THE DEVELOPMENT OF

PROBIOTICS FOR USE IN THE OSTRICH

FARMING INDUSTRY IN SOUTH AFRICA

By

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CERTIFICATION OF SUPERVISOR

In terms of paragraph GP 7 of "General Rules for the degree of Doctor of Philosophy (Ph.D.)" I, as supervisor of the candidate Elloise du Toit, support the submission of this thesis for examination.

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CONTENTS

ABSTRACT…… **1**

ABBREVIATIONS…… **4**

CHAPTER 1 General introduction……...……... **6**

CHAPTER 2 Identification and characterisation of potential probiotic strains isolated from the gastro-intestinal tract of wild and farmed ostriches…….. **31**

CHAPTER 3 The effect of dietary supplementation with *L. plantarum, L. reuteri* and *B. pseudolongum* subsp. p*seudolongum* on the faecal and gut microbial populations using culture-dependent techniques……. **74**

CHAPTER 4 Molecular analysis of the ostrich gastrointestinal tract using universal, *Bifidobacterium*- and *Lactobacillus*-specific primers……..…. **111**

CHAPTER 5 Investigation of the ostrich chick intestinal bacterial communities by 16S rRNA targeted fluorescent in-situ hybridisation and the observed differences when fed tylosin or a probiotic mix…….……...**153**

CHAPTER 6 General conclusions……...**179**

LITERATURE CITED…….. **184**

ABSTRACT

Ostrich farming in South Africa is an important industry but it often suffers from high mortality rates among the ostrich chicks. This is thought to be due in part to environmental stress, leading to the delay in the development of the microbiota, thus making invasion by pathogens likely. Very little is known about the microorganisms inhabiting the ostrich gastrointestinal tract, however they do play an important role in humans and animals and disturbance of this community can be fatal. It has been established that probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* spp. play an important role in the health of the host. In order to discover novel probiotic strains, bacteria should be isolated from the host, identified, characterised *in-vitro* to screen their potential suitability, and finally these results should be confirmed *in-vivo*. The aim of this study was to follow these guidelines in order to find a successful probiotic mix to decrease the mortality observed on ostrich farms, and to compare the effects of the probiotic mix to that of the antibiotic, tylosin, on the microbiota of the gastrointestinal tract (GIT).

The isolation and identification of probiotic strains from faecal or gut samples is the first stage in the process of selection, however, the number of strains isolated may be restricted by the commonly used growth media and conditions. The most common probiotics belong to *Lactobacillus* or *Bifidobacterium* spp. In this study, four *Lactobacillus* spp. and one *Bifidobacterium* spp. were used, based on the initial screening which showed them to tolerate bile and acid, and to produce antimicrobial substances against important pathogens.

The ability of the strains to survive the harsh conditions encountered in the gut was tested *in-vitro*. The five strains used in this study showed good potential for use as probiotics, tolerating 2% bile and pH 2, as well as demonstrating autoaggregation and coaggregation with four common pathogens. All five strains and their combinations showed good adhesion capability to mucous and could inhibit and displace the pathogens in most cases. Only the probiotic combinations were able to out-compete the pathogens. The strains produced antimicrobial substances active against important pathogens and produced enzymes essential to the ostrich diet. These *in-vitro* conditions cannot, however, mimic those found in the gut, and therefore strains showing potential *in-vitro* were now tested *in-vivo* in feeding trials.

The ostrich chicks were divided into pens (n = 17) and assigned to specific treatments of probiotic, E, tylosin, T, or a combination of the two, ET, and controls, C. All chicks were fed

a normal diet and the microbial content of their faeces and mucosa was studied at intervals over nine weeks, using both culture and molecular techniques. Three methods were used here in an attempt to get a more comprehensive overview of the bacterial populations in each treatment group.

During the feeding trial no significant differences in feed conversion or weight gain were observed between treatments, however the chicks receiving the probiotic mix as well as the control group showed noticeably higher mortality rates than the chicks receiving tylosin. Culturing of faecal samples from chicks in the feeding trial did not show significant differences between treatments, however, chicks in treatment E showed visibly higher counts of *C. perfringens.* When comparing the faecal counts over time, most groups showed increasing trends. The mucosal samples showed increasing trends for total bacteria, LAB and *Enterobacteriaceae.* At slaughter one (at two weeks), *C. perfringens* was present in all areas of the gut of chicks in treatment E, and by slaughter two (at five weeks), was only detected in the caecum and colon of chicks in treatment ET. Post-mortems confirmed this pathogen to be the likely cause of death of the chicks.

DGGE analysis of faecal samples showed the diversity of total bacteria and *Lactobacillus* spp. to be unaltered by probiotics alone, but the *Bifidobacterium* spp. changed with this treatment. In combination with tylosin, the probiotics did not affect the diversity of total bacteria or the *Bifidobacterium* spp. population, however the diversity of the lactobacilli present was decreased.

Ostrich chicks were rapidly colonised by bacteria one week post-hatching as shown by FISH-FC on faecal and mucosal samples. Faecal samples were found to be initially dominated by the *C. coccoides/E. rectale* group of bacteria, but by week seven *Bacteroides* was most abundant. The caecum and colon were both found to harbour predominantly butyrate-producers belonging to the *C. coccoides/E. rectal* group and the duodenum mostly by *Bacteroides. C. perfringens* was detected throughout the gut, but its proportions were found to be highest in the duodenum.

This is the first study to confirm that *C. perfringens* is a major pathogen in ostrich chick mortality. This particular probiotic mix alone was not effective against *C. perfringens* but, together with tylosin, it not only prevented this pathogen from causing death of the chicks, but also increased the prevalence of beneficial bacterial groups in the ostrich chick. However,

a new probiotic strain or mix which is more capable of competitively excluding *C. perfringens* needs to be investigated for use in these chicks without the antibiotic tylosin.

ABBREVIATIONS

bp	:	base pair(s)
CFU	:	colony forming units
DGGE	:	denaturing gradient gel electrophoresis
DNA	:	deoxyribonucleic acid
EDTA	:	ethylenediaminetetra-acetic acid
FISH	:	fluorescent *in-situ* hybridisation
h	:	hour(s)
kb	:	kilobase pair(s)
kDa	:	kilodalton(s)
kg	:	kilogram
log	:	logarithmic
M	:	molar
mg	:	milligram
MIC	:	minimum inhibitory concentration
min	:	minute(s)
ml	:	millilitre
mM	:	millimolar
MW	:	molecular weight
NCBI	:	National Centre for Biotechnology Information
nm	:	millimetre
OD_{560}	:	optical density measured at wavelength of 560 nm

OD_{600}	:	optical density measured at wavelength of 600 nm
OD_{620}	:	optical density measured at wavelength of 620 nm
ORF	:	open reading frame
PCR	:	polymerase chain reaction
qPCR	:	quantitative real-time PCR
RNA	:	ribonucleic acid
rRNA	:	ribosomal RNA
s	:	second(s)
SDS	:	sodium dodecyl sulphate
spp.	:	species
Tris	:	tris(hydroxymethyl)aminomethane
U	:	units
v/w	:	volume per volume (in ml per 100 ml)
w/v	:	weight per volume (in grams per 100 ml)
w/w	:	weight per weight (in grams per 100 grams)
α	:	alpha
β	:	beta
γ	:	gamma
Δ	:	delta
λ	:	lambda
µ	:	micro

CHAPTER 1

General Introduction

Contents

1.1. **The ostrich**……..7

1.1.1. Diet and digestion in the ostrich..... 7

1.2. **Intestinal microbiota**..... 10

1.3. **Ostrich farming**..... 11

1.4. **Ostrich health**..... 14

1.5. **The use of growth-promoting antibiotics in animal farming**..... 15

1.6. **Effect of antibiotics on the commensal microbiota**..... 16

1.7. **Probiotics**..... 17

1.7.1. Studies in animals..... 18

1.7.2. Probiotics in poultry farming..... 19

1.8. **Prebiotics**..... 20

1.9. **The transit of probiotic strains through the gastrointestinal tract**..... 20

1.10. **Selection of appropriate probiotics**..... 21

1.10.1. Origin and identity..... 21

1.10.2. Technical properties..... 22

1.10.3. Physiological properties..... 23

1.10.4. Monostrain, multistrain or multispecies probiotics..... 28

1.10.5. The development of ostrich probiotics..... 28

1.11. **Aims of current research**..... 29

1.1. The ostrich

Ostriches (*Struthio camelus*) belong to the family Ratitae, which are the flightless or running birds. In the wild, adult ostriches reach a height of between two and three m and weigh between 100 and 150 kg (Dunning, 1993). After six months, ostriches have reached their full size and at 24 months have reached sexual maturity (Cooper *et al.*, 2004). Wild ostriches have become increasingly rare in many areas of their natural range due to over-hunting, loss of habitat due to encroaching domestic stock, over-cultivation and predation (Cooper *et al.*, 2010). They live in nomadic groups of five to fifty birds (Donegan, 2002), the larger groups having the advantage of rapid predator detection.

1.1.1. Diet and digestion in the ostrich

The ostrich is herbivorous and studies on the contents of the stomach of wild ostriches confirmed this but also showed the presence of insects, bones, teeth (presumably ingested with the plants) and antelope faeces (Williams *et al.*, 1993; Cooper 2006). The ostrich is a selective feeder, but adapts its feed intake according to food availability, ingesting green annual grasses and forbs when available, or leaves, flowers and fruits from succulents and woody plants (Milton *et al.*, 1994). Small stones and grit are ingested and used in the gizzard to grind food and thus aid digestion (Cooper *et al.*, 2004).

Ostrich chicks are born with a sterile GIT and in the wild are coprophagic, pecking at their parents' faeces, thereby receiving the first inoculum of beneficial bacteria which are thought to aid in fibre digestion as well as aid in the development of the immune system (Cooper, 2006). Ostriches are monogastric herbivores with a digestive tract comparable to other avian species but with some unique adaptations, allowing it to survive in arid and semi-arid conditions. They lack teeth and a crop (an extended muscular pouch near the gullet used for storing food) and only certain areas of the digestive tract secrete enzymes (about 25% of GIT), in comparison to other avian counterparts where enzymes are secreted by the entire inner surface of the proventriculus (Fowler, 1991). In addition, it is thought that enzyme activity of membrane-bound enzymes is low in young ostriches as the length of the villi in the

duodenum is short until around day 72. This factor is considered important in the mortality of young chicks (Iji *et al.,* 2001). The oesophagus empties into the glandular stomach (proventriculus; Fig. 1.1), where digestive enzymes and acids are secreted (Aganga *et al.,* 2003), and the food then passes into the gizzard containing grit, used to grind up the feed. This grinding, together with the acid conditions (pH 1.2 – 2.1), could play a major role in the exposure of fibre fractions to microbial fermentation in the lower intestinal tract (Swart *et al.,* 1993a).

