates are also found (Larbier and Leclercq 1994). Although these components of the

bile can be found in the Sigma catalogue, they were too expensive to justify their use in the quantity needed in this study. In addition, no protocol was found for obtaining bile salts directly from chickens for laboratory use. Therefore, oxgall bile (Sigma) was used for testing the bile salt tolerance of the selected lactobacilli.

The results showed that all the lactic acid bacteria isolated from chicken gut successfully tolerated the various pH levels they were exposed to. At pH 2.6 both the *Lact. salivarius* strains (No 6 and No 16) and the *Lact. fermentum* strain (No 5) suffered from the acid environment they were subjected to. In the small intestine, where the pH increases to 6.2, both the *Lact. salivarius* (No 6 and No 16) strains, but not *Lact. fermentum*, seemed to recover. Mojgany et al. (2007) reported that only one strain (*Lact. Fermentum* RNL 44), among the three tested (the other two were *Lact. acidophilus* RNL26 and *Lact. salivarius* RNL49) managed to survive at pH 2.0. They observed that there was a decrease in the growth of the bacteria when they were subjected to pH 2.0 or below, which is in accordance with the results of other authors (Hood and Zottola 1988; Gupta et al. 1996).

The low pH of the stomach, between 1.5 and 3.0, and the bile salts of the upper intestine is the beginning of the stressing conditions that organisms are exposed to (Shah et al. 1995). Survival at pH 3.0 for two hours, and at bile concentration of 1.000mg L^{-1}, was considered optimal for potential probiotic bacteria by Usman and Hosono (1999). Although the pH of the stomach could

be as low as 1.0, for this experiment pH 2.6 was preferred as the lowest that the lactobacilli were exposed to, though other researchers preferred pH 3 for their *in vitro* experiments (Garriga et al. 1997; Mojgani et al. 2007).

In addition to acid tolerance, successful probiotics also need to be bile tolerant in order to grow and exert their action on the GI tract. Survival in the presence of bile concentrations between 0.1 and 4.0% w/v in growth media is considered as optimal for potential probiotic strains (Gilliland and Speck 1977). For most of the studies (Sjovall 1959; Gilliland et al. 1984; Gilliland et al. 1985; Mojgani et al. 2007) including the current study the potential probiotics were subjected to the mean intestinal bile salt concentration of the GI tract which is considered as critical and is 0.3% w/v.

All the cultures were grown in MRS agar broth overnight before being exposed to the simulated chicken gut. This might have helped them to adjust to the low pH of 4.5 in the crop, as the pH of the medium used drops from an average of pH7 to pH4.

2.2.8 Cell surface hydrophobicity

Two strains Nos 3 and 16, that showed the greatest aggregation properties, antagonistic activity and ashesion to chicken epithelial cells were tested for surface hydrophobicity, as an additional test for assessing their adhesion ability to epithelial cells. The methodology used for studying the hydrophobic

interactions of cells is based on the well established MATH (Microbial adhesion to hydrocarbons) assay (Rosenberg et al. 1980), following the modifications of Gusils et al. (2002a). The bacterial cells were grown in LAPTg broth (Raibaud et al. 1961) for 12-18h. Cultures were centrifuged for 10mins at 100000g, washed twice and resuspended in physiological saline (PS) to an optical density (OD_{600}) of 0.5 - 0.7. One ml of test hydrocarbon (Hexadecane, Toluene and Xylene) was added to the suspensions. The mixtures were vortexed for 90sec and after allowing 15 min for the hydrocarbon phase and the aqueous phase to separate, the aqueous phase was carefully removed with a Pasteur pipette and transferred to a 1ml cuvette. The optical density (OD) was determined at 600 nm, using a spectrophotometer. Three replicates of each *Lactobacillus* culture were used to calculate the hydrophobicity. The percentage of hydrophobicity was expressed as the decrease in the optical density of the original bacterial suspension due to cells partitioning into the hydrocarbon layer and the equation used was

$$\%hydrophobicity = \frac{OD_{600}\,before\,mixing - OD_{600}\,after\,mixing}{OD_{600}\,before\,mixing} \times 100$$

Mycobacterium sp was used as positive control and *Lact. acidophilus* as negative control (de Ambrosini et al. 1999).

No 3 showed a greater hydrophobic activity with all the solvents (hexadecane, toluene and xylene) than No 16 (Table 12).

Table 12. Cell surface hydrophobicity

	% hydrophobicity[a]		
Strain	Hexadecane	Toluene	Xylene
No 3	80	50	91
No 16	19	24	83

[a] %hydrophobicity = ($OD_{600\ before\ mixing}$ - $OD_{600\ after\ mixing}$) x 100 / $OD_{600\ before\ mixing}$

2.2.9 Homo/heterofermentation test

A homofermentation test was performed for the LAB that were to be used to ferment feed. The homo/heterofermentation test was performed on 46 chicken *Lactobacillus* isolates. *Lactobacillus* strains were cultivated at 30 °C in MRS broth and Petri dishes were incubated in anaerobic jars (Gas Pak Anaerobic System, Oxoid, Basingstock, UK). Colonies were tested for acid and gas production from glucose after inoculation of 10ml of a modified glucose-MRS broth (containing no citrate or meat extract; and addition of $10ml^{-1}$ chlorophenol red (0.4% solution) in test tubes containing inverted Durham tubes and incubated at 30 °C (Muller, 1990). Examination of tubes after 24h and 48h were estimated as follows:

- Gas production indicated heterofermentative metabolism.
- Colour change (chlorophenol red indicator) from red to yellow indicated acid production

From the 41 lactobacilli that have been tested, 34 were found homofermentative and 7 heterofermentative (Apentix 1).

There is no evidence that heterofermentation adversely affects the palatability and intake of FMF for chickens as it does for the pigs because of the production of acetic acid and carbon dioxide. However, this test was conducted in case it was decided to use any of the bacterial strains for fermentating feed for pigs in the future. The only restriction in using an organism that utilizes the heterofermentative pathway for preparing FMF for chickens is that the production of carbon dioxide during the heterofermentation process indicates potential dry matter loss. In this experiment, 83% of the lactobacilli tested were found to be homofermentative.

2.3 Conclusions

The aim of the screening procedure was to investigate whether LAB of chicken origin could be identified that had potential as probiotics. The results indicate that of the 111 candidate organism isolates at least three strains of lactobacilli No 3 (identified as *Lact. plantarum* with API identification kit and *Lact. salivarius* Salivarius after the full 16S rRNAgene sequence identification), No 6 and No 16 (identified as *Lact. salivarius* with both the identification methods), isolated from the natural gut microflora of poultry exhibited strong potential as probiotic adjuncts and could perform effectively within the GI tract. The use of isolated and purified LAB from the GI tract of chickens provides an alternative to the use of ileal extracts as a means of introducing beneficial bacteria to the digestive tract of chicks as it circumvents the problems of potential introduction of pathogenic organisms associated with the use of ileal extracts. However, given

the complexity of the chicken GI tract, the proof of efficacy of the two probiotic bacteria in broilers requires *in vivo* studies.

Chapter 3

Preliminary studies for *in vivo* study: water resistance and fermentation ability

3.1 Introduction

For the *in vivo* assessment of the probiotic properties of the selected lactobacilli, two different methods of delivery were considered; administration of the *Lactobacillus* culture through drinking water, or through fermented moist feed (FMF). Administration of probiotics in the drinking water, or in dry feed is the most usual and easily accepted practice for producers, although it does not guarantee the intake of adequate inoculum by all birds. Added benefit may be obtained if the feed is allowed to ferment to produce a feed containing at least 150 mmol L^{-1} lactic acid and a low pH <4.5, as this has been shown to reduce contamination of feed by enteropathogens such as Salmonellae (Heres et al. 2003a). In fermented diets, both bacteria and their fermentation products are present, whereas when probiotics are included to dry diets, only the bacteria are present and the products of their metabolism only appear in the GI tract after being consumed. The numbers of bacteria also differ and moreover in the fermented diets bacteria grow fast, whereas in dry diets they have to be reactivated (Scholten et al. 1999). The benefits of fermented feed are well documented in pig production (Canibe and Jensen 2003; Dung et al. 2005; Brooks 2008) and if a number of practical and engineering problems relating to the production and delivery of fermented moist feed (FMF) to chickens can be

solved, it could benefit both consumers and producers by improving of poultry health and poultry product safety.

Two cereal grains were chosen for assessing the fermentation characteristics of selected lactobacilli; barley and red sorghum. Barley is widely grown around the world for animal feed and can be included in broiler and layer rations at 70 and 55%, respectively. Sorghum is widely grown in Africa and Asia and the semi-arid tropics world-wide (Ragaee et al. 2006). Sorghum and particularly by-products from sorghum processing are reported as upalatable and not widely used in Europe. The recommended maximum rate for breeders and layers is 5% (Ewing 1997).

The aim of this study was to determine the survival of two selected *Lactobacillus* strains (reffered to as No3 and No6 in chapter 2), in water and their capacity to produce a good quality FMF that could be used in *in vivo* experiments to assess their potential probiotic effect. The effect of temperature and water type on the survival of LAB was also assessed. Two studies were conducted:

- Study 1. Survival of selected organisms in water, and
- Study 2. Fermentation ability of selected organisms

3.2 Materials and methods

3.2.1 Study 1. Survival of selected organisms in water

The study was designed as a three factor factorial experiment:

Factor 1. LAB strains: (No 3-*Lactobacillus salivarius* Salivarius (NCIMB 41606) or No 16-*Lactobacillus salivarius* Salivarius (NCIMB 41610);

Factor 2. Type of water: distilled water as control or hard water and

Factor 3. Temperature: 20 °C or 35 °C.

Each treatment was replicated three times.

Two different temperatures were used in this study; 20 °C and 35 °C. As it was planned to conduct the *in vivo* experiments at the Veterinary Laboratory Agency (VLA, Weybridge, Surrey, UK), the treatments were designed to conform with the environmental conditions in the VLA experimental facilities; the room temperature where the birds are held is usually 20-23 °C; also, the temperature under the heaters that are used for the first week posthatch are approximately 35 °C. LAB was also monitored in distilled and hard water to check whether water type has any effect on the efficiency of LAB. Hard water was used, because in the UK many places such as Surrey, where the VLA is located, have hard water. Hard water was prepared by adding 0.05 mg L^{-1} of sodium hypochlorite solution 12% w/v (BDH Laboratories England) and 0.04 mg L^{-1} of calcium carbonate to distilled water. None of the samples were sterilised.

3.2.1.1 Preparation of the sample

The *Lactobacillus* cultures were grown overnight in 10 ml of MRS broth (Oxoid) at 37 °C, in 5% CO_2 atmosphere, giving a suspension of 10^9 CFU ml^{-1}. The next day the cultures were centrifuged for 10 min at 10000xg, washed once with 10 ml of PBS, and centrifuged a second time. They were then resuspended in the same initial volume of phosphate-buffered saline (PBS) at a concentration of 10^9 CFU ml^{-1} (pH 6.0). One ml of the suspension was added to 9 ml of water and then incubated at 20 °C or 35 °C.

3.2.1.2 Inoculation and incubation

After each time interval, samples were diluted using serial dilution technique. One ml of each sample was diluted in 9ml of PBS (Dulbecco A, OXOID Ltd; England), giving a concentration of 10^8 CFU ml^{-1} of lactic acid bacteria. The dilution continued to 10^{-4} fold. Appropriate dilutions were plated on MRS Agar (CM0361 OXOID Ltd) for enumeration of LAB (Collins et al. 1995). Plates were incubated at 37 °C, for 24h. Colonies were counted after 24 h using a Colony Counter (Gallenkamp) (Collins et al. 1995).

3.2.2 Study 2. Fermentation ability of selected organisms

The fermentation study was designed as a two factor factorial experiment:

Factor 1. LAB strains: No 3-*Lactobacillus salivarius* Salivarius (NCIMB 41606) or No 16-*Lactobacillus salivarius* Salivarius (NCIMB 41610);

Factor 2. Fermentation substrate: barley or red sorgum

Additionally, *Lactobacillus* strain No 3-*Lactobacillus salivarius* Salivarius (NCIMB 41606) was also tested for its ability to ferment a commercial pelleted chicken feed (as used at VLA).

The composition and proximate analysis of the pelleted feed used (Saracen chick crumbs, J & W Attlee, Personage Mills, Dorking) is presented in Table 13. Barley was obtained from Edwin Tucker and Sons, StonePark, Ashburton, Devon and red sorgum (*Sorgum bicolour* L. Moench) from Northern Nigeria. The two cereal grains were hammer milled through a 3mm screen and together with the pelleted feed were irradiated (25kGy from Co^{60}) in 100g sachets prior to fermentation (Becton and Dickinson, Plymouth, UK). *Lactobacillus* strains were inoculated into ten ml of MRS broth and incubated at 37 °C, for 24 h, in 5% CO_2 atmosphere, giving a suspension of 10^9 CFU ml^{-1}. Feed samples were mixed with water at a ratio of 1:1.4 and inoculated with 0.1ml of MRS broth containing one of the two *Lactobacillus* cultures and incubated at 30 °C. Pelleted feed was fermented only with *Lactobacillus* No 3. The pH was recorded after 24 and 48h (for the pelleted feed only after 24h) of fermentation.

Table 13. Declared analysis and composition of basal diet*

Proximate composition	
Oil %	4.0
Protein %	18.5
Fibre %	3.2
Ash	5.6
Methionine %	0.4
Vit A iu/kg	10000
Vit D3 iu/kg	3000
Vit E iu/kg	15
Moisture %	14.0
Copper mg/Kg (added copper as Cupric Sulphate	25

Ration Ingredients (in descending weight order)

Ingredients	% ratio
Wheat	54.5
Hipro soya	16.7
Barley	10
Wheatfeed	10
Minerals	2.7
Peas	2.5
Vegetable fat (contains BHT as anti-oxidant)	1.2
Vitamins	0.75
Methionin	0.13
Lysine	0.03

*Saracen chick crumbs, complete compound feed for chicks up to 4 weeks (J & W Attlee, Personage Mills, Dorking, Surrey RH14 1EL)

Samples of fermented liquid feed (0.5 ml) were taken at three intervals, immediately after the inoculation of the feed, after 24 h and after 48 h of fermentation of the feed and stored at −20 °C prior to extraction and chromatographic analysis, using the method of Niven et al. (2004). When required, samples were allowed to defrost and 20 µml of 7% sulphuric acid was added (giving a pH ca. 1.8). The addition of a mineral acid helps denaturation of dissolved proteins shifts the acid equilibrium of weak acids to the protonated form and helps maintain the functionality of the column. The samples were shaken in a vortex mixer at 1300 rpm for 10min. The supernatant was decanted using 1ml polypropylene disposable syringes (Fischer Scientific, SZR-150-011Q) and filtered through 0.45 µm NYL polypropylene syringe filters (Whatman

International Ltd. Maidstone, England) to exclude any particulate material left. Standards containing three concentrations of analytical grade lactic acid (300, 150 and 75 mmol L^{-1}), acetic acid (100, 50 and 25 mmol L^{-1}) maltose (100, 50 and 25 mmol L^{-1}) glucose (50, 25 and 12.5 mmol L^{-1}) and fructose (50, 25 and 12.5 mmol L^{-1}) were run. All authentic reference compounds (maltose, glucose, fructose, lactic acid, acetic acid,) were of analytical grade, or higher, (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) and were dissolved in Milli-Q water (Millipore Corp., Bedford, MA, USA). After 24h, 1g of the FMF sample was added to 9mls of MRS broth (OXOID) and was subjected to serial dilutions. 0.1ml of each dilution was plated on fresh MRS agar for the enumeration of lactobacilli present in the FMF. The IEC system consisted of a Gynkotek (Dionex Corp., Sunnyvale, CA, USA) P580A gradient pump, Gynkotek GINA 50 autosampler and a Shodex RI-71 refractive index detector (Showa Denko K.K., Tokyo, Japan). The system and data capture was controlled by a personal computer running ChromeleonTM software (Dionex Corp., Sunnyvale, CA, USA). Separation was achieved on a MetaCarb 87H 300mm×7.8mm (i.d.) column (MetaChem Technologies Inc., Torrance, CA, USA) at room temperature. A guard column, of the same phase, was placed in front of the analytical column. The eluent was 5mmol L^{-1} sulphuric acid (AR grade (Sigma–Aldrich) degassed by helium displacement and ultrasonication and used in isocratic mode at 0.5ml min^{-1}.

3.2.3 Statistical analysis

The data was subjected to ANOVA using the General Linear Model (GLM) procedure of MINITAB v.15.0. Death rate of LAB at different temperatures and different time intervals was converted to log_{10} before applying statistical analysis.

3.3 Results and discussion

3.3.1 Study 1. Survival of selected organisms in water

The number of the two *Lactobacillus* strains after 24h was 8.30 and 8.65 Log_{10} CFU ml^{-1} for *Lactobacillus* strain No 3 and No 16 respectively, and did not significantly reduced or differ between organisms.