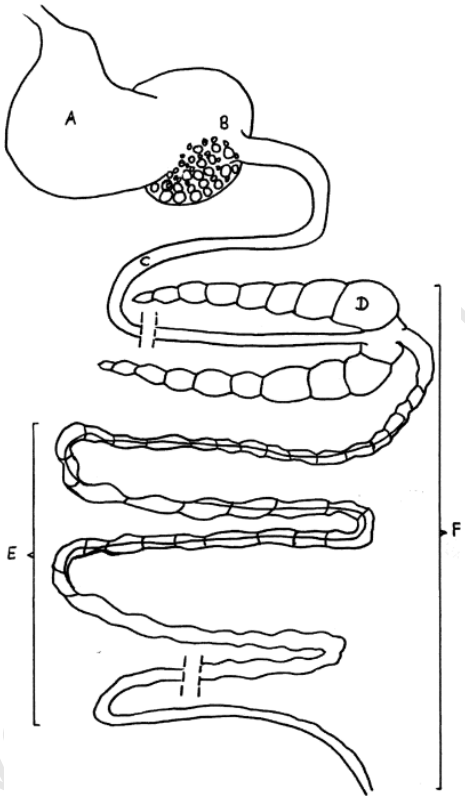

Figure 1.1. The digestive system of an adult ostrich (Cooper *et al.,* 2004). (A), proventriculus (glandular stomach); (B), ventriculus (gizzard); (C), small intestine; (D) caeca; (E) colon; (F) hindgut.

The ratites have both similarities and differences in their GIT (Table 1.1). None have a crop, a feed storage organ in other avian species (Angel *et al.,* 1995). Ostriches have a relatively small proportion of their GIT represented by the small intestine (36% compared to 90% of the chicken and 88.5% in the emu; Table 1.1). In comparison, the caecum represents only 7% of the intestine, as in the chicken. The most prominent difference between the ostrich and other ratites and chickens is the size of the colon, represented by 57% of the ostrich intestine,

3% in the chicken, and below 15% for the emu and rhea. The modifications of the hindgut in the ostrich include an extremely long, capacious, haustrated colon (57% of digestive tract; Table 1.1) and a well-developed sacculated caeca (Bezuidenhout, 1986), which indicates the use of fermentative digestion of plant fibre (Swart *et al.*, 1993b).

Table 1.1. Comparison between the intestinal lengths of ratites (adapted from Angel *et al.*, (1995))

Length	Ostrich[a]		Chicken[b]		Emu[c]		Rhea[d]	
	cm	%[e]	cm	%[e]	cm	%[e]	cm	%[e]
Small intestine	512	36	61	90	315	88.5	132	63.2
Cecum	94	7	5	7	12	3.3	46	22
Colon	800	57	2	3	29	8.2	31	14.8

[a] adult 122kg female ostrich

[b] adult chicken

[c] adult emu 38.3kg

[d] Cho *et al.* (1984)

[e] % of total length of intestinal tract (from duodenum to end of colon)

Ostriches are known as hindgut fermenters like donkeys, horses and rabbits. It is therefore critical that their intestines are colonized with the beneficial microorganisms as soon as possible (Aganga *et al.*, 2003). High concentrations of volatile fatty acids (VFAs) are produced in these fermentation chambers and digestion of hemicellulose and cellulose is carried out here by the resident bacteria present. The VFAs detected in the ostrich include acetate, found in the proventriculus, gizzard and small intestine, and acetate, butyrate, propionate, isobutyrate, isovalerate and valerate, found in the hindgut (Swart *et al.*, 1993b). The VFAs are absorbed and metabolized as an energy source, providing as much as 76% metabolizable energy requirements of a growing ostrich (Swart *et al.*, 1993b). Ammonia is the product of microbial proteolytic activity, deamination of amino acids and urease activity, and is found at the highest concentration in the colon and lowest concentration in the caeca

(Swart *et al.,* 1993b). An increase in pH is noted from the proventriculus and gizzard (pH 2 - 3) to the small intestine (pH7 - 8). The retention time of food in ostriches has been known to be as long as 76 hours, compared to 5.5 hours for emus (Swart *et al.*, 1993a). This extended retention time, together with the advantageous pH values, is thought to create optimal anaerobic conditions in the hind gut for microbial fermentation and absorption of water and volatile fatty acids (Swart *et al.,* 1993b).

1.2. Intestinal microbiota

Intestinal flora includes a mixture of approximately 10^{14} bacteria, mostly obligate anaerobes, which play important roles in the host's metabolism (Mitsouka, 1992). These roles include synthesis of vitamins B and K, non-specific activation of the immune system, production of short-chain fatty acids which act as an energy source for the host's tissues, conversion of ingested carcinogens to inactive compounds and prodrugs to active drugs (Shanahan, 2002). The commensal flora also acts as a barrier to invading pathogens, preventing their attachment by competition for niches and nutrients in the gut (competitive exclusion) and their production of toxic compounds such as hydrogen peroxide and other bacteriocins (Havenaar and Huis, 1992). Their importance becomes obvious when an antibiotic is administered and the colonization of the gut by pathogenic bacteria occurs (Shanahan, 2002). The method by which this protection occurs is unknown but it is thought to involve cross-talk between the host cells and the commensal bacteria where the probiotics secrete soluble compounds which inhibit processes of pathogenicity such as binding of pathogens to the gut and increased production of mucous (Cook and Sellin, 1998).

The harsh conditions in the gut make survival for many bacteria a challenge and they are often flushed out by peristalsis or cannot endure the effects of the antimicrobial chemicals produced by the gut (Quigley, 2010). Bacterial survival in the gut can be achieved by either increasing the growth rate to overcome the rate of removal via peristalsis, or by their attachment to the gut wall (Fuller, 1989).

There is only limited information on the microbiota in the gastrointestinal tract of the ostrich. Matsui *et al.* (2010b) showed the ostrich caeca to have a rich and diverse microbial community, the majority of which have not yet been cultivated. The study showed *Firmicutes* to be the major phylum present (50.9% of total number of sequences), followed by *Bacteroidetes* (39.4%), *Fibrobacter* (6.5%), *Euryarchaeota* (1.9%), *Spirochaetes* (1.0%), and *Verrucomicrobia* (0.3%). *Clostridium coccoides* and *Clostridium leptum* were the predominant phylotypes in the *Firmicutes*. A faecal isolate from the study showed 93 and 90% similarity to *Fibrobacter succinogenes* and *Fibrobacter intestinalis*, respectively, suggesting a new species of *Fibrobacter* in the ostrich caeca. This report, by Matsui *et al.* (2010b) is the most extensive study, to date, on ostrich microbiota. Studies on the culturable ostrich microbiota described a *Ruminococcus flavefaciens* isolated from caecal digesta of mature ostriches (Matsui *et al.,* 2010a), as well as five fibrolytic bacteria. The latter was isolated from ostrich faecal matter and included an unknown *Fibrobacter* species, assumed to be involved in fibre digestion in the ostrich caeca, and *Propionibacteria acnes,* both having been detected in ostrich faecal matter (van Gylswyk *et al.*, 1998).

In contrast to ostriches, the chicken GIT is well studied (Zhu *et al.,* 2002; Lu *et al.,* 2003, Zhu and Joerger, 2003; Amit-Romach *et al.,* 2004*;* Apajalahti *et al.,* 2004; Apajalahti and Kettunen, 2006; Collado and Sanz, 2007a; Gong *et al.*, 2007; Rehman *et al.,* 2007; Brisbin *et al.,* 2008; Gérard *et al.,* 2008; Lee *et al.,* 2010). Even though there are significant differences between the two systems, one can use some strategic aspects of the chicken microbiota studies and findings as a platform to investigate the bacterial flora of the ostrich gut.

The caecum of chickens has been suggested to offer a relatively stable environment and to harbour the largest and most complex microbial community within the GIT (Mead, 1997; Zhu *et al.,* 2002). Microbial fermentation leads to the formation of short chain fatty acids (SCFAs) and lactate (Ricke *et al.,* 2004), as in the ostrich, however they are not important energy sources in the chicken, as they are for the ostrich (Swart *et al.,* 1993a, b).

1.3. Ostrich farming

Hunting ostriches for their meat, skin and feathers is an ancient practice, being featured in Egyptian hieroglyphs (Holtzhausen and Kotzé 1990). However, due to over-hunting and a

lack of control, the species became extinct in several natural habitats (Cooper *et al.,* 2009). To remedy this, domestication and breeding practices have been implemented and have shown successful repopulation in many countries around the world (Shanawany, 1995).

Ostrich farming began in South Africa in the middle of the 18th century and the climate conditions here are ideal for ostrich breeding. The ostrich industry is one of the few agricultural industries in the world where South Africa is regarded as the undisputed world leader (National Agricultural Marketing Council, 2003), rearing and slaughtering approximately 340 000 ostriches in 2002. In 2005 the export of ostrich products amounted to ZAR1 200 000 accounting for approximately 5% of South African agricultural exports (Brand and Jordaan, 2010). The Western Cape is the leading producer of ostrich products, contributing 75% to the total ostrich production. Ostrich meat is increasingly popular in Europe where it is considered to be a healthier meat because it contains less cholesterol, sodium and fat, and more protein and iron compared to other meats (Table 1.2; Thomson, 1997; South African Ostrich Business Chamber, 2010). Importantly the Western Cape ostrich industry employs approximately 20 000 workers, representing 14% of labourers (McDonald and Punt 2001). Hence, this industry has a significant impact on the economy of South Africa and a decrease in ostrich production would have severe social and economic consequences.

Ostriches were originally domesticated for gathering of the feathers, but now it offers a range of products in addition to the meat and feathers, which include durable high quality hide, curios and agri-tourism services (Table 1.2; South African Ostrich Business Chamber, 2002).

In order for ostrich farming to be successful, the farmer needs to raise sufficient numbers of viable and healthy ostrich chicks to slaughter age. This can be a challenge given the high mortality rates reported around the world for ostrich chicks up to three months of age (Smith *et al.*, 1995; Adams and Revell, 1998; Glatz and Miao, 2008, Jarvis, personal communication). The mortality rate can reach up to 80% (Cloete *et al.,* 2001) and could be due to inadequate knowledge on feeding and management of young chicks, stress or the delayed development of the microbiota (Glatz and Miao, 2008). Ostriches are naturally coprophagous but due to the conditions under which they are farmed, they are separated from their mothers at birth and can therefore not acquire the natural flora usually obtained from the parent's faeces (Schneitz, 2005).

Table 1.2. Comparison of nutritional value of ostrich, beef, pork, chicken and lamb meat

Nutritional information/100g raw, extra-trim meat			
Type of Meat	Energy Value KJ	Protein (g)	Fat (g)
Ostrich fillet	392 KJ	21.3 g	0.80 g
Beef fillet	543 KJ	19.0 g	5.95 g
Pork fillet	498 KJ	19.5 g	4.50 g
Chicken breast fillet	458 KJ	21.5 g	2.50 g
Lamb stir-fry	619 KJ	19.0 g	8.00 g

Source: South African Ostrich Business Chamber

An unbalanced microbiota can lead to the colonization of the gut by pathogens such as *Salmonella*, *Campylobacter* and *Escherichia coli* which are the main pathogens in chickens and cause serious illness in humans if consumed (Garriga *et al.*, 1998). Not much is known about the diseases affecting ostriches, but gastrointestinal pathogens are known to have a highly detrimental effect on these birds (Shivaprasad, 1993). To this end it has been suggested to include rabbits in the raising of ostrich chicks (Madeiros, 1998). Not only does the rabbit supplement the mother figure, but also improves the welfare of the newly hatched chick by providing faeces which the chicks eat, obtaining vitamins B and K and receiving the beneficial inoculums to promote a healthy gut microbiota.

The growth of the ostrich chicks depends on their food intake, which is determined by their current weight and size and the energy content and ration of the food (Kreibech and Sommer, 1995). The ostrich chick loses weight in the first seven days post-hatching due to utilisation of the yolk sac, but hereafter gains weight at 1.3kg/week up to 12 weeks old (Mushi *et al.*, 1998). Ostrich chicks have highly efficient feed conversion to body mass ratio up until the age of two months, and it is therefore necessary to have accurate and careful chick nutrition

management (Cooper, 2005). The diet of the ostrich requires evaluation and knowledge of the farming environment for good growth and nutrition (Cooper *et al.*, 2004). Verwoerd *et al.* (1999) recommended feeding chicks *ad libitum* with a high quality diet at least for the first four months. Mushi *et al.* (1998) added that rearing chicks of the similar weight together can improve chick growth. The rapid growth rate of the chicks in the first four months is thought to lead to leg deformities which in turn can lead to death of the chicks (Musa *et al.*, 2005). This can be avoided by feeding the chicks a high fibre diet and allowing them more exercise. However, there are conflicting reports on feeding fibre to ostrich chicks. Angel (1993) reported that ostrich chicks are able to forage in the wild 48 h after hatching. Poultry chicks are born without gut microbes but these appear within a few days (Mead, 2000). More research is needed into the quantity and source of fibre fed to ostrich chicks (Ganz and Miao, 2008).

The main expenses in ostrich farming are feed and health control, contributing 80% (Brand and Gous, 2006) and 10% (Bhiya, 2006) of the gross expenditure per bird, respectively. The farmer therefore needs to ensure an adequate number of birds are slaughtered to cover this cost (Cooper *et al.*, 2004). The average age of slaughtering ostriches is nine to 12 months, as this is when their food conversion ratio declines, and when the ostrich is small enough to make skinning and eviscerating cost-effective (Cooper *et al.*, 2004). However, there has been a suggestion to lower the slaughter age to seven months to decrease the cost involved in raising the chick to slaughter age (Mike Jarvis, personal communication). This would require effective management and facilities, knowledge on the diet required, and good genetic selection. Growth promoting supplements (such as probiotics), excluding antibiotics, could also be beneficial.

1.4. Ostrich health

In the first few months of life, ostrich chicks are particularly susceptible to disease and infection, various disorders and stress (Huchzermeyer, 1994; Samson, 1997). It is imperative to keep young ostrich chicks warm, with the correct diet, in a stress free environment and plenty of space to exercise. Ostrich chick fading syndrome may develop if chicks continue to

lose weight after hatching, and may require individual attention in a stress-free environment if they are to have a chance of surviving (Glatz and Miao, 2008). Once clinical symptoms have developed, there is little chance of the chick being saved. Navel and yolk sac infections are other major contributors to the high mortality rates seen in chicks (Glatz and Miao, 2008). Ostrich chicks are sensitive to stress, originating from transport, handling, noise, heat, toe trimming and over-crowding in pens, amongst other things (Glatz and Miao, 2008) and these factors should be kept to a minimum for optimal health of the chicks. Ostriches are susceptible to diseases found in avian species including *Salmonella typhimurium, Escherichia coli* and *Clostridium* spp. infection. Other diseases that can affect ostriches include Newcastle disease, various strains of avian influenza that have been isolated from ostriches in Zimbabwe and South Africa, fading chick syndrome, enteritis and Tibiotarsal rotation (Huchzermeyer, 2002).

1.5. The use of growth-promoting antibiotics in animal farming

Farm animals are susceptible to bacterial imbalances which may lead to detrimental effects such as inefficient digestion of food, poor nutrient assimilation, the risk of pathogenic infections and increased mortality. To minimise economic losses and mortality, antibiotics are commonly used in animal feed as supplements for disease control, for their growth promoting properties and to increase feed conversion. These attributes are thought to be due to induced changes in the gut microbiota (Singer and Hofacre, 2006). Antibiotics such as tylosin are thought to reduce the microbial load in the gut and therefore make more nutrients available to the host. Tylosin is a macrolide-class antibiotic commonly used in veterinary medicine. It has a broad spectrum of activity against gram positive bacteria (*Staphylococcus* spp., *Streptococcus* spp. and *Clostridum* spp.), as well as some *Mycoplasma* and *Chlamydia*, and a narrower range against gram negative bacteria (*Campylobacter* spp., *Helicobacter pylori*, and *Pasteurella* spp). It is used for both treatment of bacterial infections, as well as its growth promoting properties. However, antibiotic resistance has increased and is becoming a worldwide problem and a threat to public health. Antibiotic resistant enterococci have been isolated from animals, water, food and soil (Aarestrup, 2000; Abriouel *et al.*, 2008). The isolation of multi-drug resistant bacteria, such as vancomycin-resistant enterococci (VRE) intensifies the public health concern (Novais *et al.*, 2005). Due to these facts, the European

Union banned the use of antibiotics as growth promotants in animal production (Bager *et al.*, 1997).

1.6. Effect of antibiotics on the commensal microbiota

The use of antimicrobials may lead to alterations in intestinal microbiota, although faecal microbiota has been shown to be resilient to short-term administration of antibiotics albeit some species remaining depressed for several months (Collier *et al.,* 2003). The mode of action of growth-promoting antibiotics is not known, but given the fact that germ-free chickens did not show any change in growth rate when fed these antibiotics, it has been suggested that its growth-promoting properties are due to their inhibitory effect against certain intestinal bacteria that produce toxins or compete with the host for available nutrients (Coates *et al.,* 1963; Engberg *et al.,* 2000). The effect the antibiotic has on the commensal microbiota is antibiotic-specific. Tylosin, used in this study, is commonly used in animal husbandry, and is often used for the treatment of chronic enteropathies in dogs (Suchodolski *et al.,* 2009). It is currently unknown if the therapeutic use of tylosin has a direct effect on intestinal pathogens or if it leads to a more general alteration of the intestinal microbiota in dogs with diarrhoea, with the subsequent improvement in digestion and absorption. It is also used to treat canine small-intestine bacterial overgrowth and tylosin-responsive diarrhoea, and *Clostridium perfringens* and *Campylobacter* spp. are generally sensitive to tylosin (Marks and Kather, 2003). In addition, an anti-inflammatory property has been proposed for tylosin due to the modulation of cyclooxygenase-2, nitric oxidase synthase, and several cytokines (Cao *et al.,* 2006). Suchodolski *et al.* (2009) found that treating dogs with tylosin for 28 days resulted in a significant shift in the intestinal microbial population, with an increase in *Proteobacteria* and a decrease in *Firmicutes, Actinobacteria, Bacteroidetes* and *Tenericutes,* to mention a few. Macrolide antibiotics, including tylosin, showed high minimum inhibitory concentrations (MICs) against several *C. perfringens* strains isolated from chickens with necrotic enteritis (Gharaibeh *et al.,* 2010). Although tylosin is usually reported to be effective against *C. perfringens* (Suchodolski *et al.,* 2009), Gharaibeh *et al.* (2010) suggested this resistance was due to the prophylactic use of macrolides in the treatment of *Mycoplasma gallisepticum,* rampant in chickens in Jordan. Poole *et al.* (2003) showed that the addition of tylosin to a mixed anaerobe continuous-flow culture of chicken

microbiota aided in the persistence of pathogenic *E. coli* 0157:H7 and suggested that the use of low-level, growth-promoting antimicrobials may compromise the ability of normal microbiota that serve as a natural host defence against infection. Tylosin has also been shown to significantly reduce total bacteria counts, while selecting for *Lactobacillus* species, commensals known to competitively exclude potential pathogens from colonising the intestine (Collier *et al.,* 2003). It is evident that the effect of antibiotics on the commensal microbiota is strain specific and should be examined to avoid detrimental side effects when used. To avoid antibiotic-resistance developing in these bacterial communities, it would be beneficial to find a natural alternative to antibiotics.

1.7. Probiotics

Probiotics have been defined in several ways, initially by Kollath in 1953 who defined them as being all organic and inorganic food complexes in contrast to harmful antibiotics (Kollath, 1953). A more recent and more commonly used definition regards probiotics as live microbial feed substances which beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). They are now defined as "live organisms that, when administered in adequate amounts, confer a health benefit on the host" (Sanders, 2008). Research into probiotics has taken a large leap forward, as their potential for use in health supplements for humans and animals is being realised. As early as the 19th century, Escherich (1885) described the microbiota and the advantages to digestion of the colonization of the gastrointestinal tract (GIT) of young children. Metchnikoff (1907) published ideas on the benefits of lactic acid bacteria (LAB) in fermented milk products, and proposed that normal gut flora was detrimental to the health of the host, but that their replacement by yoghurt bacteria was beneficial. Since then, health benefits of probiotics have been shown to include prevention of lactose intolerance in humans, treatment of diarrhoea and antibiotic-related gastrointestinal side effects, treatment of allergies, treatment of *Clostridium difficile* colitis and *Helicobacter pylori* infection, amongst many others (Doron and Gorbach, 2006). Those used in animal husbandry usually include *Lactobacillus* spp., *Bifidobacterium* spp., *Bacillus subtilis* and *Saccharomyces* spp. (Lim and Tan, 2009) and are thought to provide protection by the production of antimicrobial substances like bacteriocins and lactic acid, and the colonization of the gut to prevent attachment of undesirable bacteria (Ehrmann *et al.*, 2002).

1.7.1. Studies in animals

Probiotics are useful in the treatment of disorders caused by disturbed intestinal microbiota and increased gut permeability and have been shown to encourage growth and feed utilisation, decrease mortality and increase feed conversion in farmed animals such as pigs, chickens and calves (Lim and Tan, 2009). The use of probiotics in ostriches has not been studied, their effects on other farmed animals such as pigs and calves, and the closest model animal, the chicken, is reported here. In animals, probiotics have been shown to improve body weight gain and feed conversion as well as decrease the number of cases of diarrhoea in pigs and calves (Abe *et al.*, 1995). Probiotics are also thought to neutralise the toxin produced by *E. coli* which is pathogenic for pigs (De Mitchell and Kenworhty, 1976). A combination of live yeast and *Pediococccus acidilactici* in piglet diets was found to improve the piglet's intestinal mucosa, which is critical to nutrient absorption and immunity against pathogens (Lim and Tan, 2009). Varying success has been obtained since the 1970s using fungi, yeasts, and bacteria to increase milk production and weight gain, improve health status and resistance to diseases in ruminants.