The present study demonstrated that the water type did not affect the survival of lactobacilli, as the strains used in this experiment survived up to 24 h in both hard and distilled water. These results are in agreement with Azhar (2005), who found that the mineral content of water did not affect the survival of LAB. He reported that both *Lact. Plantarum* and the commercial probiotic Stabicil (Medipharm,) survived up to 24 h in both hard and soft water, suggesting that water type does not significantly affect the number of live lactic acid bacteria.

According to Azhar (2005), LAB died quicker, at 20 °C than 4 °C. In the current study, water temperature (20 or 35 °C) did not have a significant influence upon

the survival of the LAB tested. LAB are classified as mesophiles, which means that they preferably grow under moderate temperature (Adams and Moss 2003). LAB can easily grow and be metabolically active up to 30 °C, though at 4 °C, due to low temperature, organisms become metabolically inactive, which results in their prolonged survival (Berry and Foegeding 1997).

Factors other than environmental temperature and hardness of the water, may affect the survival of LAB in water. 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 (i.e. the chemical relationship that might occur between a copper pipe and the water for example) and the resident microflora present in biofilms on the surface of the delivery systems.

3.3.2 Study 2. Fermentation ability of selected organisms

The two organisms produced FMF containing 10^9-10^{10} CFU ml^{-1} lactobacilli in both grains and pelleted feed.

The pH of the four treatments (*Lactobacillus* strain No 3 x barley, No 3 x red sorghum, *Lactobacillus* No 16 x barley and No 16 x red sorghum) after 24 and 48h is presented in Tables 14 and 15. At T=24h and T=48h the pH of both feeds fermented with both *Lactobacillus* strains decreased significantly ($P<0.05$)

from pH 5.6±0.18 to pH 3.66±0.04 and pH 3.50±0.05, respectively. The pH was significantly affected by the type of grain ($P<0.01$), the time of fermentation ($P<0.01$) and the interaction between the grain used and the time of fermentation ($P<0.01$). The pH of the pelleted feed fermented with *Lactobacillus* No 3 was 3.99±0.05.

The residual concentration of maltose, glucose and fructose of each of the treatments are shown in Tables 14 and 15. There were no significant differences between the two organisms for any of the parameters measured. The concentration of maltose was significantly affected by the type of grain ($P= P<0.01$), the time of fermentation ($P<0.01$), the interaction of grain and time as well as the interaction between three factors the: strain, the grain and the time of fermentation ($P=0.017$). The glucose concentration was significantly affected by the type of grain ($P<0.01$), the time of fermentation ($P= 0.04$) and the interaction of: the grain and the time of fermentation ($P<0.01$). However, the concentration of fructose was significantly affected only by the type of grain ($P<0.01$) and the interaction of: the grain and the time of fermentation ($P<0.01$). Residual sugar levels were significant lower in fermented sorhum than fermented barley.

Lactic and acetic acid production was significantly affected by the type of grain ($P=0.007$ and $P=0.001$ respectively) and the length of fermentation ($P<0.01$). There was no significance difference in lactic acid and acetic levels between the cereal grains at 24h, but at 48h both lactic and acetic acid were significant lower

in fermented sorghum. The lactic acid concentration of the pelleted feed (355.6 mmol L^{-1}) after 24h was not significant different from the lactic acid concentration of barley (302.9 mmol L^{-1}) and sorghum (223.1 mmol L^{-1}) after 24h of fermentation. However, the pH of pelleted feed after 24h of fermentation (pH 3.7) was significantly higher ($P<0.05$) than the pH of the fermented barley (pH 3.7) and sorgum (pH 3.63).

Table 14. Mean values of the pH and the concentration (mmol L^{-1}) of Maltose, Glucose and Fructose, Lactic acid and Acetic acid of Barley fermented with *Lactobacillus* strain No3 and No16 and Red Sorgum fermented with *Lactobacillus* strain No3 and No16 after 24 and 48h

		Mean values for both lactobacilli			Mean values for both cereals		
		Cereal type			Organism		
		Barley	Sorgum	Significance	No 3	No 16	Significance
Maltose	0	66.07	30.10	P<0.01	47.78	48.39	NS
	24	48.02	2.865	P<0.01	25.70	25.19	NS
	48	31.02	2.871	P<0.01	17.18	16.72	NS
Mean		48.37	11.95	P<0.01	30.22	30.10	NS
Glucose	0	22.76	40.15	NS	31.75	31.17	NS
	24	93.75	17.75	P<0.01	55.78	55.71	NS
	48	78.55	23.06	P<0.01	52.16	49.45	NS
Mean		65.02	26.99	P<0.01	46.56	45.45	NS
Fructose	0	27.47	21.47	NS	24.87	24.07	NS
	24	53.86	6.660	P<0.01	27.65	32.88	NS
	48	40.89	10.35	P<0.01	26.52	24.72	NS
Mean		40.74	12.83	P<0.01	26.34	27.22	NS
Lactic acid	0	10.66	3.69	NS	7.20	7.16	NS
	24	293.39	234.12	NS	263.01	264.50	NS
	48	445.63	343.75	P<0.01	390.02	399.36	NS
Mean		249.89	193.86	P<0.01	220.08	223.67	NS
Acetic acid	0	3.79	0.53	NS	2.42	1.90	NS
	24	5.91	3.10	NS	4.45	4.57	NS
	48	15.00	7.44	P<0.01	9.60	12.83	NS
Mean		8.23	3.686	P<0.01	5.49	6.43	NS
pH	0	5.45	5.76	P<0.01	5.60	5.60	NS
	24	3.70	3.62	NS	3.68	3.65	NS
	48	3.46	3.54	NS	3.50	3.50	NS
Mean		4.20	4.30	P<0.01	4.25	4.25	NS

Each value is the mean of the triplicate of each sample

Table 15. pH and concentration (mmol L^{-1}) of Maltose, Glucose and Fructose, Lactic acid and Acetic acid of Barley fermented with *Lactobacillus* strain No3 and No16 and Red Sorgum fermented with *Lactobacillus* strain No3 and No16 after 24 and 48h*

Time	Lactobacilli	Feed	Maltose	Glucose	Fructose	Lactic acid	Acetic acid	pH
0	No 3	Barley	59.32±4.70	21.61±14.47	26.37±10.70	10.77±2.60	4.15±4.45	5.45±0.10^{m}
		Sorgum	36.24±1.06	41.88±1.60	23.37±2.94	3.62±2.25	0.69±0.00	5.74±0.03^{m}
	No 16	Barley	72.82±4.72^{a}	23.91±1.41	28.57±3.88	10.55±3.49	3.44±1.85	5.45±0.14^{n}
		Sorgum	23.96±2.39^{a}	38.43±3.24	19.56±2.86	3.76±3.49	0.36±0.00	5.77±0.03^{n}
24	No 3	Barley	48.61±8.57^{d}	94.65±56.26^{f}	48.84±15.87^{n}	302.90±171.35	6.02±2.14	3.71±0.12
		Sorgum	2.79±1.72d	16.91±5.07f	6.45±0.94^{h,l}	223.12±7.51	2.87±1.21	3.63±0.03
	No 16	Barley	47.44±16.47^{b}	92.84±34.11^{g}	58.89±17.29^{k}	283.88±31.63	5.80±3.40	3.68±0.11
		Sorgum	2.94±1.28^{b}	18.59±8.23^{g}	6.87±2.52^{i,k}	245.12±19.13	3.32±2.05	3.61±0.10
48	No 3	Barley	31.96±4.21^{e}	82.63±11.82	42.97±6.66^{j}	447.90±61.50	14.06±1.78	3.45±0.03
		Sorgum	2.39±0.82e	21.69±2.04	10.07±0.69^{j}	332.14±16.88	5.15±1.41	3.55±0.03
	No 16	Barley	30.09±10.78^{c}	74.47±43.69	38.82±5.06^{l}	443.35±43.21	12.11±6.97	3.46±0.07
		Sorgum	3.35±0.58^{c}	24.43±1.97	10.62±0.78^{l}	355.37±38.47	9.72±8.50	3.54±0.01

*Each value is the mean of the triplicate of each sample. Same superscripts show significant (P<0.005) different values

For the current study, distilled water was used for the preparation of the fermented feeds. However, according to Wee et al. (2004) $CaCO_3$ affected lactic acid production under culture conditions. Bhatt and Srivasta (2008) found that low concentrations of $CaCO_3$ in the fermentation medium increased lactic acid production when cane molasses was fermented with *Lact. delbrueckii* NCIM 2025.

The survival of the two *Lactobacillus* strains Nos 3 and 16 was not affected significantly by the type of water and temperature and both were capable of producing a good quality FMF that could be used in subsequent *in vivo* experiments for the assessment of their of their potential probiotic effect.

According to Heres et al. (2003a), high numbers of lactobacilli (>10^9 CFU ml^{-1}), a high concentration of lactic acid (>150 mmol) and a low pH (<4.5) in the fermented feed could make chickens less susceptible to *Salmonella* infections. In this experiment, both *Lactobacillus* strains tested produced fermented feed, containing 10^9 CFU ml^{-1} lactobacilli, 263.76 ± 36.22 and 394.69 ± 59.6 mmol L^{-1} lactic acid and a pH of 3.66 ± 0.04 and 3.50 ± 0.05 after 24 and 48h incubation at 30°C, respectively. When Beal et al. (2005) examined wheat and barley samples after spontaneous fermentation for 24 h at 30 °C, they found that the mean concentrations (mmol L^{-1}) of lactic acid, acetic acid, butyric acid and ethanol were 59.6 ± 40.0 (range 0.14-134.9), 23.2 ± 11.1 (range 2.9-51.4), 17.2 ± 16.8 (range 0.0-62.2) and 15.0 ± 9.0 (range 4.6-53.7) respectively. After fermentation for 24 h only 9 of 300 fermentations produced more than 75 mmol

L^{-1} lactic acid, which is the level considered to be bactericidal against *Salmonella* in liquid pig feed (Beal et al. 2002). This suggested that natural fermentation cannot be depended upon to produce sufficient SCFAs to prevent the proliferation of enteropathogens (Beal et al. 2005).

The concentration of three carbohydrates maltose, glucose and fructose and the production of lactic and acetic acid during fermentation by lactobacilli were measured. Maltose, which is a disaccharide, as well as glucose and fructose, which are monosaccharides, play an important role during the fermentation of grains, as they are converted predominantly into lactic and acetic acid among others (like propionic and butyric acid). Results from the fermentation test (Tables 14 and 15) show that sugar (maltose, glucose and fructose) concentration of barley was significantly higher than that of sorghum. The concentration of the sugars and acids was affected by the type of grain. The utilization of the sugars by the LAB present for the production of lactic and acetic acid affects the variation of the concentration of sugars in the fermented feed. Charalampopoulos et al. (2002) suggest that sugar fermentation by LAB is strain specific. However, this was not the case in the current experiment, as the concentration of sugars was not affected by the type of organism used for fermentation. This might suggest that both the LAB tested could be considered as good inoculants for fermenting feed.

The barley fermentation had a significantly higher concentration of organic acids (lactic acid and acetic acid) than sorghum. As far as lactic acid is concerned, it

was found that barley produced more lactic acid (302.90 ± 171.35 mmol L^{-1}) than red sorgum (223.12 ± 7.51 mmol L^{-1}) or commercial pelleted feed (118.53 ± 5.83 mmol L^{-1}), after 24h fermentation at 30 °C when fermented with *Lactobacillus* No3. These values are higher than those reported by Beal et al. (2005). One reason might be that Beal et al. (2005) used a feed to water ratio of feed to 2.5 (ideal for pigs), whereas in this study a 1 feed to 1.4 water ratio was used (as suggested by Heres (2002) for chickens). Also, instead of the controlled fermentation used in this experiment, they used spontaneous fermentation, which might be inconsistent and could not result in the production of desired SCFA in the feed.

The barley fermentation resulted in a significantly lower pH than sorghum, which may be attributed to its higher concentrations of acids. There is evidence that a low pH and the presence of organic acids reduces the emptying rates of the stomach (Mayer 1994), improves the ileal digestibility of amino acids through the stimulation of the secreted proteolytic enzymes (Harada et al. 1986) and enhances digestion (Lyberg et al. 2008). However, care should be taken as fermentation of FF by hetefermentative LAB can result in the production of undesirable SCFAs such as acetic acid, propionic and butyric, and affect negatively the palatability and the nutritional value of the feed and consequently the feed intake in pigs (Niven et al. 2004). As far as chicken nutrition is concerned there is no evidence that a high concentration of acetic acid affects negatively the palatability of the feed.

3.4 Conclusions

Both *Lactobacillus* strains, No 3-*Lactobacillus salivarius* Salivarius (NCIMB 41606) and No 16-*Lactobacillus salivarius* Salivarius (NCIMB 41610) were found to survive in water after 24h hour and to produce fermented feed, of low pH (<4.5) that contain more than 150 mmol L^{-1} lactic acid during a 24h fementation at 30°C, which make them good candidates for the assessment of their efficacy as potential probiotic bacteria in *in vivo* studies. However, *Lactobacillus* No 3 that performed better at the screening programme, described at chapter 2, was selected for further *in vivo* studies.

Chapter 4

Moist feed, fermented with *Lactobacillus salivarius* Salivarius NCIMB 41606, reduces susceptibility of one day old chicks to *Salmonella enterica* serovar Typhimurium Sal 1344 nal^r, during their 40 day development

4.1 Introduction

Consumption of poultry meat is associated with human *Salmonella* infections (Revolledo et al. 2006). One way to control the presence of these bacteria in broiler flocks is to make chickens less susceptible to colonisation. Probiotics and liquid feed may be a potential tool to reduce *Salmonella* carriage in broiler chickens. Delivery of probiotic strains of lactic acid bacteria (LAB) to poultry may be acheived by addition to the drinking water, though added benefit may be obtained if the feed is allowed to ferment to produce a feed containing at least 150 mmol L^{-1} lactic acid and a low pH <4.5, as this has been shown to reduce contamination of feed by enteropathogens such as Salmonellae (Heres et al. 2003a). Spontaneous fermentation of FLF is considered unreliable as it failed 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). It would be advantageous if the selected strain also had probiotic properties. Selection of potential probiotic strains, using *in vitro* models is cost- and time-effective, easily controlled and has no ethical constraints. However, given the complexity of the chicken GI tract, the proof of

efficacy of the probiotic bacteria in broilers requires *in vivo* studies. In the present experiment, a strain identified as *Lactobacillus salivarius* Salivarius NCIMB 41606 *(Lact.* Salivarius*)*, reffered as No 3 in chapters 2 and 3, that was isolated from chicken gut, and was selected for its probiotic and fermentation properties, was assessed for its efficacy in reducing the shedding of *Salmonella enterica serovar* Typhimurium Sal 1344 nal^r (*Salm.* Typhimurium,) in one day old chicks, and during their 40 day development. Additional aims of the study were the examination of changes in the intestinal microflora by culture-based and molecular methods, and an assessment of histological alterations of intestinal villi and liver.

4.2 Materials and methods

4.2.1 Ethical approval

The *in vivo* studies were conducted at the Veterinary Laboratory Agency (VLA, Weybridge, UK.). The protocol and procedures were approved by the VLA ethical committee and the studies were conducted according to and within UK Home Office regulations (Animal Scientific procedure act 1986).

4.2.2 Experimental design and treatments

The experiment was a randomised block design with four treatments. A total of 68 hatchlings were randomly divided into four groups of 17 birds each. The four groups received one of four treatments namely:

1. Control (**CON**): birds fed dry diets with no addition of LAB or acid.
2. Water supplemented with Lactic Acid Bacteria (**WAT**): birds fed as CON, but provided with drinking water containing 10^7 CFU ml^{-1} of *Lact.* Salivarius NCIMB 41606 from day one of age.
3. Fermented Moist Feed (**FMF**): birds fed the same basal diet as CON, but fermented with *Lact.* Salivarius NCIMB 41606 from day one of age. The FMF contained 10^9 CFU ml^{-1} of *Lact.* Salivarius.
4. Acidified Moist Feed (**AMF**): birds fed the same basal diet as CON, but acidified with 30.3 ml of lactic acid (88% food-grade; PURAC. America, Inc) per kg of wet feed, from one day of age, to provide the same acid concentration as FMF.

4.2.3 Strains and culture conditions

A *Lactobacillus* strain identified as *Lactobacillus salivarius* Salivarius (NCIMB 41606) that had been isolated from chicken gut and had been selected for its probiotic and fermentation properties in our laboratory (reffered to as strain No 3 in chapter 2 and 3), was stored at -80°C and inocula were cultured in MRS broth. *Lactobacillus* cultures needed for inoculation of the feed and the water were prepared by inoculating MRS broth and incubating it overnight at 37 °C.

A nalidixic acid-resistant derivative of *Salmonella enterica serovar* Typhimurium *Sal 1344 nal^r (Salm.* Typhimurium*)* from the colection of Veterinary Laboratory Agency (Weybridge, UK) was obtained and stored at -80 °C in heart infusion

broth supplemented with glycerol (30% w/v). When needed, the pathogen was grown on blood agar and stored at 4 °C. For challenging the chickens, broth cultures of *Salm.* Typhimurium were grown overnight in LB broth (Luria Broth, Oxoid, Basingstoke, UK) at 37 °C, aerobically with gentle agitation.

For selection and enumeration of lactobacilli, samples were suspended in phosphate-buffered saline (PBS, Oxoid, Basingstoke, UK), plated on MRS agar and finally were incubated in anaerobic jars, at 37 °C, for 48h. For determining *Salm.* Typhimurium numbers, samples were suspended in PBS and plated on brilliant green agar (BGA, Oxoid, Basingstoke, UK) supplemented with nalidixic acid (15μg ml^{-1}) and incubated under aerobic conditions, at 37 °C, for 24h.

4.2.4 Environment and daily management practices

Newly hatched chicks from a specific pathogen free (SPF) White Leghorn flock specific pathogen free avian supply (SPAFAS) were obtained and housed in negative pressure isolators on wood shavings. Housing and care of the birds conformed to the VLA guidelines. Supplemental light and heat were provided for the first two weeks. Heaters were placed in each isolator from the first day, adjusting the temperature to approximately 35 °C for the first week. The second week the temperature was reduced gradually to 20-23 °C, which was the room temperature. Water and feed were provided in a conical, 10 litre, plastic poultry waterer and a plastic tray, respectively. Fresh feed and water was provided once every day, early in the morning. At day 3, 10 chickens per group were wing tagged.

4.2.5 Preparation of diets

For the WAT treatment, birds were provided with a 24h culture of *Lact.* Salivarius in 100ml MRS broth, washed with PBS and reconstituted in 100ml PBS and mixed in 10 L of drinking water to obtain approximately 10^7 CFU ml^{-1}.

FMF was prepared by mixing commercial, unmedicated, pelleted feed (Saracen chick crumbs, obtained from J & W Attlee, Parsonage Mills, Dorking; Table 16) with water at a ratio of 1 feed: 1.2 water, inoculated with 10^6 CFU ml^{-1} of *Lact.* Salivarius resuspended in PBS (0.1 M, pH 7.2). The 24h culture was prepared by inoculating 20 ml MRS broth with *Lact.* Salivarius, to give a concentration of 10^9 CFU ml^{-1} of broth. The culture was centrifuged in 50ml centrifuge tubes, at 4000 rpm, for 10 minutes at a temperature of 20 °C. The pellet was resuspended in the same volume of PBS and was used to inoculate the moist feed at a volume of 0.1% of the dry feed in the mixture to give a concentration of 10^6 CFU g^{-1} dry feed. The moist feed was placed in polythene bags and incubated for 30 °C, for 24 h, prior to feeding.

The pH of the FMF and the AMF was recorded using a pH electrode (pH 213 microprocessor pH meter, Hanna instruments, Portugal). The mean pH of the fermented feed before inoculation was 5.97 ± 0.07 mmol L^{-1} and when delivered to chickens was 4.45 ± 0.19. The mean lactic acid concentration was measured by HPLC and was 361.8±162.6 mmol L^{-1}. The AMF was formulated to contain the same amount of lactic acid as measured in FMF; feed and water were also mixed in a 1:1.2 ratio and prepared shortly before they were provided to broilers. The mean pH of the AMF when delivered to chickens was 4.18 ± 0.18

and the mean lactic acid concentration was 459.3±165.0 mmol L^{-1}. Both feed and water were provided *ad libitum*.

Table 16. Declared analysis and composition of basal diet*

Proximate composition	
Oil %	4.0
Protein %	18.5
Fibre %	3.2
Ash	5.6
Methionine %	0.4
Vit A iu/kg	10000
Vit D3 iu/kg	3000
Vit E iu/kg	15
Moisture %	14.0
Copper mg/Kg (added copper as Cupric Sulphate	25

Ration Ingredients (in descending weight order)

Ingredients	% ratio
Wheat	54.5
Hipro soya	16.7
Barley	10
Wheatfeed	10
Minerals	2.7
Peas	2.5
Vegetable fat (contains BHT as anti-oxidant)	1.2
Vitamins	0.75
Methionin	0.13
Lysine	0.03

*Saracen chick crumbs, complete compound feed for chicks up to 4 weeks (J & W Attlee, Personage Mills, Dorking, Surrey RH14 1EL)

4.2.6 Challenge of the chickens with *Salmonella enterica* serovar Typhimurium Sal 1344 nal^r

Salm. Typhimurium cultures in LB broth, were centrifuged and suspended in 0.1 ml of PBS. All groups were challenged by oral gavage with 10^6 ml^{-1} of a nalidixic acid resistant mutant of *Salm.* Typhimurium at two weeks of age, using a dosing catheter of size 4.5g and length 60mm (Harvard Apparatus Limited, USA). Before challenge, birds were dosed with 0.2ml of sodium bicarbonate to neutralise the acidic environment in the upper GI tract of the bird. After challenge the remaining culture was plated on BGA agar plates supplemented with nalidixic acid (15 µg ml^{-1}) and incubated aerobically at 37 °C for 24h to confirm the level of the bacterium dosed.

4.2.7 Performance Parameters

Chickens in each pen were weighed on a weekly basis (i.e. wk 1 to 6) to determine average BW and weight gain (WG). Feed intake per pen was recorded daily, and FCR was also calculated weekly (FI/WG). In addition, overall BW gain (BWG), FI, and FCR were calculated for the whole duration of the experiment. The average feed intake per bird was calculated using the following equation:

$$FI = \frac{weight\ of\ feed\ T(24) - weight\ of\ feed\ T(0)}{number\ of\ birds}$$

where $weight\ of\ feed\ T(0) = the\ weight\ of\ feed\ when\ provided\ to\ birds$

$weight\ of\ feed\ T(24) = the\ weight\ of\ feed\ after\ 24\ h\ of\ feeding$

For the fermented and the acidified feed the equation was transformed as follows:

$$FI = \frac{[weight\ of\ feed\ T(24) - weight\ of\ feed\ T(0)] \times \frac{1}{1.2}}{number\ of\ birds}$$

where $\frac{1}{1.2} = \frac{parts\ of\ the\ dry\ feed\ in\ the\ moist\ feed}{parts\ of\ water\ in\ the\ moist\ feed}$

4.2.8 Collection of feed samples

Samples of the FMF and AMF were collected daily and frozen at -20 °C until used to determine of the concentration of the maltose, fructose, glucose, lactic acid and acetic acid by HPLC. The pH of the fermented feed was recorded twice before inoculation and after 24 hours of fermentation at 30 °C. The pH of the acidified feed was recorded immediately after the addition of the lactic acid.

4.2.9 Bacteriological analysis of cloacal swabs

All media used were obtained from OXOID (Basingstoke, UK). Cloacal swabs were taken from the same 10 tagged birds per group at least twice per week for 2 weeks prior to and 4 weeks after challenge, to determine the total lactobacilli counts and the shedding of *Salmonella*. Enumeration of *S.* Typhimurium and LAB was conducted according to a semiquantitative method described by La Ragione *et al.* (2004). Swabs were weighed before and after the swabbing process to determine the weight of sample taken. Swabs were resuspended in 9mls of PBS, vortexed for 50 seconds, and the suspension was subjected to serial 10 fold dilutions (until dilution 10^5). Suspensions were plated on MRS agar, using the method described by Miles, Misra and Irwin (1938) and

incubated in anaerobic jars, at 37 °C, for 48h, for the enumeration of lactobacilli. For the *Salmonella* counts, the suspension was plated on BGA (Brilliant Green Agar) agar plates supplemented with nalidixic acid (15 μg ml^{-1}) and incubated aerobically at 37 °C for 24h. Colonies were visualised using a Petri Viewer MK2 camera and counted with Sorcerer Image Analysis System software version 2.2 (Perceptive Instruments Ltd). In addition, 1ml of the suspension was added to 9ml of selenite broth (enrichment) and incubated, aerobically, for 6 days. After one day of incubation, the suspension in selenite broth was plated on BGA agar and further incubated, aerobically, for 24h. The next day the samples were observed for being *Salmonella* positive. Those samples that were scored as *Salmonella* negative were spread for a second time, at day 6 of incubation.

4.2.10 Post mortems, collection of samples and bacteriological analysis

At 26 and 40 days postinoculation, six birds were selected randomly from each of the four groups and killed by cervical dislocation. Tissue samples of ileum, caecum, liver and spleen were removed aseptically, for subsequent bacteriological analysis. Post-mortem enumeration of *S.* Typhimurium and LAB was conducted according to the method described by La Ragione *et al.* (2004).

One gram of tissue samples of ileum, caecum, liver, spleen and muscle were removed aseptically from each bird weighed and placed separately in sterile 1oz MacCartney glass bottles. Each organ was homogenised in PBS and was subjected to bacteriological analysis. Total lactobacilli from ileum and caecum, as well as *Salmonella* counts from ileum, caecum, liver, spleen and meat were determined by plating the serial dilutions made in PBS (0.1M, pH 7.2). One ml

of the suspension was added to 9ml of selenite broth (enrichment medium) and incubated aerobically for 6 days. After one day of incubation, the suspension was plated on BGA agar and further incubated aerobically for 24h. The following day the samples were observed for being *Salmonella* positive. Those samples that were scored as *Salmonella* negative were spread for a second time, at day 6 of incubation.

4.2.11 Bacteriological analysis of environmental samples

At day 32, sawdust samples from the floor of the isolators were collected in MacCartney bottles for bacteriological analysis from 5 different points of each negative pressure isolator where the birds were housed. The position of the environmental sampling points is presented in Figure 11.

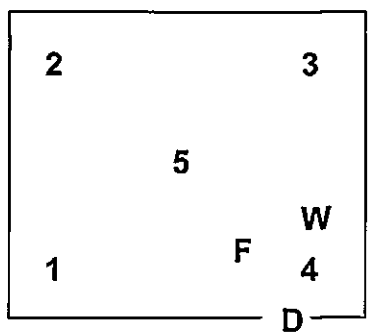


Figure 11. Negative pressure isolator. 1, 2, 3, 4, and 5: environmental samples collection points, F: feed tray, W: waterer, D: door.

One gram of each sample was added to 9ml of PBS to give a 1 in 10 dilution factor. The viable counts of *Salm.* Typhimurium were determined by plating dilutions made in PBS (0.1 M, pH 7.2) on BGA supplemented with nalidixic acid (15μg ml^{-1}). A gram of each sample was also enriched by addition to selenite

broth and incubated for 6 days, aerobically, at 37°C. The *Salmonella* negative and positive samples were then recorded at day 1 and 6 of incubation.

4.2.12 Antibody response - Enzyme Linked Immunosorbent Assay (ELISA)

Circulating plasma *Salmonella* antibodies were determined according to the method described by Dibb-Fuller *et al.*, (1999). Blood samples were also collected from the jugular vein of six birds per group at 26 and 40 days post mortem (using syringes without anticoagulant). Enzyme Linked Immunosorbent Assay (ELISA) was performed on the serum of blood samples to determine levels of *Salm.* Typhimurium antibodies within the sample.

The samples that were planned to be used as a control for the ELISA test were collected at day 13, i.e. before *Salmonella* challenge; Five birds from the control group were selected randomly and 5ml blood samples were taken by jugular puncture. After a few hours samples were poured into Eppendorf tubes and were centrifuged at 20 °C and 13rpm. The plasma was removed from the Eppendorf tubes with a pipette and the samples were refrigerated at -20 °C.

4.2.12.1 Protein concentration from whole cell Salmonella antigen

Salm. Typhimurium *Sal* 1344 *nal*^{r} was plated on sheep blood agar and incubated at 37 °C overnight. Twenty ml of LBG broth was inoculated with a loop of the culture and incubated at 37 °C overnight in a bench top shaker. The inoculum was centrifuged at 4000 rpm, for 10 minutes, to remove the supernatant and re-suspended in 20 ml PBS. The suspension was placed in a

water bath at 65 °C for 30 minutes, in order the bacteria to be killed. The protein concentration was estimated using a commercial assay (Bio-Rad Dc protein Assay), following the manufacturers' instructions. A standard curve was obtained using dilutions of Bovine Serum Albumin (2 mg, 1.5, 0.5, and 0.25). Test samples were diluted 1 in 5 and 1 in 10 for the assay. In flat bottom plates (Nunc, Denmark), 5 µl of standards and samples were plated in triplicate.

The plates were left to incubate on the bench for 15 minutes and then read at a wavelength of 630 nm on a plate reader. The protein concentration of the total *Salmonella* bacteria in the samples was calculated using a standard curve derived from the results of the BSA standards. The standard curve was defined by a linear regression as follows 0.25 (average row b M^+) 0.5 (average row c M^+) 1 (average row d M^+) 1.5 (average row e M^+) 2 (average row b M^+). The result obtained was 0.961164 (approximately 1) to show a linear fit in the dilution concentrations of protein. The concentration of the 1/5 and 1/10 dilutions in mg ml^{-1} were estimated from the standard curve.

4.2.12.2 Antibody determination using enzyme Linked Immunoabsorbent assay (ELISA)

Five µg ml^{-1} of crude, whole cell antigen, obtained by VLA, was diluted in bicarbonate coating buffer (Sigma). One hundred µl of the coating antigen was added to each well of a microtitre plate (Polysorb, Nunc) and incubated overnight at room temperature. The following day, the microtitre plate coated with antigen was washed three times by filling the wells with ELISA wash (0.1M

PBS, pH 7.2, 0.05%, v/v_0 Tween-20) and blotted with paper tissue. The blocking step that followed consisted of the addition of 200µl of 3% (w/v) dried skimmed milk in ELISA wash and incubation for 30 min at 37 °C. The microtitre plate was washed three times by filling the wells with ELISA wash and tapping dry on paper tissue for a second time. The next two steps were the primary and the secondary antibody reaction. First, the initial dilutions of the test samples in ELISA wash were prepared and 200 µl were added to appropriate wells on the plate and diluted with ELISA wash. Test control without antigen, samples and no-conjugate wells were included on each plate and each sample was tested in duplicate. Plates were left in the incubator, at 37 °C, for 1h, and were washed three times by filling the wells with ELISA wash and tapping dry on paper tissue. For the second phase of the antibody reaction, 100µl of species–specific anti-immunoglobulins, conjugated to horseradish peroxidise was added to each well (except the no conjugate control wells) and plates were incubated at 37 °C, for 30min, and washed three times by filling the wells with ELISA wash and tapping dry on paper tissue. One hundred µl of tetramethyl benzidine substrate (Sigma) was added to each well and incubated at room temperature, in the dark for 10min. The reaction was stopped by adding 50µl of 10% (v/v) H_2SO_4 to all wells. The absorbencies were read using a spectrophotometer, at 450 nm.

The colour change of the substrate to blue is caused by the bound antibodies. The intensity of the colour increases with the antibody concentration. Addition of H_2SO_4 turned the solution to yellow. The color was recorded using a spectrophotometer.

4.2.13 Examination of changes in the intestinal microflora

At 40 days *post mortem*, caecal digesta from each bird was collected aseptically in Eppendorf tubes containing 50% of glycerol and kept at -80 °C until used for examination of microflora by cultivation on selective media. Ileal and caecal content was also collected, for estimation of species (Risatype) diversity using rRNA Intergenic Spacer Analysis (RISA).

4.2.13.1 Determination of microflora of the caecum by cultivation on selective media

Each caecal digesta sample from the 40 day chickens was mixed with PBS and diluted serially from an initial 10^{-1} dilution to 10^{-9}. For each dilution, 0.1ml was subsequently plated on selective agar media for enumeration of target bacterial groups. All samples were incubated at 37 °C for 72h on the following media: MRS agar for lactobacilli, incubation in anaerobic jars (OXOID, England); Sheep's blood agar (SBA), incubated in an anaerobic cabinet for total anaerobes and incubated aerobically for total aerobes; MacConkey agar, incubated aerobically for total coliforms; Wilkins Chalgren agar, incubated in an anaerobic cabinet for bifidobacteria; Slanetz and Bartley, incubated aerobically for enterococci, and Clostridial agar incubated in an anaerobic cabinet for total numbers of clostridia. All media used were obtained from OXOID, England. Colonies were counted using a Sorcerer Automatic Colony Counter (Perceptive Instruments).

4.2.13.2 Estimation of species (Risatype) diversity using rRNA Intergenic Spacer Analysis (RISA).

The method described by Borneman and Tripplet (1997) and Acinas et al. (1999) was followed.

4.2.13.2.1 Bacterial DNA extraction from chicken caecal and ileum contents

All solutions needed were prepared using sterile water for molecular use:

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

The TE was autoclaved and the remaining solutions were kept at room temperature until used.