Probiotics do not only result in positive outcomes however. They have been known to cause detrimental effects such as sepsis in murine models. Wagner *et al.* (1997) colonised neonatal mice with various probiotic strains and reported LGG and *L. reuteri* to be fatal, suggesting the presence of immune-deficiency in neonates put them at risk of sepsis. Cilieborg *et al.* (2011) found that a mixture of *Lactobacillus paracasei, Bifidobacterium animalis* and *Streptococcus thermophilus* (2.4 X 10^{10}/day) fed to new-born piglets, as dead or live bacteria, resulted in intestinal weights being lower and the incidences of necrotizing enterocolitis to be higher in pigs receiving the probiotics compared to the control group. In addition, the probiotics were found to minimally affect the colonisation of the gut and resulted in lower bacterial diversity in the distal intestinal mucosa and lower SCFA concentrations in the colon. They suggest these detrimental effects were due to the specific strain and dose combination, and the immature gut immune system of these new-born piglets. In a rat model of indomethacin-induced enteropathy, provision of LGG increased intestinal ulceration (Kamil *et al.*, 2007). It is therefore clear that in some circumstances, certain probiotic strain(s) may be detrimental for immunocompromised patients with an unstable gut microbiota.

1.7.2. Probiotics in poultry farming

Chicken farming experiences the same problems as ostrich farming concerning enteric diseases which lead to loss of productivity, increased mortality and contamination of poultry products for human consumption (Patterson and Burkholder,, 2003). Probiotics have been shown to protect chickens from diseases such as *Salmonella,* as well as increase the growth rate, decrease mortality rate by competitive exclusion and improve the feed conversion rate (Abe *et al.,* 1995; Schneitz, 2004). Lee *et al.* (2006) investigated the use of *Aspergillus oryzae* as a potential probiotic and found it increased the number of *Lactobacillus* which consequently lowered the number of S*almonella* and *E. coli* in the faeces of chickens fed *Aspergillus oryzae.* Han *et al.* (1999) found probiotics to improve digestion in broilers, and postulated that amylolytic and proteolytic enzymes produced by the probiotics were affecting digestion of the layers. Kim *et al.* (2003) showed a decrease in serum cholesterol levels in broilers fed *Aspergillus oryzae* which is thought to produce a compound which inhibits cholesterol biosynthesis. *Bacillus subtilis* was found to significantly increase body weight gain and feed efficiency in turkeys, as well as increase the crop and caecal counts of this strain (Jiraphocakul *et al.,* 1990). Nahashon *et al.* (1994) reported increases in body weight gain, passage rates of digesta and retention of calcium, phosphate, cobalt and magnesium, as well as increased egg weight and size in layers that were fed *Lactobacillus* (Lacto). Protexin (*Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Streptococcus thermophilus, Enterococcus faecium, A. oryzae, Candida pintolopessi*) is a probiotic mix commonly used in animals, and was shown to increase live weight gains as well as the carcass yield, breast and leg weights and antibody titre in broilers (Kabir *et al.,* 2004). An increase in feed conversion ratios and body weight gain was observed in broilers fed *Bacillus coagulans* (Cavazzoni *et al.,* 1998). Chickens and ducks fed 4 X 10^{10} *Lactobacillus* sp./day were found to gain weight significantly faster than the control groups (Angelakis and Raoult, 2010). Liver mass was also found to be higher in those birds receiving the inoculums and *Lactobacillus* sp. was detected more abundantly in the faeces of these birds compared to the control birds.

1.8. Prebiotics

Gibson and Roberfroid (1995) described prebiotics as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Common prebiotics include fructooligosaccharide products (inulin for example), lactulose, glucooligosaccharides, raffinose and sucrose (Patterson and Burkholder, 2003). They have shown potential for inclusion into food products for humans as they increase the efficiency of probiotics (Holzapfel and Schillinger, 2002), and it has been shown that they increase the faecal Bifidobacterial count (Bouhnik *et al.*, 2004). The combination of a probiotic with a prebiotic is known as a synbiotic, and is thought to improve its transit through the GIT, reaching the caecum to benefit bacterial groups (Holzapfel *et al.,* 2002). Synbiotics have been shown to have increased performance on the host compared to either probiotics or prebiotics alone (Rowland *et al.,* 1998). Prebiotics are also considered to offer protection to the probiotic during processing, drying and storage. Conrad *et al.* (2000) demonstrated the thermoprotectant effect of trehalose, non-fat milk solids and adonitol on *Lactobacillus acidophilus* before spray drying, and Carvalho *et al.* (2004) showed the cryoprotection offered by fructose, lactose or mannose on *Lactobacillus bulgaricus* before freeze drying. In both cases, viability of the test strains was improved. Thus prebiotics can be seen to have beneficial effects on probiotics during processing and during transit through the GIT.

1.9. The transit of probiotic strains through the gastrointestinal tract

The beneficial effects of probiotics have been realised, but their suitability for use as probiotics requires further research. The probiotics need to survive the transit through the GIT, remaining viable after exposure to bile and stomach acids, and be able to colonize the GIT adequately to provide a barrier to incoming pathogens, as well as surviving the manufacturing and storage process. Some of their beneficial properties include their non-pathogenic nature, oxygen and heat tolerance, bile and acid tolerance, ability to adhere to intestinal cells, bacteriocin production and antibiotic resistance. Suitable probiotic strains should ideally be selected from a homologous species (Ross *et al.,* 2005). To aid their

survival through the GIT, probiotics can be encapsulated in a protectant, or be administered with prebiotics.

1.10. Selection of appropriate probiotics

Several factors need to be taken into consideration when choosing a probiotic for therapeutic use. To be beneficial, the bacterial strain must have the following properties:

1.10.1. Origin and identity

A probiotic should be chosen on its history of non-pathogenicity and on the knowledge of strain-specific risk factors. Identifying the bacteria is therefore important. Lactobacilli and Bifidobacteria very rarely cause disease and can therefore be assumed to be non-pathogenic in use as a probiotic (Holzapfel, 2005). Administering a bacterial strain from a homologous species increases the chances of the bacteria's survival in the GIT and also decreases the chances of causing disease in the animal due to host specificity (Garriga *et al.*, 1998).

The amplification of the 16S rRNA region is among the most widely used methods of identification used (O'Sullivan, 1999). Depending on the level of discriminatory power needed, there are several regions of the rRNA genes which can be studied. Sequencing of half of this gene (750bp) is sufficient for reliable identification of the strain, however, it is recommended to use the full gene to establish phylogenetic relationships (Tannock, 1999). Less than 97% homology between the two 16S rRNA genes being compared means they have less than 60-70% DNA similarity over the whole chromosome and are therefore unlikely to belong to the same species (Stackebrandt and Goebel, 1994). A drawback of this method is that species within a genus often show very high relatedness of 16S rRNA sequences and thus exact identification is not always ensured (Botina *et al.*, 2005). When looking at phlyogenetic trees derived from 16S rRNA data, it is important to remember that the branching of the tree is dependent on the difference of the nucleotides, evolution rates, gene

region analyzed, and the number and choice of organisms analyzed (Stachebrandt and Goebel, 1994). It is therefore necessary to include as wide a range of organisms, related or not, in a phylogenetic tree.

Another more discriminating method of identification is to use the 16S-23S intergenic spacer region (ITS), which can resolve the difference between closely related species such as *Lactobacillus plantarum* and *Lactobacillus pentosus* (Tannock *et al.,* 1999). The spacer region between the 16S and 23S genes is extremely variable in length and sequence (Garcia-Martinez *et al.,* 1999). In comparison to the 16S rRNA gene, where the entire gene (approximately 1500 bp) should preferentially be sequenced for accurate identification, only 200 bp of the intergenic region (if the short spacer region is used) needs to be sequenced (Tilsala-Timisjarvi and Alatossava, 1998; Berthier *et al.,* 1998, Nour, 1998, and Nakagawa *et al*., 1994). The variable spacer region, which is responsible for the species-specific banding pattern obtained, is due to the presence of several functional units, tRNA genes for example, or sequences required for recognition of enzymes such as the ribonuclease III, or *boxA* (Garcia-Martinez *et al.,* 1999). *Lactobacillus* species characteristically show 3 bands when the spacer region is amplified. This is due to the presence, absence and number of tRNA molecules. The common tRNA genes in this spacer region are $tRNA^{glu}$, $tRNA^{ala}$ and $tRNA^{ile}$.

1.10.2. Technical properties

Growth features of the strain in vitro are important in assessing the potential for use as a probiotic. A suitable strain should have a relatively high growth rate to make production of large volumes feasible and more economical (Margolles *et al.,* 2009). Methods to keep the strains viable during transport and storage are still under investigation, but include freeze- and spray-drying, and encapsulation in complex carbohydrates (Ross *et al,* 2005).

1.10.3. Physiological properties

1.10.3.1. Acid tolerance

The number of bacteria ingested is greatly diminished by the bactericidal effects of gastric juice and bile acid (Begley *et al.*, 2004), and although dead bacteria do exert probiotic effects (Sanders and Veld, 1999), the delivery of live bacteria would be preferable for the maximum effect to be seen. The pH in the stomach of humans is approximately 2 – 2.5 and may reach as low as 1.5 (Waterman and Small, 1998) and in ostriches reaches as low as 1.9 in the gizzard (Swart *et al*, 1993a; Jarvis, 2002, unpublished). The ability of bacteria to survive transit through the GIT is therefore based on its acid tolerance. Dunne (1999) showed that Bifidobacteria are less acid tolerant than Lactobacilli. *Lactobacillus acidophilus* has been shown to survive exposure at pH 4 for 2 hours and at pH2 for several minutes but not for 45 minutes (Hood *et al.*, 1988). The use of a protective food carrier such as cheddar cheese has shown to enhance microbial survival in acid environments and a buffering effect has been proposed (Standton *et al.*, 1998). Charteris *et al.* (1998) demonstrated that milk or milk proteins added to gastric juice increase the pH, thereby increasing survival of some *Bifidobacterium* and *Lactobacillus* spp., and again, a buffering effect was suggested. Isolating LAB from harsh environments like the GIT, often results in the isolation of particularly acid-resistant strains (McLauchlan *et al.*, 1998). This method can isolate strains which are both acid and bile tolerant and may be more resistant to other stresses like hydrogen peroxide and storage at low temperatures. Lorca *et al.* (2002) showed that exposing *L. acidophilus* CRL 639 to sub lethal acid condition (pH 5.0) increased their survival when exposed to lethal conditions at pH 3.0. The acid tolerance response of *Streptococcus mutans* has been thoroughly studied (Matsui and Cvitkovitch, 2010). Bacteria living in acidic environments risk the acidification of the internal cytoplasm which could lead to loss of glycolytic enzyme activity, structural damage to the cell membrane, DNA and proteins (Lindahl and Nyberg, 1972). *S. mutans* was found to be able to survive these acidic conditions by repairing and protecting cellular macromolecules (Quivey *et al.*, 1995), altering its metabolic activity in response to pH fluctuations (Len *et al.*, 2004), increase cell numbers and form biofilms (Li *et al.*, 2008) and by maintaining the internal pH homeostasis by altering the composition of the membrane fatty acids, end product efflux via lactic acid and increasing the expression of proton pumps (Kuhnert *et al.*, 2004; Shibata *et al.*, 2009). Other LAB may also utilise one or more of these methods to tolerate low pH.