Approximately 0.5-1g samples of caecal and ileal contents, that had been removed aseptically during the second *post mortem* and kept in Eppendorf tubes at -20 °C until used, were transferred to Falcon tubes. Then, fresh lysozyme (50mg/ml in TE) was added, mixed, and incubated at 37 °C for 30 min. 105µl of lysis solution was added to each sample and mixed gently. Subsequently, 1.2µl of warmed extraction solution, which had been kept in a waterbath at 60 °C, was added to each sample and mixed gently. An equal volume, approximately 3-4ml, of ice cold phenol (Sigma molecular grade, pre-

equilibrated with TE) was added and the mixture was left in ice for 10min. Then, 2ml of chloroform was added and mixed gently. The samples were centrifuged at 4000rpm, for 10mins, in a Hettich centrifuge (Rotina 46, Tuttlingen, Germany). The clear upper layer was carefully removed to a fresh tube, on ice, taking care not to take any of the protein precipitate at the interface. The chloroform extraction step was repeated 2 more times for the caecal contents and one more time for the ileum contents, to improve purity, although it lowered the yield. The lower chloroform phase was discarded carefully to waste. 0.54 of the total volume of the ice-cold isopropanol was added to each sample, to precipitate DNA. The samples were left for 10mins and were spun at 4000rpm for 10 min. The supernatant was removed leaving the pellet on the bottom of the tube. The pellet was washed by adding about 1ml 70% ethanol. The samples were centrifuged and the supernatant was removed. That step was repeated three times in total. The samples were dried, with the top off, under vacuum, for 20mins. Lastly, the pellet was redissolved in a small volume TE and stored at 4 °C.

4.2.13.2.2 Spectrophotometric assay

The optical density of the DNA mixture was tested using 426 Nanodrop software. The protein and the humic purity was considered as good when A260/A280> 1.8 and A260/A230> 1.7. The yield, in μg, of the DNA was calculated by multiplying the optical density at A_{260} with the number 47 (47μg/ml DNA has an OD of 1.0 at 260nm.

4.2.13.2.3 Polymerase chain reaction - PCR

Bacterial DNA was amplified using PCR primers;

B1055 AATG GCTG TCGT CAGC TCGT (20 bases) and

23SOR TGCC AAGG CATC CACC GT (18 bases),

obtained from Eurofins, London, UK

All the reagents needed for PCR reaction were supplied with the PCR kit (Qiagen, Ltd., West Sussex). A mixture of 2 µl of each bacterial DNA extraction, with 18µl of reaction mix, containing 10µl master mix, 1µl B1055, 1µl 23SOR and 6µl H_2O was prepared in an ultraviolet cabinet (Labcaire) and left overnight at 4°C. Samples were amplified in a PCR thermacycler (TECHNE, Model TC-312) for a period of 4 h. The PCR program was then run as follows: at 95°C for 1min, 55°C for 1 min, 72°C for 2 min plus an extension of 10min at 72°C (Borneman and Tripplet 1997; Acinas et al. 1999).

PCR products were then separated by electrophoresis on a 2%, 'metaphor' agarose gel (Lonza, Rockland ME, USA). A mixture of 0.8g of agarose nondenaturating gel and 60ml of 1xTBE (Tris/Borate/EDTA buffer, Metaphor, Lonza) and 30ml of ethidium bromide was prepared and left overnight, at 4 °C. To prepare the samples for electrophoresis, 5 µl of DNA loading buffer (Blue Bioline) was added to each of the PCR products. The gel was run at 70 volts overnight and the bands were visualised and photographed using a camera on a UV transilluminator (Universal Hood II, BIO-RAD Laboratories, Segrate, Milan,

Italy) and Quantity 1 BIO-RAD Software version 4.6.3 (California, USA). Bands were manually labeled and scanned by densitometer

4.2.14 Intestinal mucosa and liver histology

For histological examination, samples of caecum, ileum and liver were taken from 6 chickens from each group (3 from each of the two *post mortems*). Samples were removed and fixed in neutral buffered formalin until used.

Histology was undertaken in the Electron Microscopy Centre of the University of Plymouth. The material was dehydrated by immersion in a graded series of alcohols of increasing concentration (from 70% to absolute) infiltrated with xylene, and embedded in paraffin. A microtome was used to make 4µl thick sections that were mounted on glass slides and stained with Haematoxylin and Eosin as well as Mallory's trichrome stain.

Slides containing paraffin sections were placed in a slide holder, and deparaffinised and rehydrated (xylene 3 times for 3 min, 100% ethanol 3 times for 3 min, 95% ethanol once for 3 min, 80% ethanol once for 3min and lastly deionised, H_2O once for 5 min). Excess water was blotted before immersing in haematoxylin. For haematoxylin staining, sections were placed in haematoxylin solution for 3 min, rinsed with deionised water, and placed in tap water to allow stain to develop for 5 min. To be destained, samples were dipped in acid ethanol fast, 8–12 times and rinsed with tap water and deionised water, twice for a minute and once for 2 min respectively. After the excess water was blotted

from the slide holder, the slides were placed in eosin for 30min, and then steeped in ethanol 95% and 100% three times, for 5min each, and xylene three times for 15 min. Haematoxylin has a deep blue-purple colour and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins non-specifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.

According to Johnson (1954) Mallory's trichrome stain is used primarily for muscle and connective tissue. After bringing sections to water via xylene and ethanol, they were placed into a solution made by acid fuschin (1g $100ml^{-1}$ distilled water) for 2 min, rinsed with distilled water and placed in phosphomolybdic acid solution (1g $100ml^{-1}$ distilled water) for 2 min. The samples were rinsed quickly with distilled water and placed in another solution made of orange G, methyl blue and oxalic acid (2g, 0.5ml and 2g respectively) in 100ml distilled water. After 15 min, they were washed well with distilled water and dehydrated and differentiated with ethanol. Lastly, they were cleared with xylene and mounted with a resinous medium.

Mallory's trichrome stain stains nuclei red, muscle (cytoplasm), pale red, red blood cells (erythrocytes) orange and collagen fibres deep blue.

The aim was to measure the villus height and crypt depth of the caecum and ileum and the congestion of the liver using an image analyser. Theoretically, the congestion of the liver, i.e. the accumulation of excessive blood in the organ, reflects the intensity of the liver activity, due to either killing the pathogens, or detoxifying the acid (C. Kudi personal communication). Light microscopy was used to observe and take pictures of the samples.

4.2.15 Statistical analysis

The results of the bacteriology studies were analysed using the Minitab statistical package. Linear Statistical Models of analysis of variance (ANOVA) were performed. The Mean and S.D. of the *Salmonella* counts were transformed to their logarithm to base 10, after adding one to prevent zero counts becoming minus infinity. Statistical analysis of swab data for total lactobacilli and *Salm.* Typhimurium nal^r and microflora counts was conducted. Comparisons were made between levels of shedding separately, at all time points, for the same group and for each time point for each group. For *post mortem* data, total lactobacilli counts for caecum and ileum as well as *Salm.* Typhimurium nal^r counts for caecum, ileum, liver and spleen, were determined and comparisons were made between levels of shedding separately at each *post mortem* and for each Group. For ELISA data, absorbencies of the plasma samples at each *post mortem* and control samples for each group were compared.

For the statistical analysis of the estimation of species diversity (band presence/absence and density) of the microflora of caecum and ileum samples using rRNA Intergenic Spacer Analysis (RISA), the Plymouth Routines In Multivariate Ecological Research (PRIMER 6, Primer-E Ltd, Plymouth Marine Laboratory, Plymouth, UK) software package was used. Differences between treatments were analysed by Hierchical clustering (CLUSTER), ordination by non-metric multidimensional scaling (MDS) and permutation-based hypothesis testing (ANOSIM) (Clarke and Warwick 2001).

4.3 Results

4.3.1 Analysis and pH of feed samples

The pH of the moist feed before inoculation or addition of lactic acid was 5.95±0.07. After 24h of fermentation with *Lact.* Salivarius the pH of the FMF decreased to 4.45±0.19, which was significantly higher than the pH of the AMF (4.18±0.18). The lactic acid and acetic acid concentration of the moist feeds did not differ significantly Table 17.

Table 17. Concentrations (mmol L^{-1}) of maltose, glucose, fructose, lactic and acetic acid and pH of AMF and FMF when delivered to chickens*

	Maltose	Glucose	Fructose	Lactic acid	Acetic acid	pH
AMF	106.5±30.5	76.4±20.7	70.0±31.8[a]	459.3±165.0	24.09±15.8	4.18±0.18[b]
FMF	109.3±39.2	73.72±15.0	110.58±27.37[a]	361.8±162.6	77.22±36.3	4.45±0.19[b]

*Each value is the average of eight samples. Same superscripts show significantly different values $P< 0.05$.

4.3.2 Performance Parameters

The average weight gain and feed intake of the birds, during the experiment are shown in Table 18 and 19. There was an increase of average feed intake for all the treatments from week 1 to week 6. The average feed intake per bird of both moist feed groups, i.e. FMF and AMF, was significantly higher ($P<0.01$) than that of birds in the dry feed groups, i.e. CON and WAT. Also, the average feed intake of birds in the AMF group was significantly higher ($P<0.01$) than the birds in the WAT and CON groups. There was an increase of average weight gain for all the treatments from week 1 to week 6. Dietary treatment had a significant effect on the daily live weight gain per bird from days 3-38 of the experiment. Birds in the FMF group had a significantly higher overall average weight gain than birds in the CON and WAT group. At week 3 and 6, chickens fed FMF were significantly heavier than the CON chickens. Birds in the CON group had lower average weight gain than birds in the WAT and AMF treatments.

Table 18. Average feed intake (grams/bird/day dry feed basis) during the 40 days of the experiment for the four groups: CON, WAT, FMF, AMF*

	Week								
Treatment	1	2	3	4	5	6	Mean	SED	SEM
CON	25.20	22.63	45.43	44.92^{ab}	53.28^{cd}	54.95^{fh}	41.07^{AC}	15.16	2.66
WAT	26.56	31.56	41.90	53.41	64.18^{e}	73.86^{gi}	48.58^{BD}	19.36	2.66
FMF	11.20	44.88	64.25	80.09^{b}	114.28^{de}	127.82^{hi}	73.75^{CD}	41.51	2.66
AMF	14.21	38.17	60.60	76.45^{a}	98.54^{c}	114.79^{fg}	67.13^{AB}	35.95	2.66
Mean	19.30^{EFGHI}	34.3^{E}	53.05^{FJK}	63.72^{GLM}	82.57^{HJL}	92.86^{IKM}			
SED	10.83	12.42	14.30	18.26	26.07	31.14			
SEM	2.16	2.16	2.16	2.16	2.16	2.16	$5.31^{\#}$		
P_{value}	P>0.05	P>0.05	P>0.05	P<0.05	P<0.05	P<0.05	$P<0.05^{\#}$		

*Same superscripts show significant different values P<0.05. #SEM and Pvalue for the Treatment x Day interaction

Table 19. Average weight gain (grams/bird/day) during the 40 days of the experiment for the four groups: CON, WAT, FMF, AMF*

	Week								
Treatment	1	2	3	4	5	6	Mean	SED	SEM
CON	50.80	109.56	121.80^{abc}	271.80	384.80	507.00^{d}	240.96^{ABC}	1.55	3.93
WAT	55.70	112.05	186.90^{c}	277.90	392.50	513.10	256.36^{BD}	1.72	3.93
FMF	51.70	117.90	195.20^{b}	301.00	412.00	560.20^{d}	273.00^{CD}	1.36	3.93
AMF	55.20	114.73	196.20^{a}	293.70	409.00	548.90	269.62^{A}	1.41	3.93
Mean	53.35^{EFGHJ}	113.56^{EJKLM}	175.03^{FJNOP}	286.10^{GKNQR}	399.58^{HLOQS}	532.30^{IMPRS}			
SED	4.40	8.63	41.78	26.54	35.42	58.13			
SEM	4.82	4.82	4.82	4.82	4.82	4.82	9.63^{**}		
P_{value}	P>0.05	P>0.05	P<0.05	P>0.05	P>0.05	P<0.05	$P<0.05^{**}$		

*Same superscripts show significant different values P<0.05. **SEM and Pvalue for the Treatment x Day interaction

4.3.3 Bacteriological analysis of cloacal swabs

The mean lactobacilli counts of the birds in FMF treatment at the end of the experiment (8.4 $\log_{10}$ CFU ml^{-1}) was significantly higher ($P<0.05$) than the mean lactobacilli counts of birds in the CON, WAT and AMF treatments (approximately 7.82 $\log_{10}$ CFU ml^{-1}). Birds provided with *Lact.* Salivarius either by water of FMF had significantly higher numbers of LAB than the controls (Table 20). The data in Table 20 show the mean of the total lactobacilli counts of the 10 tagged birds per group for each day of sampling and the significance between the different values in detail.

The percentage of the *Salmonella* negative cloacal swab samples per group during the whole study was 52% for the FMF group and this was significantly greater than the AMF group (25%), the group provided with *Lact.* Salivarius via water (19%) and the CON group (7%). Also, the percentage of salmonella-free days was significantly ($P<0.05$) greater in the AMF group (25%) than the CON group (7%) (Figure 12).

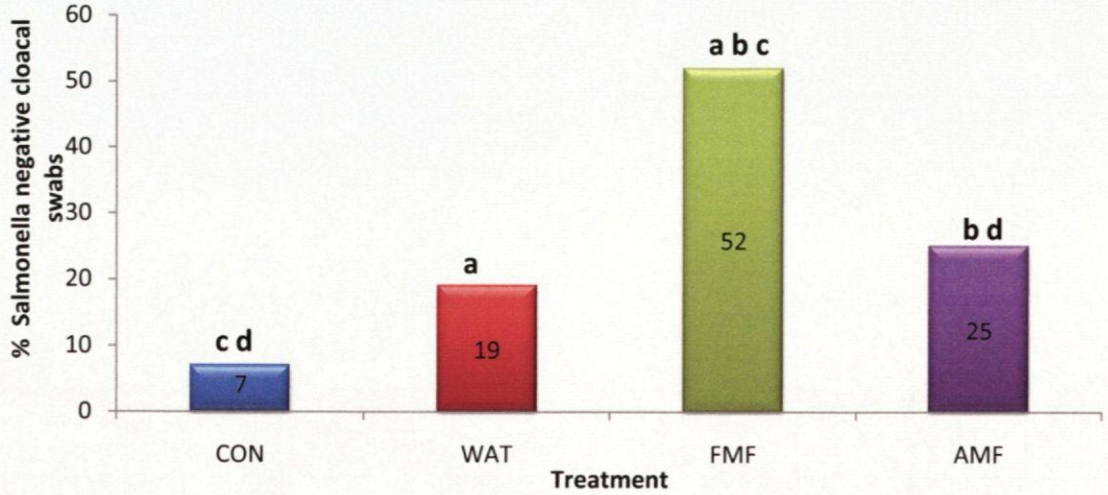
At day 17, three days after they were challenged with *Salm.* Typhimurium, 10, 9, 3 and 2 of the 10 swabbed birds from the WAT, FMF, AMF and CON groups respectively, were found to be *Salmonella* negative. For the WAT treatment there was a sudden and high decrease in the number of *Salmonella* negative birds to 20%, which worsened in the following days, then increased until the last two samplings, where 60-70% of the birds were *Salmonella* positive (Table 21). The data in Figure 13 demonstrate that the number of *Salmonella* negative

birds was more stable in the FMF group since, on most of the days, more than 50% of the swabbed birds were *Salmonella* negative; with the exception of days 33 and 36, when the number of the *Salmonella* negative birds dropped to 30 and 40% respectively. For almost the whole week after challenge, 30% of the AMF chickens were found to be *Salmonella* negative. At day 24, all of the birds were *Salmonella* positive, but the number of *Salmonella* negative birds started to increase gradually until the last sampling, when 60% of the birds were found to be *Salmonella* negative. For the CON group all the chickens were found to be *Salmonella* positive for the majority of the experimental period; with the exception of the last sampling; at which 4 to 10 swabbed birds were found to be negative (Table 21).

Table 20. The mean of the total lactobacilli counts (Log_{10} CFU ml^{-1}) of the cloacal swabs of ten tagged birds for each group

Days	Treatments				Mean	SED	P_{value}
	CON	WAT	FMF	AMF			
5	$8.99^{abcdefgnijA}$	7.97	7.75^{abcA}	7.88^{a}	7.96	0.59	<0.01
8	7.24^{b}	7.26^{a}	8.05^{d}	7.46^{bcd}	7.12	0.34	0.07
12	$7.27^{a\,A}$	B7.90^{b}	8.18^{ABC}	$8.41^{e\,C}$	7.59	0.72	<0.01
15	7.48^{AB}	7.53^{c}	9.00^{adeA}	7.67^{fB}	7.64	0.47	<0.01
17	7.48^{c}	7.75^{d}	8.49b	8.39^{bg}	7.80	0.66	<0.01
19	7.79^{d}	8.02	8.32	8.65^{ch}	7.83	0.66	0.15
22	$7.39^{e,A}$	7.55e	8.81^{A}	7.66^{i}	7.53	0.23	<0.01
24	8.62	$8.56^{abcdefgh}$	8.70^{c}	9.08^{defijk}	8.54	0.53	0.22
26	7.63^{f}	7.77^{f}	8.13	7.65	7.59	0.21	0.02
29	7.88^{g}	7.962^{i}	8.36	8.09^{l}	7.83	0.30	0.85
33	7.17^{hA}	7.82^{g}	8.12^{e}	7.80^{jA}	7.41	0.08	0.02
36	7.74^{i}	8.048	8.33	8.12^{m}	7.83	0.36	0.27
39	7.07^{jA}	$7.01^{h,i,B}$	8.30^{ABC}	$7.03^{aghklmC}$	7.14	0.21	<0.01
Mean	7.67^{ACD}	7.78^{DE}	8.35^{BCE}	8.00^{AB}			
SED	0.63	0.41	0.37	0.50			

Same lowercase superscripts show significantly (P<0.05) different values in the same column and same uppercase superscripts show significantly (P<0.05) different values in the same row



*Same letters show significantly different values (P<0.05). Standard error= 0.3983

Figure 12. Percentage of the *Salmonella* negative cloacal swabs per group during the whole experimental time

Table 21. Number of birds negative for *Salmonella* of the CON, WAT, FMF and AMF groups

Day	Treatment				Mean	SED
	CON	WAT	FMF	AMF		
17	2	10	9	3	6	4
19	1	2	3	5	3	2
22	0	1	5	1	2	2
24	0	1	6	0	2	3
26	0	1	7	1	2	3
29	0	0	5	2	2	2
33	0	1	3	4	2	2
36	0	6	4	5	4	3
39	4	7	7	6	6	1
Mean	1	3	5	3		
SED	1	4	2	2		

The numbers of *Salm.* Typhimurium *nal*^{r} recovered from the cloacal samples of ten chickens from each group on days 17, 19, 22, 24, 26, 29, 33, 36 and 39 are shown in Figure 14 and their P values are presented in Table 22. *Salm.* Typhimurium counts obtained from the cloacal swabs of the *Salmonella* positive chickens of each group were affected significantly by the time, the treatment, as

4.3.4 Post mortem bacteriological analysis

The numbers of lactobacilli in the ileum and caecum of 6 birds from each group killed at day 26 and 40 are presented in Table 23. The mean lactobacilli numbers in the ileum of the FMF and CON treatments were 7.73 and 8.69 log_{10} CFU ml^{-1} respectively, and this was significantly higher ($P<0.05$) than those of birds in the WAT and AMF groups (7.60 and 7.67 log_{10} CFU ml^{-1}, respectively). For the 40 day PM there was no significant difference between them. Mean lactobacilli numbers were significantly higher ($P<0.05$) in the caecal samples of the WAT and FMF group (8.29 and 8.65 log_{10} CFU ml^{-1}, respectively) than the AMF and CON treatments (7.27 and 7.87 log_{10} CFU ml^{-1}, respectively).

Table 23. Lactobacilli counts (Log_{10} CFU ml^{-1}) of ileum and caecum the 26 and 40 PM*

Tissue	Group	*Post mortem*		Treatment Mean
		26 day	40 day	
Ileum	CON	9.08^{hj}	7.94	8.69^{AC}
	SED	3.78	3.38	
	WAT	8.78^{ik}	8.70	7.60^{CD}
	SED	2.91	3.3	
	FMF	6.82^{ghi}	8.51^{g}	7.73^{BD}
	SED	3.56	3.63	
	AMF	7.26^{jk}	8.30	7.67^{AB}
	SED	3.83	3.54	
	P_{value}			<0.01
	SEM			0.13
Caecum	CON	7.46^{gh}	8.29^{f}	7.87^{EHI}
	SED	3.14	3.52	
	WAT	8.45^{bg}	8.12^{di}	8.29^{FH}
	SED	3.34	3.25	
	FMF	8.44^{ah}	8.87^{ci}	8.65^{GI}
	SED	3.54	3.38	
	AMF	7.19^{ab}	7.35^{cdf}	7.27^{EFG}
	SED	3.08	3.08	
	P_{value}			<0.01
	SEM			0.11

*Same superscripts show significantly different values $P< 0.05$.

The numbers of *Salm.* Typhimurium *nal*^{r} negative birds in the six birds slaughtered from each group at the 26 and 40 day *post mortem* and their counts are presented in Table 24 and 25, respectively. Most of the ileal and caecal samples were found to be *Salmonella* positive. The FMF and WAT groups, had significantly lower ($P<0.05$) *Salmonella* counts (mean of the two *post mortems*) for ileal samples (1.23 $\log_{10}$ CFU ml^{-1} and 1.71 $\log_{10}$ CFU ml^{-1}, respectively), compared with the CON (3.39 $\log_{10}$ CFU ml^{-1}). The FMF group at the 26 day *post mortem* had significantly ($P<0.05$) lower *Salmonella* counts (1.08 $\log_{10}$ CFU ml^{-1}) compared to the CON (6.61 $\log_{10}$ CFU ml^{-}1)and AMF groups (6.10 $\log_{10}$ CFU ml^{-1}). Two of the six birds from the FMF group were found to have *Salmonella* negative caecal samples and the mean *Salmonella* counts for the caecum (for the first PM) of FMF and WAT were significantly lower than the CON. At the 26 day PM, each of the WAT and AMF groups had 1 bird with a *Salmonella* free liver, however, the FLF and CON treatments all had positive livers. The enumeration of the mean liver *Salmonella* counts revealed that the AMF group had significantly ($P<0.05$) higher *Salmonella* counts (0.42 $\log_{10}$ CFU ml^{-1}) than the WAT group (1.17 $\log_{10}$ CFU ml^{-1}). The second *post mortem* liver sampling resulted in *Salmonella* negative samples; except for two birds from the AMF treatment. For the spleen there appeared to be a trend toward lower recovery of *Salmonella* from birds supplied with *Lact.* Salivarius through water and FMF than those fed the acidified and control diets. Six of the ten birds in the AMF group had *Salmonella* negative spleens, which had significantly higher *Salmonella* counts than the WAT and FMF groups. With regard to recovery of *Salm.* Typhimurium from the muscle, there were significantly ($P<0.05$) fewer organisms from the WAT group than the AMF group (Table 25).

Table 24. Number of *Salmonella* negative birds at 26 and 40 day PM

Post mortem	Treatment	Tissue					Average
		Ileum	Caecum	Liver	Spleen	Meat	
26 day PM	CON	0	0	0	0	1	1
	WAT	0	0	1	0	4	5
	FMF	1	0	0	1	2	4
	AMF	2	0	1	1	3	7
40 day PM	CON	0	0	6	1	5	12
	WAT	2	0	6	6	5	19
	FMF	0	2	6	3	6	17
	AMF	1	0	2	0	1	4
Mean		1	0	3	2	3	

Table 25. *Salmonella* counts (Log_{10} CFU ml^{-1}) in tissue samples at 26 and 40 day PM*

		Treatment				P_{value}	SEM
Tissue	*Post mortem*	CON	WAT	FMF	AMF		
Caecum	26 day PM	5.03^{ef}	1.20^{f}	1.05^{e}	3.96		
	40 day PM	4.79	4.55	2.91	4.53		
	Mean	4.01	4.00	2.21	3.64	>0.05	0.56
	SED	0.17	2.44	1.31	0.40		
Ileum	26 day PM	6.61^{cd}	6.09	1.08^{ac}	6.10^{ab}		
	40 day PM	4.18^{d}	6.33	6.68	3.77^{b}		
	Mean	3.39^{AB}	1.71^{A}	1.23^{B}	2.27	<0.05	0.43
	SED	1.72	0.17	3.96	1.65		
Liver	26 day PM	2.51	0.96	1.13	0.98		
	40 day PM	0.00	0.00	0.00	3.15		
	Mean	1.04	0.42^{C}	0.56	1.17^{C}	<0.05	0.20
	SED	1.78	0.68	0.80	1.54		
Spleen	26 day PM	4.14	1.64	1.63	3.47		
	40 day PM	3.54	0.00	0.98	4.88		
	Mean	2.05^{F}	0.81^{EF}	1.02^{D}	2.39^{DE}	<0.05	0.33
	SED	0.42	1.16	0.46	1.00		
Muscle	26 day PM	0.93	0.57	0.88	0.85		
	40 day PM	0.30	0.33	0.00	2.08		
	Mean	0.48	0.23^{G}	0.35	0.81^{G}	<0.05	0.15
	SED	0.45	0.17	0.62	0.87		

*Same superscripts show significantly different values P<0.05

4.3.6 Immunology- Enzyme linked Immunosorbent Assay (ELISA) – Antibody capture

The negative control was found to have some background colour, which might have been caused by insufficient washing of plates or plates being left too long before reading on the plate reader and the colour continuing to develop (though at a slow rate because the stop solution had been added).

The average of the values before challenge and the blank values were used as the minimum value of the optical density (OD 450nm). There was no indication of production of *Salm.* Typhimurium antibodies within the samples of all the groups at 28 days of sampling as the optical density of the serum was similar to or lower than that of the non-challenged birds. At 39 days sampling, birds on the FMF and AMF threatments had no production of antibodies for *Salm.* Typhimurium (corresponding to optical density values of 0.00 OD_{450nm}). The mean OD_{450nm} of the 39 days PM of the FMF and AMF groups was significantly ($P<0.05$) lower than the mean OD_{450nm} of the CON group (0.11 OD_{450nm}). The mean optical density of the WAT (0.06 OD_{450nm} units) did not differ significantly compared to the CON, FMF and AMF treatments.

4.3.7 Examination of changes in the intestinal microflora

4.3.7.1 Determination of microflora of the caecum by cultivation on selective media

There were no statistically significant differences between treatments for total aerobes, total anaerobes, coliforms, bifidobacteria and enterococci. When a general linear model of anova was performed for all the types of bacteria tested, it was found that the caecal microflora of birds fed AMF had significantly lower ($P<0.01$) concentrations of lactobacilli (7.35 log_{10} CFU ml^{-1}) than birds from the FLF (8.87 log_{10} CFU ml^{-1}), WAT (8.12 log_{10} CFU ml^{-1}) and CON (8.28 log_{10} CFU ml^{-1}) groups. Significantly higher *Lactobacillus* counts were found in the caecum of the birds of the FMF group than those from the CON and the WAT group. In addition, the numbers of clostridia and coliforms for the WAT (9.85 and 8.34 log_{10} CFU ml^{-1}, respectively), FLF (9.81 and 8.35 log_{10} CFU ml^{-1}, respectively) and CON (9.61 and 8.09 log_{10} CFU ml^{-1}, respectively) groups were significantly higher than those of the AMF group (8.72 and 7.23 log_{10} CFU ml^{-1}, respectively) (Table 27).

Table 27. Caecum microflora (microbial counts in Log_{10} CFU $ml^{-1)}$ at day 40*

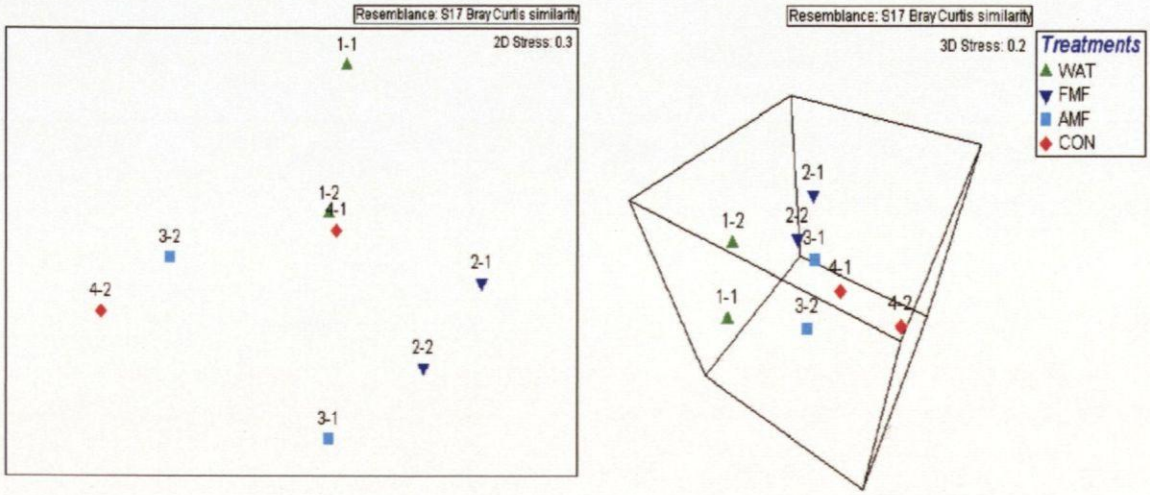
	CON		WAT		FMF		AMF		SEM	P_{values}
	Mean	SED	Mean	SED	Mean	SED	Mean	SED		
Total anaerobes	8.37	0.31	8.41	0.23	8.33	0.05	8.40	0.56	0.14	0.98
Total aerobes	8.07	0.58	8.53	0.22	8.27	0.21	8.42	1.38	0.31	0.75
Bifidobacteria	8.68	0.13	8.49	0.36	8.45	0.18	8.51	0.46	0.13	0.61
Lactobacilli	8.28^{ad}	0.43	8.12^{ce}	0.31	8.87^{bde}	0.28	7.35^{abc}	0.38	0.15	<0.05
Enterococci	7.86	0.29	8.19	0.35	8.30	0.19	8.08	0.40	0.13	0.13
Clostridia	9.61^{h}	0.56	9.85^{f}	0.21	9.81^{g}	0.11	8.72^{fgh}	0.71	0.19	<0.05
Coliforms	8.09^{k}	0.45	8.34^{l}	0.28	8.35^{j}	0.14	7.23^{ijk}	0.39	0.14	<0.01

*Same superscripts show significant ($P<0.05$) values

4.3.7.2 Estimation of species (Risatype) diversity using rRNA Intergenic Spacer Analysis (RISA).

A photograph of the high resolution gels containing samples obtained from caecum and ileum from chickens of each treatment is presented in Figure 16. Not all the PCR products were run successfully in the gel, probably because the cells of the gel were contaminated, so two of the caecum samples and five of the ileum samples from each group were selected for statistical analysis. S17 Bray Curtis similarity dendrogram and two and three dimensional multidimensional scaling ordinations based on Curtis similarities for caecum and ileum are shown in Figures 17-20.

Within treatment similarity (SIMPER) and between treatments dissimilarity percentages are presented in Table 28. As far as the ileum was concerned, the Bray Curtis similarity dendrogram (Figure 17) demonstrated that treatments with *Lact.* Salivarius (WAT and FMF) showed high similarity between them. The CON and AMF groups also showed high similarity. For the caecal samples, the FMF and AMF groups showed high similarity (Figure 19).



*Each sample name consists of two numbers; the 1st one is the Treatment (1-WAT, 2-FMF, 3-AMF, 4-CON) and the second one is the replicate for each sample (1-2-3-4-5) found in each sample

Figure 20. Two and three dimensional multidimensional scaling ordinations based on Curtis similarities (2D and 3D Stress-0.3; 1-WAT, 2-FMF, 3-AMF, 4-CON) for caecum*

Table 28. Within treatment similarity (SIMPER) and between treatments dissimilarity percentages

	Groups	Average similarity (%)	Average dissimilarity (%)		
			CON	WAT	FMF
Ileum	CON	74.77	-		
	WAT	69.96	28.68	-	
	FMF	98.83	27.16	25.75	-
	AMF	90.93	29.13	27.19	5.24
Caecum	CON	44.41	-		
	WAT	61.75	60.33	-	
	FMF	50.22	62.62	46.10	-
	AMF	43.29	56.81	55.26	59.07

Table 29. Level of congestion of liver samples

Treatment	*Post mortem*	Sample	*Salmonella* Positive: + Negative: -	Congestion Low: L Medium: M High: H
CON	28 day	1	+	M
		2	+	H
		3	+	M
	39 day	1	-	L
		2	-	M
		3	-	M
WAT	28 day	1	+	M
		2	+	M
		3	+	M
	39 day	1	-	M
		2	-	L
		3	-	M
FMF	28 day	1	+	M
		2	+	H
		3	+	M
	39 day	1	-	L
		2	-	L
		3	-	M
AMF	28 day	1	+	L
		2	+	M
		3	+	M
	39 day	1	+	M
		2	+	H
		3	-	L

concentrations, through water, had no significant effect on Salmonella colonisation. Providing the same amount of lactic acid in AMF as found in FMF resulted in an intermediate reduction in Salmonella. Salmonella counts in the CON group were significantly higher than the groups provided *Lact.* Salivarius, either via feed or water, or lactic acid (Table 22).