1.10.3.2. Bile tolerance

Bile acids are synthesized from cholesterol in the liver and are secreted from the gall-bladder into the duodenum in their conjugated form where they are involved in emulsification, digestion and absorption of lipids (Hoffman *et al.,* 1983). Microbial activity in the colon modifies the bile acids by deconjugation, dehydroxylation and dehydrogenation. Deconjugation of bile salts is carried out by bile salt hydrolases, an enzyme present in *Lactobacillus* and *Bifidobacterium* spp. and catalyses the hydrolysis of glycine and/or taurine conjugated bile salts, resulting in a free amino acid and an unconjugated bile acid molecule (Tannock, 1998). The connection between deconjugation of bile acid and colonization of the gut is not apparent as neither product of deconjugation is used by the bacteria. Bile salts act as a detergent, permeabilizing the bacterial cell wall and leading to cell death (Pumbwe *et al.,* 2007). On the other hand, bile salts have been shown to increase biofilm formation, stimulate bacterial co-aggregation and adhesion to human cells (Pumbwe *et al.,* 2007). *B. fragilis* was shown to have increased fimbria and cellular vesicle formation as a signal for attachment to host cells and other bacteria, suggesting that bile salts increase bacterial adhesion properties (Pumbwe *et al.,* 2007). Both conjugated and non-conjugated bile salts are toxic to bacteria, but some species are more tolerant to the deconjugated product than others (Tannock, 1998). It has been suggested that there may be differences in efficiency of cell membrane-associated transport mechanisms between strains that maintain intercellular levels of unconjugated bile acid at non-toxic concentrations. McAuliffe *et al.* (2005) reported the identification of two genes encoding bile salt hydrolysis in the genome of *L. acidophilus,* having different substrate specificities. While *bshA* activity is dependent on the steroid nucleus, *bshB* specificity is dependent on the absence of taurine in the bile salt structure.

1.10.3.3. Adhesion potential

For probiotics to have an effect on their host, it is necessary for them to adhere to the intestinal mucosa (Dunne *et al.,* 2001). This is important for temporary colonization, antagonism against pathogens and modulation of the immune system (Salminen *et al,* 1998), and has been said to be host species specific (Fuller, 1973). This is a desirable property of potential probiotics, but the reasons for this have been debated. The differences in adhesion

of interspecies probiotics has not been fully studied, but it has been shown that LAB of human origin can adhere well to canine (Rinkinen *et al.*, 2000) and fish mucosa (Nikoskelainen *et al.*, 2001), and some dairy species have relatively good adhesion in humans (Rinkinen *et al.*, 2000). Probiotics intended for animal use are often used for more than one species (Rinkinen *et al*, 2003). Rinkinen *et al.* (2003) suggest that LAB adhesion to the mucosa is more dependent on strain species and the intestinal microbiota than on host specificity.

The adhesion of probiotic strains to the intestine prevents the subsequent attachment of pathogens (Patterson and Burkholder, 2003). This is known as competitive exclusion (CE) and has been shown to reduce the colonization and shedding of *Salmonella* and *Campylobacter* spp. in day-old chicks (Patterson and Burkholder, 2003). CE is 100% biological, does not leave any residues and one treatment on the first day of hatching was reported to be sufficient, either by spraying the hatchery or applying the probiotics to the drinking water (Schneitz, 2005). CE is thought to work via production of volatile fatty acids (VFAs) and the occupation of attachment sites on the mucosa. VFAs decrease the growth rate and maximum optical densities of pathogens, as shown by the decrease in numbers of *Enterobacteriaceae* in the presence of VFAs (Schneitz, 2005). Seuna (1979) showed that chicks challenged with *Salmonella* one to two hours after CE treatment were well protected from colonisation of the pathogen. Administration of *Lactobacillus plantarum* has lead to the inhibition of enterotoxigenic *Escherichia coli* (Pretzer *et al.*, 2005). This is thought to be due to the competitive exclusion of *E. coli* by recognition of the same binding sites on the intestinal membrane.

The host epithelium has mannose-containing glycoconjugates which are targets for many pathogenic bacteria having type one fimbriae. These fimbriae are responsible for mannose-specific adherence to the host epithelial cells. *L. plantarum* has been shown to specifically bind to mannose-containing sugar moieties and to human intestinal cell lines, leading to competitive exclusion of pathogens (Adlerberth *et al.*, 1996). This adhesion is thought to be due to a lectin-like element in the bacterial cell wall (Kinoshita *et al.*, 2005). Apart from this protein, lactobacilli cell surfaces contain lipoteichoic acid, teichoic acid, extracellular polysaccharide and other cell wall surface proteins. It is speculated that the lectin-like protein

in the bacterial cell wall recognizes the sugar chains on human colonic mucin (HCM), a high molecular weight glycoprotein. *L. plantarum* was shown to have a higher adhesion value to HCM, containing sugar moieties, than to BSA which has no sugar chains.

Other possible adhesion factors include a mucin-binding protein, a fibronectin-binding protein and a surface layer protein (Buck *et al.,* 2005). Inactivation of the gene, *slpA*, encoding the surface layer protein, resulted in an 84% decrease in adherence (Buck *et al.,* 2005). The function of the surface layer of a bacterial cell involves cell shape, protection, epitope display and epithelial-cell attachment. It is therefore not surprising that the inactivation of this gene leads to such a significant decrease. Fibronectin is a secreted protein, thought to be responsible for *Lactobacillus* spp. adhesion (Kapczynski *et al.,* 2000). It has been shown that inactivation of a surface-associated protein, FbpA, leads to a 76% reduction in *L. acidophilus.* Glycoproteins called mucins are present in the mucus surrounding host intestinal cells (Kapczynski *et al.,* 2000). Mub, a mucin-binding protein in *L. reuteri*, has been shown to be associated with the cell surface (Roos and Jonsson, 2002). A strain having a mutation in this protein showed a decrease in adherence of 65%.

1.10.3.4. Oxygen and temperature tolerance

During processing of the bacterial culture to produce a probiotic in a form suitable for the host to consume, the strain undergoes various environmental stresses such as high temperature and oxygen exposure (Crittenden, 2009). Since most potential probiotics are anaerobic, they need to be able to survive processing and storage conditions without losing viability. Thermal inactivation of bacteria is thought to come about by denaturation of cell components like RNA and DNA. Oxygen-induced inactivation is brought about when there are high levels of reactive oxygen species (ROS) present (Kullisaar *et al.,* 2002). ROS cause DNA, protein and lipid damage. Aerobic and facultative anaerobic bacteria produce the enzymes catalase and superoxide dismutase (SOD), amongst others, which reduce ROS to harmless substances. Bifidobacteria are strict anaerobes, but differ slightly in their oxygen tolerance. Kaizu *et al.* (1993) showed that some Lactobacilli have antioxidative activity and can reduce the accumulation of ROS during ingestion of food. Screening microorganisms from extreme conditions seems to be a plausible method of finding heat tolerant species, as

reported by Niamsup *et al.* (2003), who isolated *Lactobacillus* species from chicken faeces under high temperature.

1.10.3.5. Enzyme production

An effective probiotic is one which has functional properties useful to the host. One of these properties is the production of enzymes to aid in the digestion of food and therefore increasing the availability of minerals to the host (Guarner and Schaafsma, 1998). A good example is the hydrolysis of phytic acid. Mono-gastric animals do not produce phytases (Brindch-Pederson *et al.,* 2002) which break down phytic acid to phosphates and minerals, essential to animal health. They therefore depend on the microbiota to produce phytases. If the gut flora is not established, due to the use of antibiotics, problems such as bone diseases appear. Many bacteria, *Bifidobacterium* in particular have been identified as phytase producers (Haros *et al.,* 2005). Interestingly, many phytase-producing bacteria are found in wild birds, suggesting that their diet supports these bacteria more readily than does the artificial diet, where phytic acid-rich food is prevalent. Enzymes that aid in the digestion of fibre, such as cellulases and xylanases, would also be beneficial to the ostrich as they live on a high fibre diet which is a major source of energy to growing chicks (Swart *et al.*, 1993a).

1.10.3.6. Antibiotic resistance

There are several mechanisms employed by antibiotic resistant bacteria. These include: decreased uptake of antibiotic, increased export of the antibiotic, inactivation/modification of antibiotic target, introduction of new antibiotic-resistant target, hydrolysis of antibiotic, modification of antibiotic, or prevention of activation of the antibiotic (Ammor *et al.,* 2007). The wide use of antibiotics worldwide has lead to the appearance of antibiotic-resistant bacterial strains. Resistant bacteria can have intrinsic or acquired resistance. Acquired resistant is achieved by either one or more sequential mutations or by the incorporation of new genes (Ammor *et al.,* 2007). The latter presents the highest risk of horizontal spread. Teuber *et al.*, (1999) have proposed that beneficial commensal bacteria may be responsible for the transfer of resistance genes to pathogenic bacteria. Probiotic strains have shown sensitivities to different antibiotics, allowing for the use of various antibiotics in media for selection of certain strains from a mix of strains (Ammor *et al.,* 2007). The range of

resistance seen also allows for the use of antibiotic treatment while using a multistrain probiotic preparation as not all strains will be affected. These advantageous properties may lead to the uncertainty of the safety of the probiotics. For human consumption, all strains need to be graded as Generally Regarded As Safe (GRAS). With the presence of resistance genes, the risk of gene transfer to pathogenic bacteria is possible. When characterising a possible probiotic strain, it is important to check for signs of transferable antibiotic resistance.

1.10.4. Monostrain, multistrain or multispecies probiotics

Probiotics have been shown to prevent a variety of diseases, but their modes of action against these various diseases differ and it has been postulated that a multispecies preparation would be more efficacious. Monostrain preparations contain a single strain and may have less chance of successfully colonizing the gut (Famularo *et al.*, 1999) whereas since probiotic responses are strain-specific, multistrain preparations, containing more than one strain of the same species or genus may overcome the challenges faced in the gut more efficiently (Timmerman *et al.*, 2004). In addition, a multispecies mixture, containing strains of more than one genus, would be more appropriate (Timmerman *et al.*, 2004). Mixed cultures are also thought to have synergistic effects and to complement each strain's health effect. A downside of a multistrain or multispecies probiotic mix is that secreted antimicrobial compounds may not only affect the target organism, but each other too (Kailasapathy and Chin, 2000). Therefore, in the development of a multistrain mixture, the effect of each strain must be thoroughly investigated, and the safety of the overall effect of the blend must be known.

1.10.5. The development of ostrich probiotics

The five strains used in this study were originally isolated by a PhD student (Law-Brown, 2005, unpublished) as part of a study where 71 strains were isolated from faecal or intestinal samples from healthy ostriches. The samples were isolated by culturing from three sources:

- Intestinal extracts of ostriches fed on 15 different artificial diets containing no antibiotics
- Intestinal extracts from culled wild ostriches (Law-Brown, 2005, unpublished)
- Freshly excreted faeces from ostriches on a commercial farm to which antibiotics were regularly administered.

The sampling points for the intestinal samples included the caecum, the small intestine and the large intestine or rectum.