No previous studies were found comparing the administration of probiotics through water and FMF and consequently to compare with the results of the present study, which showed that prophylactic treatment of chickens against *Salm.* Typhimurium with FMF provides superior and more consistent protection against *Salmonella* colonization than treatment via water. The WAT treatment was not only used to evaluate the efficiency of this mode of delivering the *Lact.* Salivarius strain to chickens, but also to compare the extent of the *Salmonella* reduction in comparison to FMF. It was also designed to investigate whether the antimicrobial properties of the FMF are due to the presence of *Lact.* Salivarius alone, or depend also on liquid feeding and the presence of lactic acid produced by LAB.

Chickens fed FMF were provided continually with much higher numbers of LAB (10^9 CFU ml^{-1}). An attempt was made to design WAT and FMF treatments in such a way that birds of each group received, on average, the same amount of lactobacilli. The hypothesis was that, considering that during its lifetime, a 2.3-kg broiler will consume about 8.2kg of water, compared with approximately 4.5 kg of feed (Lacy 2002), thus the chickens of the WAT group should have

received 100 times more *Lact.* Salivarius, than the FMF group. However, it was found that getting 10^9 CFU ml^{-1} water in a practical situation was impossible and eventually chickens of the WAT treatment were provided with only 10^7 CFU ml^{-1} of *Lact.* Salivarius per ml of water. The FMF fed chickens were provided with a higher dose (10^9 CFU ml^{-1}) of *Lact.* Salivarius per g of feed.

In this experiment, LAB were administered through drinking water, which is a practice that producers could more readily use in practice than application of probiotics directly into the crop of the chick by gavage, which is mainly used in laboratory studies. Also, it was also hypothesised that continual treatment with a high number of LAB could have better results than a single treatment. Generally, the use of a single oral dose of isolated lactic acid bacteria has not always been successful. For example, La Ragione et al. (2004) showed that a single oral dose of 10^9 CFU ml^{-1} *Lact. johnsonii* F19758 was not sufficient to suppress *Salm. enteritidis*. Zhang et al. (2007) observed a reduction of *Salm.* Typhimurium prevalence from 33 to 54% compared with controls and a decrease of the average *Salm.* Typhimurium counts in positive chickens by 1.5 to 2.5 log CFU g^{-1}, when each of 6 strains typed as *Lact.* Salivarius were administered by gavage (10^7 to 10^8 CFU ml^{-1} per chicken) two times, i.e. on day of hatch and the following day. Additionally, Bezckorovainy (2001), following clinical pharmacokinetic studies, suggested that in order to have a continuing probiotic effect the probiotic culture must be constantly ingested.

The results obtained from this study showed that the percentage of the *Salmonella* negative cloacal swab samples per group during the whole study was not significantly different for the WAT group provided with *Lact.* Salivarius via water (19%) and the CON (7%). However, the caecal Salmonella counts of the WAT group were significantly ($P<0.05$) lower than those of the CON group (1.20 and 5.03 $\log_{10}$ CFU ml^{-1}, respectively) at the 26 day *post mortem.* The results obtained in this study are in agreement with those from Promsopone et al. (1998). Chickens were administered *Avian Pac Plus*, by spray, at the hatchery, in the water for two days and were challenged, six hours postplacement, with 10^7 CFU ml^{-1} of *Salm.* Typhimurium. The mean caecal and colonic concentration of *Salm.* Typhimurium from the *Avian Pac Plus*-treated group was significantly lower at days 31, 38 and 43 than the nontreated control group. Although, the positive results against *Salmonella* of the above experiment might be attributed to the fact that there is some evidence (Stavric and D'aoust 1993) suggesting that the use of simple defined and complex undefined competitive exclusion mixtures have been more successful than using a single isolated lactic acid bacterium, the use of the single strain of *Lact.* Salivarius NCIMB 41606 in water reduced significantly the caecal *Salm.* Typhimurium counts compared to the nontreated CON group.

There is some evidence that probiotic effects are dose dependent. Galdeano and Perdigon (2006) reported a probiotic, dose-dependent, stimulation of gut immune cells to release inflammatory and regulatory cytokines such as gamma interferon, interleukin- 12 (IL-12), IL-14 and IL -10. According to Mack (2005)

the dose dependence of the probiotic effect is controversial, as most of the studies are based on *in vitro* experiments. The usual effective dosage in humans is 10^7 to 10^9 CFU g^{-1} per day (Mack 2005). As far as chicken nutrition is concerned, the inclusion levels required to have any probiotic effect and specifically antibacterial properties vary between 10^6 and 10^9 CFU ml^{-1} (Promsopone et al. 1998; Tellez et al. 2001; La Ragione and Woodward 2003; La Ragione et al. 2004; Higgins et al. 2007; Zhang et al. 2007; Vicente et al. 2008). In this experiment, chickens were provided with two different doses of the *Lactobacillus* culture i.e. 10^7 CFU ml^{-1} through water for the WAT treatment and a higher one of 10^9 CFU ml^{-1} through FMF. As described above the FMF treatment significantly reduced *Salmonella* recovery, whereas the WAT treatment did not differ significantly from the CON. This might suggest that the reduction of *Salmonella* is dose-dependent and that administration of *Lact.* Salivarius culture at high levels is more effective than at low levels. This is in agreement with the conclusions of Higgins et. al (2008), who suggested that oral administration of 10^6 or 10^8 CFU ml^{-1} of a *Lactobacillus*-based probiotic culture (FM-B11) to neonatal broiler chicks, within 1h of challenge with 10^4 CFU ml^{-1} of *Salm. enteritidis*, significantly reduced *Salmonella* recovery, whereas a 100 times lower dosage had no significant effect.

The total numbers of LAB in the FMF prepared by inoculation with *Lact.* Salivarius was 9 log_{10} after 24 h incubation, though the numbers of lactobacilli from the cloacal swabs were 8 to 9 log_{10}. Whether this number reflects the *Lact.* Salivarius consumed, or an ability of the *Lact.* Salivarius to shift the numbers of

the total resident lactic acid bacteria in comparison to the CON group, was not established. Similarly, in this study it was not possible to establish whether the *Lact.* Salivarius adhered to epithelial cells *in vivo*. The average number of lactobacilli recovered from the cloacal swabs through the experiment was significantly ($P<0.05$) higher for the FMF group (8.4 $\log_{10}$ CFU ml^{-1}) in comparison to the WAT (7.8 $\log_{10}$ CFU ml^{-1}) and CON (7.7 $\log_{10}$ CFU ml^{-1}) groups, which might reflect the fact that the *Salmonella* negative cloacal swabs of the FMF treatment were significantly higher ($P<0.05$) (52%) than the WAT (19%) and the CON (7%) treatment. The difference in the dose of *Lact.* Salivarius that chickens received through water and FMF is an obstacle to assuming that *Lact.* Salivarius via FMF might be more probiotic than *Lact.* Salivarius delivered via water. Similarly, the treatment does not resolve the mechanism by which *Lact.* Salivarius might eliminate the *Salmonella*; whether it is competitive exclusion, based on its ability to aggregate and coaggregate with *Salmonella* or/and its antimicrobial activity against *Salmonella* as demonstrated in the screening process.

The anti-*Salmonella* property of FMF in poultry and pigs has been demonstrated in several papers by Heres et al. (2002; 2003a; 2003c; 2004; 2004b) and by van Winsen et al. (2001a; 2001b), respectively. Heres (2004) reported that 14 out of 18 pens in the chickens fed dry feed and only 4 out of 18 of chickens fed FMF were found to be positive for *Salmonella* when chickens were inoculated with a dose of 10^3 CFU ml^{-1} *Salm. enteritidis*. Heres et. al (2003b) have also shown that broiler chickens fed FMF are less susceptible to a

single oral inoculation with *Salmonella* and *Campylobacter* than chickens fed a normal dry feed. In another experiment, they found that chickens fed FMF required a longer time after inoculation with *Salm. enteritidis,* or a higher inoculation dose, to get the same number of infected chickens in comparison with chickens fed dry feed, indicating that there is a 1 to 10 times difference in *Salm. enteritidis* susceptibility between FMF–fed chickens and dry feed-fed chickens (Heres et al. 2003a). In all of his experiments, Heres added 1.4 kg water per kg dry feed and the feed was inoculated with a *Lact. Plantarum* culture and incubated at 30 °C, for 24 to 48h. In the current experiment, it was found that this ratio of water to dry feed was creating practical problems. The FMF was too liquid and was forming a crust on the top of the feed, when placed on the feed tray, so the chickens could not picked it up easily. So, the 'the water to feed' ratio was reduced to 1.2 kg water: 1 kg dry feed. As can be seen from the feed intake data, FMF of this moisture content was readily accepted by the chickens and feed intake and growth rate were improved as a consequence.

The AMF treatment was designed to elucidate whether the effect of the FMF is just due to the production of acid. The two treatments were designed to contain the same amount of lactic acid. In practice, after 24 h, although the lactic acid values did not differ significantly (the FMF incubated with *Lact.* Salivarius contained 361.8±162.6 mmol L^{-1} of lactic acid and the AMF contained 459.3±165.0 mmol L^{-1}), the pH of the feeds differed significantly ($P<0.05$); 4.45 ± 0.19 and 4.18 ± 0.18 for the FMF and AMF, respectively. These differences might be attributed to the buffering capacity of the pelleted feed. In this

experiment, 30.3 ml of lactic acid was added per kg of wet feed, or in other words AMF consisted of 7.2% of lactic acid per kg dry pelleted feed. Commercially available poultry feed acidifiers, such as BIO-add® NC Liquid (formic acid 32%, ammonium formate 27%-total formic acid 52% and propionic acid 15%), are used at levels between 0.25 and 1.3% to control *Salmonella* incidence. Dibner and Buttin (2002) suggest that usage of formic acid at levels between 0.5 and 1.5% can have the most reproducible effects on poultry performance. These recommended levels are much lower than those used in this experiment, although Heres et al. (2004a) also used high levels of organic acids, 5.7% lactic acid or 0.7% acetic acid in dry feed to reduce *Campylobacter*, but not *Salmonella*, in poultry. They reduced *Campylobacter* and *Salmonella* to undetectable levels in contaminated broiler feed within 20min and 2 h, respectively.

AMF exerted some anti-*Salmonella* ability, as 25% of the cloacal swabs were found to be *Salmonella* negative and significantly lower than the negative cloacal swabs of the FMF treatment (52%). So, the high concentration of lactic acid may have contributed to the improved resistance of FMF-fed chickens to *Salmonella*.

According to Heres et al. (2003c), the high concentration of lactic acid reduces further the pH in the crop and gizzard. According to Cherrington et al. (1991), the full action of organic acids is expressed in the crop due to its high moisture content. When the pH decreases, the nondissociated form of organic acid

passes through the cell membranes and once inside the cell dissociates the molecule and decreases intracellular pH. Consequently, the enzymatic processes are interrupted, the proton motive force collapses and the cell dies. According to Heres et al. (2003c), lactic acid acts as a permeabiliser of the gram-negative bacterial outer membrane and may operate as a potentiator of the effects of other antimicrobial substances. Thus the probability that *Salmonella* passes the crop and gizzard and reaches the caecum is reduced (Heres 2002; Heres et al. 2003a). In addition to lactic acid, other organic acids (such as butyric, caprylic etc) are produced during fermentation and might also act against *Salmonella*. Doyle and Erickson (2006) found that 50 mmol L^{-1} of caprylic acid reduced by 5 log CFU ml^{-1} and 100 mmol L^{-1} of it completely inactivated *Salm. enteritidis* in autoclaved chicken caecal contents within 1 min and 24 h, respectively. The mechanism of action of the acids against *Salmonella* is also related to their ability to change the expression of invasion genes in *Salmonella*. Specifically, the short-chain fatty acid butyrate down-regulates expression of invasion genes in *Salmonella* spp. at low doses (Van Immerseel et al. 2006b). Also, medium-chain fatty acids and propionate decrease the ability of *Salmonella* spp. to invade epithelial cells, in contrast to acetic acid. The antibacterial effect of dietary organic acids in chickens is believed to take place mainly in the upper part of the digestive tract (crop and gizzard). When high concentrations of a combination of formic and propionic acid (Bio-Add) were provided to chickens, the acids were recovered only from the crop and gizzard (Thompson and Hinton 1997). Additionally, only little if any dietary propionic acid reaches the lower digestive tract and the caeca when provided to chickens (Hume et al. 1993). This might explain partly the high

number of positive *post mortem* samples from the caecum, ileum, spleen and liver of the AMF group. Increasing amounts of dietary lactic acid (0.25, 0.5, 1.0 and 2.0%) did not decrease caecal *Salmonella* colonisation when broiler chickens were challenged with *Salm.* Typhimurium (Waldroup et al. 1995), which is in agreement with the results of the current experiment. Recently, there have been attempts to prevent absorption of the acids in the upper gastrointestinal tract by micro-encapsulation of acid, which guarantees their release further down in the gastrointestinal tract (Van Immerseel et al. 2005; Van Immerseel et al. 2006b).

It has to be mentioned that lactic acid is not considered a cost effective way of acidifying poultry feed and in this experiment the AMF was used as a means of comparing the FMF with the AMF and to investigate whether or not the acid alone is responsible for the suppression of *Salmonella.* The benefits of the FMF cannot only be attributed to its lactic acid content. The percentage of the *Salmonella* negative cloacal swab samples per group during the whole study was 52% for the FMF group and this was significantly greater than the AMF group (25%) and the group provided with *Lact.* Salivarius via water (19%). It appears that there is a synergistic effect of both the high numbers of lactobacilli and the production of lactic acid in the FMF on the control of *Salmonella* status of chickens. Fermentation of liquid diets for poultry could provide a more cost effective means of acidifying diets than the use of organic acids.

Once *Salmonella* bacteria enter the intestinal tract of the chicken they attach to the epithelium by attachment structures, such as fimbriae, and afterwards penetrate the mucus layer (Revolledo et al. 2006). The upper intestinal tract of chickens is regarded as an acidic barrier to pathogens (pH 2.6 for the gizzard). However, the passage through the intestinal tract is a dynamic process, and although some bacteria stay in contact with antimicrobial substances in the crop and gizzard for a long time, other pass directly to the duodenum (Heres et al. 2003c). In poultry, caeca are favoured sites for *Salmonella* colonization. Even the presence of low numbers of *Salmonella* allow fresh caecal contents to become inoculated after periodic emptying and refilling of the caecum (Revolledo et al. 2006). When *Salmonellae* cross the intestinal epithelium, they contact the lamina propria, where they can multiply or enter into deeper tissues, and after reaching the blood stream, they infect internal organs such as liver and spleen (Van Hemert et al. 2007). In the current experiment, the liver was less contaminated than the ileum, caecum or spleen. Although, at the first *post mortem* the liver samples were mainly *Salmonella* positive, at the second *post mortem* 20 of the 24 samples (of all groups) were found to be negative to *Salmonella* bacteria. This might be explained by the fact that the blood stream leads directly to the liver after passing from the small intestine and spleen, which acts as a mechanical filter of the blood and competes against infections.

Heres et al. (2003c) found that FMF decreased *Salmonella* in the anterior parts of the gastrointestinal tract in chickens, i.e. crop and gizzard, more than the posterior intestinal compartment, i.e. duodenum, caecum and colon/rectum.

Salmonella colonises the intestinal lumen of the caecum due to the slow flow rate and attachment to epithelial cells. Heres (2003c) suggests that it is questionable whether probiotics could prevent colonization of *Salmonella* in the caeca, unless there are specific lactobacilli that have particular negative interactions with *Salmonella*. In this experiment, the *Lact.* Salivarius used had been selected for its probiotic properties, and this resulted in significantly lower *Salmonella* counts (1.078 $\log_{10}$) in comparison to the other three groups. The improvement was almost 6 $\log_{10}$ at the first *post mortem* and even more at the second *post mortem*, at which 2 of the 6 birds were found to have *Salmonella* negative caeca. The other groups all had *Salmonella* positive caeca. However, the ilea of these birds were found to be *Salmonella* positive.

The sensitivity of detection *Salmonella* in cloacal swabs was not 100%, as it was observed that chickens that showed no *Salmonella* in the cloacal swabs had *Salmonella* positive caeca, or/and ileum, or/and liver, or/and spleen colonized at a low level. These findings are in agreement with those of Heres et al. (2003a). It takes some time before levels of colony-forming units of *Salmonella* in the caecum are sufficiently high to detect *Salmonella* by cloacal swabbing. Enrichment helps to detect the presence of even one bacterium of *Salmonella* and consequently makes the method more accurate.