The samples were cultured on media known to favour the growth of Gram positive bacteria especially *Bifidobacterium* spp. and lactic acid bacteria. In addition, samples were screened on media designed for the detection of useful enzymes such as cellulases and xylanases (Law-Brown, 2005, unpublished). The 71 strains were presumptively identified and 34 were subjected to preliminary screening for bile tolerance, aggregation capabilities and the production of antimicrobial compounds (Juste-Poinepen, 2008). In this way, the best ten strains were selected for more intensive investigation of probiotic properties. Five of these strains have been further characterised in this thesis.

1.11. Aims of current research

The main aim of this project was to develop a probiotic mixture to improve the health and survival rates of farmed ostrich chicks in South Africa. The probiotic mixture would be tested in feeding trials on ostrich chicks over nine weeks. The aims fall under three main categories, namely:

1. To identify and characterise the five probiotic strains previously isolated from ostrich faecal and gastrointestinal samples. These five strains were chosen based on preliminary screening of their functionality as potential probiotics, particularly the acid and bile tolerance (Law-Brown, 2005, unpublished; Juste-Poinapen, 2008). The identification of these strains will be confirmed using molecular techniques and further characterised *in-vitro* to assess their ability to survive the transit through the harsh conditions of the gut of an ostrich and be able to colonize the GIT.

2. To plan and carry out two feeding trials to test the probiotic effects of these five strains *in-vivo* in the ostrich chick in the presence and absence of the growth promoter, tylosin. This will include the development of a mode of delivering the probiotic mix to the ostrich chicks successfully, monitoring of the weights, feed conversion and mortality rates of the chicks, and collection of fresh faecal samples as well as gut mucosal scrapings, and the processing and storage thereof.
3. To analyse the effects of the probiotics on the diversity of the faecal and mucosa-associated microbes in the ostrich chicks and to monitor the transition of the microbiota with age using culture-dependent and independent methods. This is to get an overview of the effect of probiotics and tylosin on the microbiota of young ostrich chicks and assess whether the probiotic mix is able to successfully colonise the gut or control pathogenicity.

CHAPTER 2

Identification and characterisation of potential probiotic strains isolated from the gastro-intestinal tract of wild and farmed ostriches

Contents

2.1. **Summary** 32

2.2. **Introduction** 32

2.2.1 Aims and objectives of this study..... 37

2.3. **Materials and methods** 35

2.3.1. Bacterial strains and growth condition 35

2.3.2. Extraction of genomic DNA 36

2.3.3. Identification of strains 37

2.3.4. Characterisation of strains 40

2.4. **Results and Discussion** 46

2.4.1. Identification of isolates 46

2.4.2. Characterisation of isolates 54

2.4.2.1. Acid and bile tolerance assays 54

2.4.2.2. Measurement of aggregation properties between probiotic and pathogens 56

2.4.2.3. Adhesion of the probiotic strains to chicken mucous and their ability to displace and inhibit pathogens 58

2.4.3. Adhesion mechanisms 61

2.4.3.1. Inhibition of pathogen adhesion to intestinal mucous 61

2.4.3.2. Displacement of pathogens adhered to intestinal mucous 61

2.4.3.3. Competitive exclusion to intestinal mucous 62

2.4.3.4. Antimicrobial action against selected pathogens 65

2.4.3.5. Enzyme assays 66

2.4.3.6. Antibiotic resistance profiles of the strains 68

2.4.3.7. Thermotolerance assay 70

2.5. **Conclusion** 72

2.1. Summary

The identification of five potential probiotic strains, isolated from ostriches, was confirmed using various molecular techniques and they were screened for some of the advantageous properties of probiotics. Four *Lactobacillus* spp. and one *Bifidobacterium* spp. were shown to tolerate up to 2% bile and a pH of 2 and to have the ability to auto-aggregate and co-aggregate with four tested pathogens. The single strains and their various combinations were all able to inhibit these pathogens to different extents, and all were able to displace the pathogens except the strains alone against *C. difficile*. However, the probiotic combinations, as well as Lp6039.3, were unable to out-compete all the pathogens. This highlights the strain-specificity of these mechanisms. All five strains produced antimicrobial compounds against the important ostrich pathogens, *E. coli* and *S. typhimurium*. However, only Lr136.2.2 was active against *C. perfringens*. The potential probiotics produced enzymes for hydrolysis of plant fibre material and would be capable of playing a role in the digestion of food in the ostrich gut. The antibiotic resistance profile of the strains against ten common antibiotics, as well as tylosin, the growth promoting antibiotic used in this feeding trial, was also investigated. All probiotics were found to be resistant to kanamycin, streptomycin and tylosin. Only the *Bifidobacterium* Bp303.3.2 was sensitive to vancomycin and resistant to erythromycin. All five strains were sensitive to cephalothin, ampicillin, penicillin G, rifampicin, tetracycline and chloramphenicol. All strains were able to maintain a sufficient viability after incubation at 60°C for 10min. The above characteristics are some of the beneficial properties required for the success of a probiotic and the tested strains show promise as good candidates for use in the ostrich farming industry in replacing growth promoting antibiotics such as tylosin.

2.2. Introduction

In recent years there has been an increase in the demand for the correct strain identification of probiotics, especially for *Lactobacillus* spp. and *Bifidobacterium* spp., which are thought to exert health benefits to their consumers (Ammor e*t al.*, 2007). Correct identification of these bacteria to the species/strain level is mandatory as probiotic effects are known to be species specific (FAO/WHO, 2002). It is recommended to use a combination of phenotypic and

genetic techniques to accomplish the identification and classification of the potential probiotics. Further characterisation is needed to study the functional aspects of the strain as well as safety assessment. Figure 2.1 shows the process by which a novel potential probiotic strain is isolated and screened by *in vitro* methods in order to gain knowledge of the mechanisms by which the strain exerts its probiotic effect. Hereafter, *in vivo* tests are performed.

2.2.1. Isolation of probiotic strains

As described in Chapter one, 74 original isolates were screened for functionality as probiotics. Of these, five strains including four *Lactobacillus* spp. and one *Bifidobacterium* spp. were chosen based on their beneficial characteristics for inclusion in this study, following the outline described in Fig. 2.1. This process is described in detail in Chapter one.

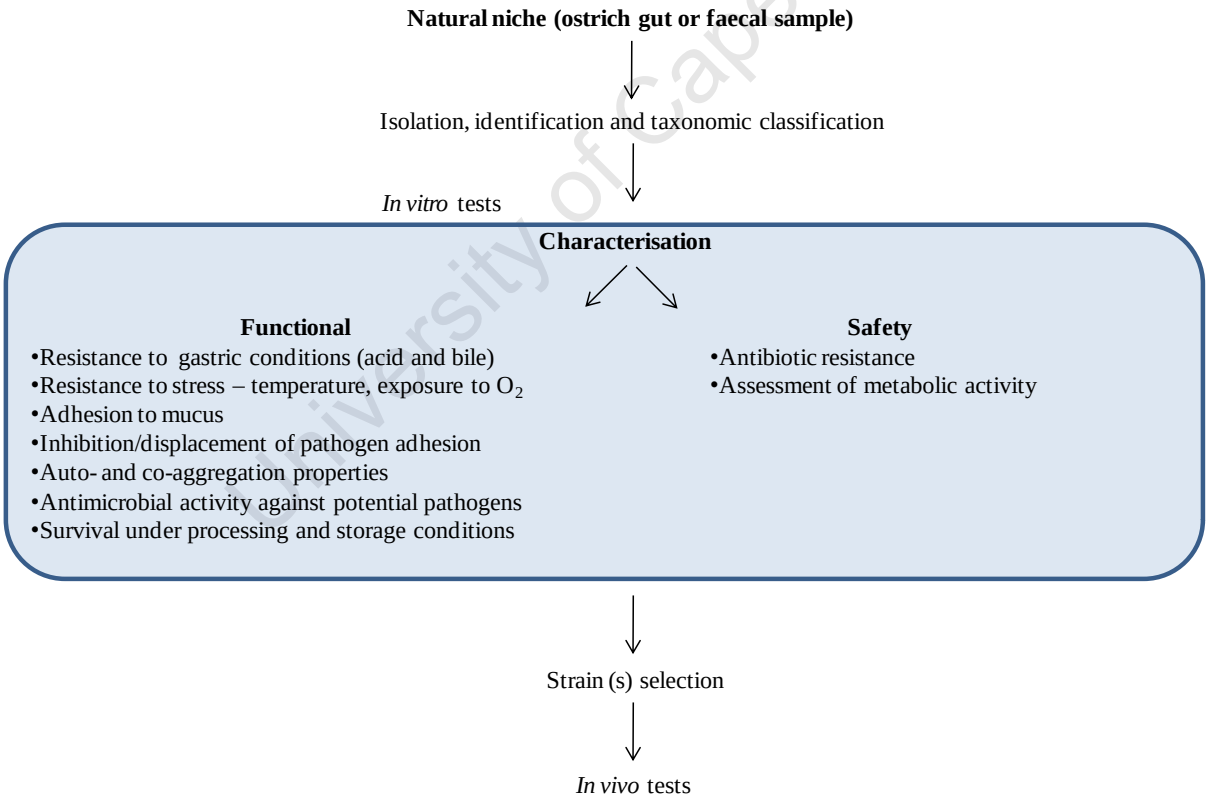

Figure 2.1. Process followed in this study to isolate and characterise novel probiotic strains

2.2.2. Identification

In order to determine the strain specificities of the potential probiotics, a number of different approaches are taken. Morphological studies under the microscope, growth studies and biochemical testing are usually performed. Further phenotypic identification methods include the Gram stain, enzyme production profile, determination of metabolites and the ability to utilise sugars. Although necessary, phenotypic identification is not always reproducible due to culture conditions, metabolic status of the cell and sometimes the lack of stability of certain genetic determinants for the resulting phenotype (Margolles *et al.,* 2009).

Molecular methods for identification are more reliable and reproducible (Tannock, 1999) and are mostly based on the 16S ribosomal RNA genes, allowing for the establishment of phylogenetic relatedness at the species and genus level. These tools can also be used to trace LAB during and post- ingestion of probiotic feed supplements, as well as from intestinal samples of subjects ingesting the probiotics.

The mucous-adhesion promoting protein (MapA) has been identified as one of the adhesion factors on the surface of *L. reuteri,* allowing it to adhere to mucous, as well as to the intestinal epithelial cells (Miyoshi *et al.*, 2006). In addition to providing information on the adhesion probability of the strains, the *map*A gene was also used for identification of the strains, and as it is less conserved between species, was hoped to be useful for differentiation of strains of the same species.

Another rapid and convenient method of identification of strains is the use of the random amplified polymorphic DNA (RAPD) technique, a useful tool to study genetic polymorphisms of DNA. It allows for the differentiation between closely related bacteria through the use of PCR using short primers which bind to the two strands of DNA at several locations, thus generating a unique fingerprint (Manan *et al.,* 2009). This method has been used to distinguish closely related strains (Johansson *et al.*, 1995) as well as to identify a selected strain from a mixture of bacteria (Tilsala-Timisjarvi and Alatossava, 1998). RAPD analysis was used in this study to detect the ingested probiotics in the ostrich faecal samples.

2.2.3. Characterisation of probiotic strains

As outlined in Figure 2.1 and described in Chapter one, several criteria are used for the characterisation of potential probiotics. The main obstacles to successful colonisation of the host by probiotic bacteria is passage through the harsh conditions of the GIT, the ability to colonise the gut which is related to the adhesion ability of the strain to the mucous or epithelial cells, and the antimicrobial activity of the potential probiotic, through the production of antimicrobial substances or its ability to inhibit or displace pathogens.

Aims and objectives of this study

The aim of this study was to further characterise five chosen potential probiotic strains. These five strains were chosen from 74 originally isolated microbes of ostrich origin based on their identity, acid and bile tolerance profiles and activity against certain pathogens. The intention was to select suitable strains that could eventually be used for the development of novel gastrointestinal probiotics, capable of replacing the use of growth-promoting antibiotics.

2.3. Materials and methods

2.3.1. Bacterial strains and growth condition

Bacterial strains were previously isolated by Law-Brown (2005, unpublished) and partially characterised. Ostrich gut microbiota samples were collected from three sources, namely: faecal samples from healthy ostriches from a commercial show farm, samples from the small intestine, caecum, large intestine and rectum were collected from healthy adult birds on 15 different diets at an experimental research centre, and from wild adult birds. Five of these were chosen based on the preliminary characterisation studies and characterised further in this study.

The ostrich faecal isolates were inoculated into 5 ml MRS broth (Biolab) supplemented with 0.05% cysteine-hydrochloride (MRS-Cys) or MRS agar (1.5% w/v) supplemented with 0.05% cysteine HCL (Sigma), and incubated for 48 hr at 37°C in an anaerobic chamber (Forma Scientific) consisting of 85% Nitrogen, 10% Carbon Dioxide and 5% Hydrogen.

In addition, pathogenic strains used in the antimicrobial assay were grown as follows: *E. coli* ATCC25922, *Salmonella typhimurium*, and *Staphylococcus aureus* were incubated aerobically on Luria agar, *Clostridium botulinum, Clostridium perfringens, Enterococcus faecium* van^R and van^S were incubated anaerobically on BHI agar.

For the mucous adhesion studies, four bacterial pathogens were used: *Clostridium difficile* DSM 1296, *Clostridium perfringens* DSM 756, *E. coli* NTCT 9001 and *Salmonella enteric* serovar Typhimurium ATCC 12028 and were grown in Gifu anaerobic medium (GAM Nissui Pharmaceutical, Tokyo, Japan) under anaerobic conditions for 18 h. Bacteria were metabolically labeled by addition of 10 µl/ml tritiated thymidine (5-^{3}H-thymidine, 120 $Cimmol^{-1}$, Amersham Biosciences, Little Chalfont, UK) to the growth medium.

2.3.2. Extraction of genomic DNA

Pure bacterial cultures (10 ml) were grown for 18 hours in MRS broth under anaerobic conditions and the purity of the cultures was assessed by Gram staining. Genomic DNA was extracted using the Fermentas Genomic DNA purification kit (K0512, Fermentas Life Sciences) with some modifications. Briefly, cultures were centrifuged at 6000 rpm (Eppendorf centrifuge 5415 C) for 2 min. The pellets were resuspended in a lysozyme-containing lysis buffer (20mM TrisCl pH8, 2mM EDTA, 1.2% Triton X-100, 20mg/ml lysozyme) prepared according to the Council for Scientific and Industrial Research (CSIR) for the lysis of *Corynebacterium* species, and incubated at 37ºC for 1 hour. Proteinase K (20 mg/ml) was then added, and incubated for a further 1 hour. Hereafter, the standard kit protocol was followed. Prior to the precipitation step, RNase (10µg/ml) was added and the samples were incubated for 20 min at 37ºC. DNA concentration was measured spectophotometrically using a Nanodrop (Nanodrop Technologies) and DNA quality was assessed by agarose gel electrophoresis using a 0.8% agarose gel in TAE buffer (40mM Tris-acetate, 0.5mM EDTA) at 5.5 $V.cm^{-1}$ for one hour and visualised using ethidium bromide under short wavelength UV light in a Gel-Doc XR (Bio-Rad).

2.3.3. Identification of strains

2.3.3.1. Primers and PCR conditions

Table 2.1. Primers used in the identification of the probiotic strains

Target	Expected product size (bp)	Primer	Sequence (5' – 3')	Annealing temp (°C)	Reference
16S rRNA gene	1500	F27 (universal primer)	aga gtt tga tcc tgg ctc ag	55	Lane, 1991
		R5 (universal primer)	acg git acc ttg tta cga ctt		Wheeler *et al.*, 1996
Lactobacillus -specific 16S rRNA gene	350	Lac 16S-f	agc agt agg gaa tct tcc a	64	Walter *et al.*, 2001
		Lac 16S-r	cac cgc tac aca tgg ag		Walter *et al.*, 2001
Bifidobacteria - specific 16S rRNA gene	1400	lm26-f	gat tct ggc tca gga tga acg	57	Kaufman *et al.*, 1997
		lm3-r	cgg gtg cti ccc act ttc atg		Kaufman *et al.*, 1997
RAPD	Various bands of different size	OPL-05	acg agg cac	55	Tilsala-Timisjarvi and Alatossava, 1998
		PL-1	acg cgc cct		Tilsala-Timisjarvi and Alatossava, 1998
ITS	variable	16-1A	gaa tcg cta gta atc g	55	Tannock *et al.*, 1999
		23-1B	ggg ttc ccc cat tcg ga		Tannock *et al.*, 1999
mapA gene fragment	600	mapA-f	ttt gaa gt(ca) ga(ct) ct ggt aaa gc	53	Juste-Poinapen, 2007
		mapA-r	c(ca)c g(tg)g agt t(ag)a t(ct)g t(ct)c c		Juste-Poinapen, 2007

2.3.3.2. PCR reactions

PCR amplification of the 6 chosen strains was performed using a GeneAmp PCR System 9700 (Applied Bio Systems). A 50 µl reaction contained 25 µl Fermentas Master Mix (0.05 units of Taq DNA polymerase/µl of reaction buffer, 0.4mM of each dNTP), 12.5 pmol of each primer (Table 2.1), 200 ng of genomic DNA and 18 µl nuclease free sterile water (Fermentas) and were amplified using the conditions below (Table 2.2). PCR products were analysed on 0.8% (16S PCR) and 2 % for the remaining reactions.

Table 2.2. Polymerase chain reaction conditions

		25 cycles			
Universal 16S rRNA	96°C 2 min	95°C 30 sec	55°C 30 sec	72°C 1.5 min	72°C 3 min
		35 cycles			
Lactobacillus spp. 16S rRNA	94°C 5 min	94°C 30 sec	56°C 20 sec	68°C 40 sec	68°C 7 min
		30 cycles			
***Bifidobacterium* spp. 16S rRNA**	94°C 5 min	94°C 30 sec	57°C 30 sec	68°C 1.5 min	57°C 30 sec 68°C 7 min
		35 cycles			
ITS	94°C 2 min	94°C 30 sec	55°C 1 min	72°C 1 min	72°C 10 min
		40 cycles			
RAPD	94°C 2 min	94°C 15 sec	37°C 30 sec	72°C 2 min	72°C 10 min
		30 cycles			
***map* A**	94°C 1 min	94°C 30 sec	53°C 30 sec	72°C 30 sec	72°C 5 min

2.3.3.3. **Cloning of PCR products for sequencing**

The PCR products were purified using the Biospin PCR Purification kit (Bioflux, Japan) as per manufacturer's instructions, and the pure products were ligated into the pTZ57R/T vector (Fermentas) and transformed into *E. coli* DH5α. Colony PCR was used to confirm the presence of the correct construct and three of these plasmids were sequenced using the M13-forward and reverse primers.

In the case of the 16S-23S intergenic spacer region (ITS) PCR however, the smallest band of the *Lactobacillus* species and the only band of the *Bifidobacterium* was excised from the agarose gel and the fragment extracted using the Biospin Gel Extraction kit (Bioflux, Japan), and cloned as described above. Plasmid extraction and determination of the nucleotide sequence data was done by Macrogen Inc., Seoul, Korea. Further sequence analysis and editing was carried out using the DNAman software programme (Lynnon Biosoft, version 4.13). The closest match of the product was determined by searching the GenBank and NCBI databases using the BLAST algorithm (Altschul *et al*., 1997). A similarity of 97% or more to

the 16S rRNA gene sequences of type strains was used as a basis for identification. Phylogenetic analysis was performed using *MEGA* (version 4; Tamura *et al.*, 2007).

2.3.3.4. **Optimisation of randomly amplified polymorphic DNA (RAPD) PCR (Guan *et al.*, 2003).**

A single, pure colony was picked and resuspended in 1 ml of nuclease free water (Fermentas) and was added to a 2ml screw cap eppendorf containing 0.1 mm sterile zirconia/silica beads and bead beaten for 70, 100 or 140sec at 4800 oscillations/min using a Mini-Beadbeater-1 (Biospec Products, Oklahoma, U.S.A). 140 sec bead beating was found to be optimal for cell shearing. PCR reactions were set up using this sheared cell suspension and the standard RAPD PCR conditions and primers were used. For optimisation purposes, the reaction mixtures varied.

- A 25 µl reaction contained 2.5 µl 10 X PCR buffer, 200 µM concentration of each deoxynuleoside triphosphate, 2mM $MgCl_2$, 12.5 pmol each primer, 0.25U Taq DNA polymerase (Fermentas), and 0.5, 1 or 1.5 µl cell supernatant
- A 25 µl reaction contained 12.5 µl Kappa Ready Mix (0.05 units of Taq DNA polymerase/µl of reaction buffer, 0.4mM of each dNTP), 12.5 pmol of each primer, and 0.5, 1 or 1.5 µl cell supernatant**.**
- A 25 µl reaction contained 12.5 µl Fermentas Master Mix (0.05 units of Taq DNA polymerase/µl of reaction buffer, 0.4mM of each dNTP), 12.5 pmol of each primer, and 0.5, 1 or 1.5 µl cell supernatant.
- A 25 µl reaction contained 1x buffer B (at 1.5mM magnesium chloride), 0.2mM dNTP mix, 2mM $MgCl_2$, 12.5 pmol each primer, 0.5U Kapa2G Robust Taq (Kapa Biosystems, R.S.A.), and 0.5, 1 or 1.5 µl cell supernatant.

For reproducible results, this procedure was repeated at least twice.

2.3.3.5. **API identification**

API strips specific for anaerobic bacteria and *Lactobacillus* species, 50CHL were used to identify the six chosen strains. The methods followed the manufacturers' (Biomérieux, France) guidelines and were set-up and incubated in the anaerobic chamber (Forma

Scientific) for 48 hours, and results were analysed using the API software (API Plus V 3.3.3 software).