On average, the time shedding was longer in the FMF group than in the WAT treatment, followed by the AMF treatment and lastly by the CON group, which is in agreement with Heres and co-worker's conclusions (2003a). At day 17, three

days after challenged with *Salm.* Typhimurium, 10, 9, 3 and 2 of the 10 swabbed birds of the WAT, FMF, AMF and CON treatments respectively, were found to be negative. The sudden and large decrease in *Salmonella* negative birds to 20% in the WAT group could not be explained and needs further investigation. However, at the end of the experiment, 70, 60, 40 and 40% of the cloacally swabbed birds of the WAT and FMF, the AMF and the CON group respectively, did not shed *Salmonella*.

The detection of the presence of *Salmonella* antibodies in the chicken serum at day 39 showed significantly higher ($P<0.01$) number of detected *Salmonella* antibodies for the CON group in comparison to the FMF and AMF group. This might reflect the level of infection of each group, suggesting that the more infected the chicken is, the more *Salmonella* antibodies are detected in its blood serum. Lactobacilli usually cause the immune-modulation by stimulating the production of natural antibodies in chickens, increasing the resistance against pathogens (Jin et al. 1997; Haghighi et al. 2005; Haghighi et al. 2006). Feeding a diet containing *Bacilus subtilis* enhanced the production of antibodies against Newcastle disease virus (Khaksefidi and Ghoorchi 2006), although Dunham et al. (1993) observed improved production of anti-salmonella IgM antibodies in newly hatched chicks when fed diets containing *Lact. Reuteri* and Kabir et al. (2004) found the antibody production of chickens fed a commercial probiotic (Protexin Boost) was higher than the control. In the current experiment, the production of *Salmonella* antibodies was limited for the FMF treatment although the lactobacilli counts were high, which is probably due to the lower infection

rate compared with CON. Further experiments should be undertaken to assess the potential of *Lact.* Salivarius NCIMB 41606 to affect the host immunity.

The treatments imposed also had an influence on environmental contamination or environmental pathogen populations. In general, the isolator used for the FMF treatment was the least contaminated; as 3/5 samples collected were found to be negative in comparison to the rest of the isolators; WAT treatment followed CON and lastly AMF with 1, 0 and 0 /5 negative samples. Despite the effective treatment with FMF, *Salmonella* incidence was not excluded completely. Secondary infection transmission might occur between the chickens in the same isolator. Transmission itself, and the rate at which it occurs, depends on the susceptibility of individual birds and the infection from the environment (Heres et al. 2003a). As chickens are coprophagic, it is inevitable that they ingest *Salmonella* at levels high enough to be infective. Although, airborne transmission might also have occurred (Heres et al. 2003c) in the same isolator, bacterial transmission between the different isolators can be excluded, as there was a negative pressure system and the birds were handled with aseptic equipment and solutions.

The scale of this experiment does not permit a confident assessment of any differences in performance. The main reason for measuring feed consumption and average weight gain of the birds was to be able to demonstrate that none of the treatments seriously compromised performance. As it was, there was a significant increase in consumption of the moist feeds (FMF and AMF treatments). Several researchers have reported improved feed intake and feed

conversion of chickens provided probiotics (Kabir et al. 2004; Gil de los santos et al. 2005; Takahashi et al. 2005; Khaksefidi and Ghoorchi 2006; O'Dea et al. 2006; Timmerman et al. 2006; Apata 2008), FMF (Brooks et al. 2003b; Brooks 2008; Canibe et al. 2008) and AMF (Falkowski and Aherne 1984). However, in the current study, it is possible that feed wastage of the moist feeds was higher than the dry feeds from the circular tray feeders used. Forbes (2003) suggested that the lack of suitably designed troughs, for providing wet feed, results in considerable feed wastage.

The current study showed that exposing the chickens to FMF fermented with *Lact.* Salivarius, to drinking water containing *Lact.* Salivarius and to AMF containing lactic acid, did not result in any negative consequences for the chicken's health or performance. The average weight gain of the birds provided *Lb* Salivarius (either through FMF or water), or lactic acid, were higher than the weight gain of the birds fed a basal diet. This might be attributed to the possible improved nutrient digestibility. Ghadban (2002) reviewed several scientific papers and suggested that lactobacilli produce digestive enzymes *in vitro* that may enhance the concentration of intestinal and bacterial digestive enzymes. Enzymes of the gastrointestinal tract increase the digestion of the nutrients, especially in the lower intestine (Jin et al. 1997). Providing *Lact.* Salivarius through FMF enhanced significantly ($P<0.05$) the average weight gain compared with administration through water. Also, liquid and fermented feeds have been reported to improve feed intake and protein digestibility (Brooks 2008). Chah et al. (1975) reported significantly improved ($P<0.05$) weight gain

and feed efficiency in broilers that were fed soybeans that had been moisture conditioned to *ca.* 31% moisture and fermented in chamber for 2-3 days, at 23-30 °C, with several strains of *Aspergilli.* They concluded that this was probably due to a greater supply of the essential amino acids. More recently, Dung et al. (2005) found that organic matter and protein digestibility were significantly greater ($P<0.05$) in a FLF diet made with broken rice and fed to growing finishing pigs, than a dry feed, or a non–fermented diet acidified with lactic acid feed to pH 4. However, care should be taken, as during steeping of the feed, phosphorus is released from phytate, making it biologically available and hence, affecting the calcium-phosphorus (Ca/P) ratio. As a consequence the diet provided to chickens may need reformulation to correct the ratio of available Ca:P. Also, an increase in the numbers of beneficial bacteria in the microflora may reduce nutrient competition between the enteric bacteria and the host.

There are studies that have shown that probiotics do not significantly change the gut microflora profile of broilers (Jin et al. 1998c) and others that resulted in a beneficial modulation of the caecal microflora (Mountzouris et al. 2007). In the current study, there were no statistically significant differences among treatments for total aerobes, total anaerobes, bifidobacteria and enterococci. Though, significantly ($P<0.05$) higher lactobacilli counts were found in the caecum of the birds of the FMF group (8.87 $\log_{10}$ CFU ml^{-1}) than those of the CON (8.28 $\log_{10}$ CFU ml^{-1}), WAT (8.12 $\log_{10}$ CFU ml^{-1}) and AMF group (7.35 $\log_{10}$ CFU ml^{-1}). The AMF group had significantly lower lactobacilli, Clostridia and Coliforms than FMF, WAT and CON groups. Mountzouris et al. (2007)

found significantly ($P<0.05$) increased bifidobacteria, lactobacilli and gram-positive cocci in treatments containing a multiple strain probiotic in feed and water, and probiotic only in the feed compared with the control and antibiotic treatments.

Whether the total lactobacilli counts of each group were of the same strains, or *Lact.* Salivarius was included in these numbers, was not examined. Though, as has been mentioned, the lactobacilli colonies found in the AMF birds were morphologically different from those of the other three groups. Molecular techniques could help to elucidate further whether the population changes in treatments FMF and WAT could be solely attributed to the *Lact.* Salivarius. In general, lactic acid bacteria are able to develop at comparatively low pH, which means that they are more resistant to organic acids than other bacterial species, e. g. *Salmonella* (Van Immerseel et al. 2006b). A reason for this may be that gram-positive bacteria have a high intracellular potassium concentration, which provides a counteraction for the acid anions (Russell and Diez-Gonzalez 1998).

Recent molecular studies for the characterisation of the composition of the chicken microbial ecology using 16S ribosomal RNA for phylogenetic analysis have shown that there are a broad range of uncultured bacteria, or bacteria whose culture conditions are not known, which cannot be identified by culture dependent methods (Bjerrum et al. 2006). According to (Apajalahti et al. 2004) a very small number, probably less that 10% of species, are regarded as

previously known bacterial species and the remaining belong to new species, or even new genera. For this experiment, the differencies in the numbers of two major bacterial groups (total aerobes and anaerobes), beneficial bacteria groups (lactobacilli and bifidobacteria) and possibly pathogenic bacteria (enterococci, clostridia and coliforms) were examined to give an indication of any changes in the caecum microflora of the treated groups (WAT, FMF, AMF) compared to the untreated group (CON).

Results of the RISA analysis indicated that there was a difference in caecal microflora of the four groups. Providing *Lact.* Salivarius through water, or FMF (fermented with the same strain), or lactic acid may influence the ileal microflora of the birds in a different way than providing a dry diet without inclusion of LAB or lactic acid. Groups provided with *Lact.* Salivarius, either through water or FMF, had similar ileal, but not caecal microflora. CON and AMF groups also had a similar ileal microflora. As far as the caecal microflora is concerned, groups provided with moist feeds (FMF and AMF) were similar.

An additional aim of this study was to evaluate the effect of treatment on the histological and morphological indexes of the intestinal mucosa and liver. Probiotics have been reported to improve the intestinal mucosa development. Pelicano et al. (2005) found that birds fed *Bacillus subtilis*-based probiotic showed significantly ($P<0.01$) higher villus height in the jejunum and ileum by 6.5 and 5.6% respectively, and greater crypt depths in the duodenum, jejunum and ileum by 6.9, 15 and 9.9%, respectively, compared with the control and

Dunham et al. (1993) showed that chicks and turkeys treated with *Lact. Reuteri* had longer villi, compared with birds provided a basal diet. Although, it would be useful to relate potential histological intestinal alterations with the effect of *Lact.* Salivarius and the anti–*Salmonella* protective effect of the FMF with *Lact.* Salivarius with the changes in the intestinal function, samples have been destroyed accidentally through the process, preventing any further investigation.

No correlation between liver congestion and the incidence of *Salmonella* in the samples could be found. Also, no inflammatory and/or destructive lesions with various degrees of necrosis defined as granulomas were observed. However, it has to be mentioned that assessing the number of heterophils and the degree of necrosis and fibrosis would have been a better indicator of the liver changes than congestion with erythrocytes, which requires euthanisation of the birds using barbiturate rather than cervical dislocation (R. La Ragione personal communication).

It has been concluded that the probiotic properties of *Lact.* Salivarius NCIMB 41606 strain used for fermentation of the liquid feed and the lactic acid produced during the fermentation both play a part in the effectiveness of FMF against *Salmonella* when provided to poultry. The *Lact.* Salivarius used was shown (during the screening process described in chapter 2) to have competitive exclusion properties (coaggregation with pathogens and adhesion to epithelial cells), as well as having antibacterial properties against several enteropathogens. It is possible that other biologically active compounds are also

produced. FMF might have an advantage over water administration as it might act as a substrate for the production of all these antimicrobial compounds by LAB. FMF might also act as an appropriate substrate for the survival and proliferation of lactobacilli. The slow periodic emptying and refilling of the caecal contents provides time to lactobacilli to be in contact with or colonise the epithelium. Additionally, several changes are observed in FMF during fermentation. Hojberg et al. (2003), who evaluated the microbial catabolic capacity in digesta from the gastrointestinal tract of pigs fed FMF in their experiment, suggested that the highest rates of fermentation and the most significant effect of diet were observed for readily fermentable carbohydrates like maltose, sucrose, and lactose. LABs use much of the fermentable carbohydrate in the diet and consequently, less substrate may be available for the growth of coliforms. Another hypothesis is that the growth stage and the activity of the LAB may be affected positively in the FMF, but all these need further investigation.

The method of administration of LAB to chickens through the medium of fermented moist feed is still innovative. If a number of practical and engineering problems posed by the production and delivery of FMF to chickens can be overcome, it could benefit both consumers and producers in terms of poultry health and poultry product safety.

Chapter 5

Conclusions

The use of antibiotics over the past years as prophylactic agents or as growth promoters (AGP's), over extended periods, to achieve increased growth performance, improved feed efficiency and reduction of enteropathogens in chicken production, posed a health threat to consumers because of the possible transmission of antibiotic resistant organisms to humans. The consequent ban on the use of antibiotics as feed additives throughout the European Union and the potential ban in the US, have increased commercial interest in using alternatives to antibiotics for poultry production, that are considered as biosafe, and at the same time to improve gut and host health, such as probiotics.

Delivery of probiotic strains of lactic acid bacteria (LAB) to poultry may be achieved by addition to either the water or the feed. Added benefit may be obtained if the feed is allowed to ferment to produce a feed containing at least 150 mmol L^{-1} lactic acid and a low pH (<4.5), as this has been shown to improve bacterial ecology of the pig's GI-tract (Moran 2001) and reduce contamination of enteropathogens such as Salmonellae in chickens (Heres et al. 2003a). The use of fermented liquid feeding is a practice that is being adopted more widely by the pig industry. Previous work at our laboratory screening porcine derived LAB has identified suitable LAB to use as starter cultures for fermentation of liquid feed (Moran 2001; Demečková 2003). This

study aimed to discover whether the same approach could benefit the poultry industry.

The aim of this study was the selection of a *Lactobacillus* strain or strains that had potential probiotic properties and could be provided to chickens either by water or fermented liquid feed and to investigate the potential positive effects on chicken production.

Many researchers have been working on selecting probiotic strains from animals (O'Sullivan 2001; Mojgani et al. 2007) and plants (Chiu et al. 2008) for use in animal, or humans (Jacobsen et al. 1999) and not all have focused on the same selection criteria or followed the same screening program. For this study, the successful screening programme set up by Moran (2001) and Demečková (2003) was followed after being adjusted to take account of the different gastrointestinal conditions in poultry and to correspond to the limited budget available for the completion of the studies. After a series of *in vitro* tests (Chapter 2), it was shown that the GI-tract of chickens may act as a pool of lactic acid bacteria with potential probiotic properties. In particular, a *Lactobacillus* strain *Lactobacillus salivarius* Salivarius NCIMB 41606 (*Lact.* Salivarius) showed:

- rapid autoaggregation ability
- maximum or marked co-aggregation ability with several enteropathogens
- tolerance to acidic pH and bile salts of the chicken GI-tract

- very good antagonistic activity against several enteropathogens and
- good adhesive capacity to chicken epithelial cells

This organism was selected as a candidate that could be used *in vivo* as a chicken probiotic.

There are some considerations in regards to some of the methods used for the screening programme. It could be argued that the agar spot test that have been used for the assession of the antagonistic activity of the lactobacilli tested (2.2.4 subchapter) may possibly assess the competition for nutrients that are found in the media. Also, an indicator *Lactobacillus* strain could be used as control for the comparison of the inhibitory effect with the rest lactobacilli tested. However this method is widely used in the literature for the assessment of bacterial antagonistic activity, and moreover it has to be mentioned that each of the lactobacilli and pathogens that used are grown in different culture media, i.e lactobacilli in MRS agar and pathogens in Nutrient agar, so the competition of nutrients between them might be limited. Also the aim of the tests was to determine the strains that perform better than the others and to exclude from further screening those that perform worse. In regards to the mucus test described in the subchapter 2.2.5, it has to be mentioned that it has not be considered as a criterium for selection of potential probiotic strains as the positive control tested did not adhere to mucin, even after many repetitions of the test. Despite the possible disadvantages of the laboratorius methods used for the *in vitro* experiments, the present screening programme is considered as practical and cost effective, and the strain that has been isolated at the end of

the process (*Lact.* Salivarius NCIMB 41606) was found to have possible probiotic properties in the *in vivo* experiments (i.e. reduction of *Salmonella* incidence).

Additionally, the selected strain was tested for its capacity to cope with osmotic stress and calcium salts in water and it found to successfully survive in water for at least 24 hours (Chapter 3). Three more strains *Lactobacillus salivarius* Salivarius NCIMB 41609, *Lactobacillus salivarius* Salivarius NCIMB 41610, *Lactobacillus plantarum* NCIMB 41607 isolated from the natural gut microflora of poultry exhibited strong potential as probiotic adjuncts and could perform effectively within the GI tract. Further, *Lact.* Salivarius NCIMB 41606 was found to be a good candidate as a starter culture for controlled fermentation of chicken feed (Chapter 3). Moist feed fermented with the selected strain was found to have 10^9 -10^{10} CFU ml^{-1} lactobacilli counts, decreased the pH of the feed to less than 4 and produces lactic acid to more than 150mmol when used to ferment barley and red sorghum. The fermentation of pelleted feed resulted to the production of less lactic acid which might be attributed to the buffering capacity of the feed. The use of isolated and purified LAB from the GI tract of chickens provides an alternative to the use of ileal extracts as a means of introducing beneficial bacteria to the digestive tract of chicks as it circumvents the problems of potential introduction of pathogenic organisms associated with the use of ileal extracts. However, given the complexity of the chicken GI tract, the proof of efficacy of the two probiotic bacteria in broilers led to the next step of this work; the *in vivo* studies.

The main objective of the *in vivo* study (Chapter 4) was to investigate whether the selected *Lactobacillus* strain, provided to chickens either in water or fermented moist feed, had a beneficial effect against *Salmonella* contamination. *Lact.* Salivarius provided to chickens in fermented moist feed provided superior and more consistent protection against *Salmonella* colonisation than when provided in drinking water and both were superior to untreated controls. *Salm.* Typhimurium counts in infected birds were reduced significantly by administration of *Lact.* Salivarius in feed or water, or lactic acid in feed.

The prophylactic mechanism of the FMF, fermented with *Lact.* Salivarius was not completely elucidated. It can be assumed that a possible mechanism that *Lact.* Salivarius might use for the elimination of the *Salmonella* is competitive exclusion, based on its ability to aggregate and coaggregate with *Salmonella* or/and its antimicrobial activity against *Salmonella* as demonstrated in the screening process. Hoever, the difference in the dose of *Lact.* Salivarius that chickens received through water and FMF is an obstacle to assuming that one reason for the successful use of FMF fermented with *Lact.* Salivarius against *Salm.* Typhimurium might be probiotic properties of *Lact.* Salivarius and the lactobacilli generally.

Another suggestion is that the reduction of *Salmonella* is dose-dependent and that administration of *Lact.* Salivarius culture in high levels is more effective than in low levels. In this experiment, chickens were provided with two different doses of the *Lactobacillus* culture i.e. 10^7 CFU ml^{-1} through water for the WAT

treatment and a higher one of 10^9 CFU ml^{-1} through FMF, for the FMF treatment. This is in agreement with the conclusions of Higgins et al. (2008), who suggested that oral administration of 10^6 or 10^8 CFU ml^{-1} of a *Lactobacillus*-based probiotic culture (FM-B11) in neonatal broiler chicks, within 1h of challenge with 10^4 CFU ml^{-1} of *Salm. enteritidis*, significantly reduced *Salmonella* recovery, whereas a 100x lower dosage had no significant effect.

A healthy and balanced gastrointestinal tract plays an important role establishing good health and performance in poultry. There is some evidence that LAB supplied to chickens via water or feed affect the composition of the gastrointestinal microbial community which plays a key role in animal health and performance through its effect on gut morphology, nutrition, pathogenesis of intestinal disease and immune response. One of the most important functions of the intestinal microbiota is to suppress pathogenic bacteria by competitive exclusion and antibacterial substances. There are studies that have shown that probiotics do not significantly change the gut microflora profile of broilers (Jin et al. 1998c) and others that resulted in a beneficial modulation of the caecal microflora (Mountzouris et al. 2007). In this experiment, there were no statistically significant differences among treatments regarding total aerobes, total anaerobes, coliforms, bifidobacteria and enterococci. Though, significantly ($P<0.05$) higher lactobacilli counts were found in the caecum of the birds of the FMF group (8.938 log_{10}) than those of the CON (7.941 log_{10}), WAT (8.497 log_{10}) and AMF group (7.507 log_{10}). This change in the composition of chickens' intestinal microbiota might have made the chicken more resistant to colonisation

with *Salmonella*. Results of the RISA analysis indicate also that there is a change in ileal microflora. Providing *Lact.* Salivarius through water, or FMF (fermented with the same strain), or lactic acid may influence the ileal microflora of the birds in a different way than providing a dry diet without conclusion of LAB or lactic acid.

The high concentration of the lactic acid, may contribute to the improved resistance of FMF fed chickens against *Salmonella*. The AMF treatment was designed to answer the question is the effect of the FMF is just due to the production of acid? AMF exerted some anti-*Salmonella* ability, as 25% of the cloacal swabs were found to be *Salmonella* negative and significantly lower than the negative cloacal swabs of the FMF treatment (52%). Organic acids are known to have antibacterial properties, using mechanisms such as decreasing intestinal pH (Heres et al. 2004a), interference with bacterial metabolism by decreasing the cytoplasmatic pH , as well as inhibition of enzymatic action and DNA synthesis (Van Immerseel et al. 2006b). However, lactic acid is very expensive, and adding the quantity needed to reduce the diet pH to 4 results in considerably higher diet costs. Reducing the pH of the liquid diet to 4.00 by fermentation with *Lact.* Salivarius could be a more cost effective method of acidified the feed for inhibiting enteropathogens in the diet. However, it has to be mentioned that although the concentration of lactic acid in the FMF (361.8±162.6 mmol L^{-1}) did not differ significantly compared to that of AMF (459.3±165.0 mmol L^{-1}), the big variation of the values raises concerns on the

assumption that the two feeds were equal in regards to the lactic acid concentration.

It is possible that other products i.e. acids (acetic etc) and other biological active compounds are also produced and might contribute to the FMF effectiveness against *Salmonella*. FMF might have an advantage against WAT treatment as it might act as a substrate for the production of all these antimicrobial compounds by LAB. FMF might acts also as an appropriate substrate for the survival and proliferation of lactobacilli or/and the slow periodic emptying and refilling of the caecum contents provides time to lactobacilli to be in contact or colonise the epithelium. Additionally, several changes are observed in FMF during fermentation. Hojberg et al. (2003), who evaluated the microbial catabolic capacity in digesta from the gastrointestinal tract of pigs fed FMF in his experiment, suggests that the highest rates of fermentation and the most significant effect of diet were observed for readily fermentable carbohydrates like maltose, sucrose, and lactose. LAB use much of the fermentable carbohydrate and consequently less substrate may be available for the growth of coliforms. Hence, less fermentable carbohydrates are available as a substrate for coliforms. Another hypothesis is that the growth stage and the activity of the LAB may be affected positively in the FMF, but all these need further investigation.

It would be useful to relate potential histological intestinal alterations with the effect of *Lact.* Salivarius and the anti–*Salmonella* protective effect of the FMF

with *Lact.* Salivarius with the changes in the intestinal function. Probiotics have been reported to improve the intestinal mucosa development increasing the villus height and the number of villi per unit area (Dunham et al. 1993; Pelicano et al. 2005). Unfortunately, samples have been destroyed through the process, preventing any further investigation.

Although the European Food Safety Authority (EFSA) requires proof of efficacy and safety for probiotic additives, which have to meet the requirements for registration as technological additives, there are no standard official analytical methods for the detection of probiotic additives. This broadens the spectrum of the selection criteria of probiotic strains each researcher relies upon. The potential probiotic capacity of the selected strains, needs further investigation to elucidate the exact mechanism of their antimicrobial activity for example, or their possible anti-inflammatory effects on the mucosa and stimulation of the mucosal immunity. It would be also interesting to test the rest of the *Lactobacillus* strains as a single or mixed strain culture for their potential protective role against enteropathogens induced *in vivo*. *Lact.* Salivarius have shown a good antagonistic activity against *Cl. perfingens* as described in Chapter 2, which might be very promising if this can be demonstrated *in vivo* as well.

Defining the effective dose is another important issue. The results of this study suggest that the reduction of *Salmonella* is dose-dependent. A higher dose than

10^7 CFU ml^{-1} of *Lact.* Salivarius in the drinking water might be bactericidal against a large spectrum of enteropathogens *in vivo*.

The method of administration of fermented liquid feed to chickens is still innovating. Heres et al. suggested that providing FLF on chickens reduces susceptibility of broilers for several enteropathogens (Heres 2002; Heres et al. 2003a; Heres et al. 2003b; Heres et al. 2003c; Heres 2004; Heres et al. 2004b). Providing fermented moist feed to chickens may be easier for small producers than for large commercial poultry units. Organic poultry producers that cannot use livestock feed additives could also benefit by adopting fermented moist feeding practice. If several practical problems relating to the engineering of production and delivery FMF to chickens and regulatory problems can be solved, the use of these organisms in water, or in freeze dried culture in dry feeds, or as a starter culture for the production of fermented moist feed could benefit consumers and producers in terms of animal health and animal product safety.

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Appendices

Table 1. Total results of the screening programme

Number	Bacteria	Gram Stain	Catalase test	Homo/heterofermentation test	Autoagreggation test	Aggregation test with *Salm.* Typhimurium	Aggregation test with *Esch. coli*	Aggregation test with *C. perfringens*	Aggregation test with *S. enteritidis 5188S*	Aggregation test with *S. enteritidis* chickens	Identification API 50 CHL (% identity) * Identification 16S rRNA sequencing	Tolerence to acidic pH and bile salts	Antagonistic activity assay	Adhesion to mucus	Comments
1	1C1	(+), rods	-	homofermentative	slow										
2	1C2	(+), rods	-	homofermentative	(-)										
3	1C3	(+), rods	-												died
4	1SI1	(+), rods	-	homofermentative	(-)										
5	1SI2	(+), rods	-												died

6	1SI3	(+), cocci	-		(-)										
7	1ILEUM1	(+), rods	-												died
8	1ILEUM2	(+), rods	-												died
9	1ILEUM3	(+), rods	-												died
10	1JEJUNUM1	(+), rods	-		slow										
11	1JEJUNUM2	(+), rods	-	homofermentative	(-)										
12	1JEJUNUM3	(+), rods	-												died
13	2C1	(-), rods	-		(-)										
14-7	2C2	(+), rods	-		rapid			1	1	1	Doubtful profile				
15	2C3	(+), rods	-												died
16	2SI1	(+), rods	-												low growth
17-11	2SI2	(+), rods	-	heterofermentative	rapid			2	1	1	Doubtful profile				
18	2SI3	(+), rods	-												died
19	2SI4	(+), rods	-												died
20	2SI5	(+), rods	-												died
21	2ILEUM1	(+), rods	-												low growth
22	2ILEUM2	(+), rods	-												died
23	2ILEUM3	(+), rods	-	homofermentative	slow										
24	2JEJUNUM1	(+), rods	-												died
25	2JEJUNUM2	(+), rods	-												low growth
26	2JEJUNUM3	(+), rods	-												died
27	3C1	(+), rods	-												died
28	3C2	(+), rods	-												died
29	3C3	(+), rods	-												died
30	3SI1	(+), rods	-												low growth
31-9	3SI2	(+), rods	-	heterofermentative	normal			3	1	1	*Lact. plantarum* 1 99.9%				
32-6	3SI3	(+), rods	-	heterofermentative	rapid	2	3	2	2	2	*Lact. salivarius* 98.8% * *Lact. Salivarius* Salivarius	tolerent	4th	good	

33	3ILEUM1	(-), rods	-												
34	3ILEUM2	(+), rods	-												died
35-17	3ILEUM3	(+), rods	-	heterofermentative	rapid				1	1	*Lact. salivarius* 99.2%				
36	3JEJUNUM1	(+), rods	-												low growth
37-19	3JEJUNUM2	(+), rods	-	homofermentative	(-)										
38	3JEJUNUM3	(+), rods	-	homofermentative	(-)										
39	1CROPSCRAPE1	(+), rods	-												died
40	1CROPSCRAPE2	(+), rods	-												low growth
41	1CROPSCRAPE3	(+), rods	-												died
42	2CROPSCRAPE1	(+), rods	-												low growth
43	2CROPSCRAPE2	(+), rods	-	homofermentative	(-)										
44	2CROPSCRAPE3	(+), rods	-												died
45	3CROPSCRAPE1	(+), rods	-												died
46	3CROPSCRAPE2	(+), cocci	-												died
47	3CROPSCRAPE3	(+), rods	-	heterofermentative	slow										
48	1CROPCONTENT1	(-), rods	-												died
49	1CROPCONTENT2	(-), rods	-												died
50	1CROPCONTENT3	(+), rods	-												died
51	2CROPCONTENT1	(+), rods	-												died
52	2CROPCONTENT2	(+), rods	-												died
53-15	2CROPCONTENT3	(+), rods	-	homofermentative	normal	2	1	1	2	2	*Lact. fermentum* 1 99.0%	tolerent	5th	good	
54	3CROPCONTENT1	(+), rods	-												died
55	3CROPCONTENT2	(+), rods	-		(-)										
56	3CROPCONTENT3	(-), rods	-		(-)										
57	ROG-1C1	(+), rods	-	homofermentative	slow										
58	ROG-1C2	(+), rods	-												died
59	ROG-1C3	(+), rods	-												died
60-10	ROG-1SI1	(+), rods	-	homofermentative	rapid	1	1	2	2	2	*Leuconostoc lactis* 99.3%	tolerent	6th	good	

61-**20**	ROG-1SI2	(+), rods	-	homofermentative	normal				1	1	*Lact. salivarius* 99.2%				
62	ROG-1SI3	(+), rods	-												died
63	ROG-1ILEUM1	(+), rods	-												low growth
64	ROG-1ILEUM2	(+), rods	-												died
65	ROG-1ILEUM3	(+), rods	-	homofermentative	(-)										
66	ROG-1JEJUNUM1	(+), rods	-	homofermentative	(-)										
67	ROG-1JEJUNUM2	(+), rods	-	homofermentative	(-)										
68	ROG-1JEJUNUM3	(+), rods	-	homofermentative	(-)										
69	ROG-2C1	(+), rods	-												died
70	ROG-2C2	(+), rods	-	homofermentative	slow										
71-**22**	ROG-2C3	(+), rods	-		normal			2	3	3	*Lact. fermentum* 1 98.3%		8th	good	
72	ROG-2SI1	(+), rods	-												died
73-**13**	ROG-2SI2	(+), rods	-	homofermentative	rapid			1	1	1	*Lact. salivarius* 83.0%				
74-**16**	ROG-2SI3	(+), rods	-	homofermentative	normal	2	3	2	2	2	*Lact. salivarius* 99.9% * *Lact. Salivarius* Salivarius	tolerent	3rd	very good	
75	ROG-2ILEUM1	(+), rods	-												died
76-**5**	ROG-2ILEUM2	(+), rods	-	homofermentative	normal				1	1	*Lact. salivarius* 99.2%				
77	ROG-2ILEUM3	(+), rods	-												died
78	ROG-2ILEUM4	(+), rods	-		(-)										
79-**12**	ROG-2JEJUNUM1	(+), rods	-		rapid			2	1	1	*Leuconostoc lactis* 99.5%				
80-**1**	ROG-2JEJUNUM2	(+), rods	-		rapid			2	2	2	*Lact. salivarius* 98.8%				
81	ROG-2JEJUNUM3	(+), rods	-	homofermentative	(-)										
82-**23**	ROG-3C1	(+), rods	-	homofermentative	normal				0	0	Unacceptable profile				
83	ROG-3C2	(+), rods	-	homofermentative	slow										
84-**4**	ROG-3C3	(+), rods	-	homofermentative	normal			1	0	0	*Lact. salivarius* 99.9%				
85	ROG-3SI1	(+), rods	-	homofermentative	slow										

86-3	ROG-3SI2	(+), rods	-	homofermentative	rapid	3	4	3	4	4	*Lact. plantarum* 1 99.9% * *Lact. Salivarius* Salivarius	tolerent	1st	good	
87	ROG-3SI3	(+), rods	-		(-)										
88	ROG-3ILEUM1	(+), rods	-		(-)										
89-18	ROG-3ILEUM2	(+), rods	-	homofermentative	rapid			1	2	2	*Lact. plantarum* 1 99.9% * *Lact. plantarum*		2nd	very good	
90	ROG-3ILEUM3	(+), rods	-												died
91	ROG-3JEJUNUM1	(+), rods	-	homofermentative	rapid										
92-8	ROG-3JEJUNUM2	(+), rods	-	homofermentative	normal			1	2	2	*Lact. salivarius* 99.2%				
92	ROG-3JEJUNUM3	(+), rods	-												died
92	ROG-1CROPSCRAPE1	(+), rods	-												died
92	ROG-1CROPSCRAPE2	(+), rods	-		(-)										
92-21	ROG-1CROPSCRAPE3	(+), rods	-	homofermentative	normal			3	0	0	*Lact. plantarum* 1 99.9%				
93	ROG-2CROPSCRAPE1	(+), rods	-												died
94	ROG-2CROPSCRAPE2	(+), rods	-												died
95-24	ROG-2CROPSCRAPE3	(+), rods	-	heterofermentative	normal			2	3	3	*Lact. fermentum* 1 97.3%		7th	good	
96	ROG-2CROPSCRAPE4	(+), rods	-												died
97	ROG-2CROPSCRAPE5	(+), rods	-	homofermentative	(-)										
98-14	ROG-2CROPSCRAPE6	(+), rods	-		rapid				2	2	Unacceptable profile				
99	ROG-3CROPSCRAPE1	(+), rods	-												died
100-2	ROG-3CROPSCRAPE2	(+), rods	-		normal				1	1	*Lact. salivarius* 99.2%				
101	ROG-3CROPSCRAPE3	(+), rods	-												died
102	ROG-3CROPSCRAPE4	(+), rods	-	homofermentative	(-)										
103	ROG-1CROPCONTENT1	(+), rods	-		slow										
104	ROG-1CROPCONTENT2	(+), rods	-												died
105	ROG-1CROPCONTENT3	(+), rods	-		(-)										

106	ROG-2CROPCONTENT1	(+), rods	-	homofermentative	(-)										
107	ROG-2CROPCONTENT2	(+), rods	-												died
108	ROG-2CROPCONTENT3	(+), rods	-	heterofermentative	normal										
109	ROG-3CROPCONTEN1	(+), rods	-	homofermentative	slow										
110	ROG-3CROPCONTEN2	(+), rods	-												died
111	ROG-3CROPCONTEN3	(+), rods	-												died