2.3.4. Characterisation of strains

2.3.4.1. Bile salt and acid tolerance assay

The bacterial strains were tested for their ability to grow in the presence of Ox bile salts (LP0055, Oxoid – sodium glycholate and sodium taurocholate at 1%, 2% and 3% (*w/v*)) and at low pH ranging from 1-3 using a 50% solution of concentrated HCl added to MRS broth. An overnight culture of the test strain was adjusted to OD_{600} = 0.5, and used to inoculate 20 ml of MRS-Cys broths at different pH or bile salt concentrations and incubated anaerobically at 37°C. MRS-Cys broth was used as the control. The absorbance was read at 620nm and 560nm for the acid and bile tolerance assays, respectively. Readings were taken every 2 h for the first 8 h and finally at 24 h in a S1000 Diode Array Spectrophotometer (Labotec).

2.3.4.2. Autoaggregation assays with probiotic strains

Overnight cultures of the probiotic strains were harvested by centrifugation at 3,200 x *g* at 4°C for 10 min (Eppendorf, centrifuge 5415 C). The cells were washed three times with PBS buffer and resuspended in 1 ml of the same buffer and adjusted to A_{600} = 0.25 (approximately 10^8 cfu/ml). Each strain and probiotic combination was incubated at 20 °C and the absorbance was monitored after 1, 2, 4 and 20 h of incubation. When autoaggregation was measured in a combination of probiotic mixes, equal volumes of cell suspensions (A_{600} = 0.25) were mixed in a 1:1 ratio. The percentage aggregation was calculated as follows using the formula $[(A_{time} - A_{mix})/ A_{time}] \times 100$, where A_{time} represents the absorbance of the mix containing the probiotic at time 0 h, and A_{mix} represents the absorbance of the culture at different incubation times.

2.3.4.3. Bacterial coaggregation analysis

Cells of probiotic and pathogen cultures were harvested as described for autoaggregation. Equal volumes of pathogen and probiotic strains (1:1 v/v) were mixed and incubated at 20 °C without agitation. Again, the absorbance was monitored at 1, 2, 4 and 20 h and the percentages of coaggregation were calculated as $[(A_{path} + A_{prob})/2 - (A_{mix})/(A_{path} + A_{prob})/2] \times 100$, where A_{path} and A_{prob} represent A_{600} of the separate bacterial suspensions in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension at different

incubation times. The results of the auto- and coaggregation assays were expressed as the average of four experiments (*n*=4), each experiment performed in quadruplicate to correct for intra-assay variation.

2.3.4.4. **Mucous adhesion assay**

Chicken intestinal mucous from different regions of the small and large intestine (duodenum, ileum, jejunum, colon and caecum) was collected as previously described (Ouwehand, et al., 2002) and stored at -80°C until use. Before use the protein concentration of the mucous was determined and diluted to 0.5 mg/ml in HEPES (N-2-hydroxyethylpiperazine-N-2-ethanosulphonic acid)-Hanks buffer (HH; 10 mM HEPES, pH7.4). 100 µl of this was immobilized into polystyrene microtitre plate wells (Maxisorp, Nunc, Roskilde, Denmark) by incubation at 4°C for 18 h. Radiolabelled bacteria were collected by centrifugation at 6000 rpm for 7 min and washed twice with PBS and the absorbance (A600) was standardized to 0.25. Of this, 100 µl was added to the wells and incubated at 37°C for 1 h. The wells were washed twice with 200µl HH buffer to remove unattached bacteria and the adhering bacteria were released and lysed by incubation with 1% (wt/vol) sodium dodecyl sulphate in 0.1M NaOH (200 µl per well) and incubation at 60°C for 1 h. The contents of each tube were added to 1 ml of scintillation liquid (OptiPhase "HiSafe 3", Wallac, Milton Keynes, UK) in a microcentrifuge tube and the radioactivity was measured by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilized mucous. Adhesion was determined in three independent experiments, and each assay was performed in triplicate.

2.3.4.5. **Inhibition of pathogen adhesion**

To test the ability of the probiotic strains to competitively exclude the pathogens, 100 µl unlabelled probiotic strains were added to wells containing immobilized mucous and incubated for 1 h at 37°C. Nonbound probiotics were removed by washing the wells twice with PBS and adding 100 µl radiolabeled pathogens and incubating for a further hour at 37°C. The wells were then washed to remove unbound labeled bacteria, and bound bacteria were released and lysed by addition of 1% (wt/vol) SDS in 0.1 M NaOH and incubation at 60°C for an hour. Radioactivity was measured by liquid scintillation. The results were calculated as the percentage of radioactivity recovered after adhesion relative to radioactivity of the bacterial suspension prior to adhesion, and the percentage adhesion inhibition was

measured as the difference in adhesion of the pathogen in the absence versus the presence of the probiotics. This assay was done in triplicate and in three independent experiments.

2.3.4.6. **Displacement of pathogens**

The ability of the probiotics to displace already adhered pathogens was tested as follows. Radiolabeled pathogens were added to the wells containing immobilized mucous and incubated for 1 h at 37°C. The wells were washed to remove unbound pathogens, and 100 µl of non-radiolabeled probiotics were added and the plates were incubated at 37°C for 1 h. The wells were washed, the bound bacteria released and lysed and radioactivity measured with liquid scintillation. The percentage of displacement of pathogens was calculated as the difference in adhesion before versus after addition of probiotics. Experiments were carried out in triplicate.

2.3.4.7. **Inhibition by competition assay**

Equal quantities of bacterial suspension of probiotic and radiolabeled pathogens were mixed and added to the immobilized mucous. This was incubated for 1 h at 37°C. The cells of the pathogen bound to the mucous were released and lysed as above. The competitive inhibition of the probiotics was measured as the percentage of pathogens bound after the combination with probiotic combinations relative to pathogens bound in the absence of probiotic strains (control).

2.3.4.8. **Antimicrobial properties**

Five µl of a pure overnight culture of the test strain was spotted in duplicate onto MRS-Cys agar plates and incubated anaerobically for 48h at 37ºC. The colonies obtained were killed by exposure to chloroform for 20min. Hereafter, the plates were overlaid with 6ml 0.75% sloppy agar inoculated with a calculated volume of the indicator strain (Table 2.3) and incubated under the respective conditions, suitable to the indicator strain for 48h. This experiment was done in triplicate. The zones of inhibition were calculated as the distance between the total inhibition zone and the edge of the colony.

Table 2.3. Experimental conditions of indicator strains

Indicator strains	Inoculation volume calculation	Soft agar media	Incubation conditions
E. coli ATCC25922	OD_{600} x µl = 4	Luria agar	Aerobic
S. typhimurium	OD_{600} x µl = 4	Luria agar	Aerobic
S. typhimurium (sewerage)	OD_{600} x µl = 4	Luria agar	Aerobic
S. aureus	OD_{600} x µl = 160	Luria agar	Aerobic
C. botulinum	OD_{600} x µl = 160	BHI agar	Anaerobic
C. perfringens	OD_{600} x µl = 160	BHI agar	Anaerobic
E. faecium vanR	OD_{600} x µl = 160	BHI agar	Anaerobic
E. faecium vanS	OD_{600} x µl = 160	BHI agar	Anaerobic

2.3.4.9. **Enzyme assays**

Modified BYG plates (Table 2.4) were used to assess the enzymatic properties of the strains. Five µl of a pure overnight culture was spotted onto the BYG agar with various substrates added, and incubated anaerobically at 37ºC for 48 h. All colonies, except those on the casein plates, were scraped off with a glass spreader under running water and tested for enzymatic activity.

In addition to these modified BYG agar plate tests, the API strips gave information on production of selected enzymes.

Table 2.4. Analysis of enzyme activities on specific BYG substrate agar plates

Test	Substrate/100ml BYG	Treatment	Positive result
Cellulase	1 g carboxy-methyl-cellulose (CMC)	16 min with 0.1% Congo red, destain 1M NaCl/flood with Bromocresol blue 2min	Yellow zone
Xylanase	0.1% xylan, 0.025% cellobiose	16 min with 0.1% Congo red, destain 1M NaCl/flood with Bromocresol blue 2min	Yellow zone
Inulinase	1% raftiline	16 min with 0.1% Congo red, destain 1M NaCl/flood with Bromocresol blue 2min	Yellow zone
Pectinase	0.5% pectin	Flood 1% cetyl-triammonium Bromide (CTAB) 40min	Zone of clearing on clouded agar
Protease	1-2% casein	No staining	White ppt around colonies
Phytase	2% (w/v) sodium phytate	Flood 2% cobalt chloride 5min, flood with equal volume 6.25% ammonium molybdate and 0.42% ammonium vanadate	Zone of clearing on clouded agar
Amylase	0.2% soluble starch	Flood Gram's iodine	Zone of clearing on blue, black agar

2.3.4.10. **Antibiotic resistance**

The antibiotic resistance profiles of the probiotics against 10 antibiotics were determined using the standard Kirby-Bauer disk diffusion method. The following antibiotics discs were used: vancomycin (5µg), kanamycin (30µg), cephalothin (30µg), ampicillin (10µg), penicillin G (10 units), rifampicin (5µg), streptomycin (10µg), tetracycline (30µg), erythromycin (15µg) and chloramphenicol (30µg;Oxoid Ltd, Basingstoke, UK). The probiotic strains were grown in MRS-Cys broth for 18 h under anaerobic conditions. The overnight culture was adjusted to A_{600} = 0.25 and 100 µl of this was spread plated onto MRS-Cys agar. Each antibiotic was tested in duplicate and each plate was repeated in triplicate. The plates were incubated for 48 h under anaerobic conditions and the diameter of the zone of inhibition around each disk was measured.

2.3.4.11. **Tolerance of probiotic strains to antibiotic tylosin**

100µl of a pure overnight culture of the probiotics strains were separately spread plated onto MRS-Cys agar plates. An inverted blue eppendorf tip was used to bore holes into this agar and the agar plugs were removed. 100µl of various concentrations of tylosin (10, 20, 30 and 40 mg/l water) were added to these wells, in duplicate and the plates were incubated for 48h anaerobically. Each plate was done in triplicate.

2.3.4.12. **Thermotolerance assay**

A single, pure colony of the five probiotic strains were separately inoculated into 5ml MRS-Cys broth and incubated anaerobically overnight to ensure the cells were in stationary phase. The purity of the cultures was tested and the cells collected by centrifugation at 10000 g for 10 min (Eppendorf, centrifuge 5415 C). The pellets were resuspended in 2 ml PBS and incubated at 37°C, 50°C, 60°C and 80°C for 10 min. Serial dilutions were made of the control and of the heat treated suspensions and 100 µl was plated separately onto MRS-Cys agar. Each treatment was plated in triplicate.

2.3.4.13. **Statistics**

Statistical analysis was performed using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Data were subjected to one-way analysis of variance (ANOVA) and the post-hoc test as Student-Newman-Keuls (S-N-K) test was used for comparison of the means, where appropriate. $P<0.05$ was considered statistically significant.

2.4. Results and Discussion

2.4.1. Identification of isolates

The molecular identification of five potential probiotics isolated from ostrich faeces and intestines using the API 50 CHL as well as by the amplification of extracted genomic DNA using various primers is shown in Table 2.5. The API 50 CHL strips identified three of the five strains as *L. plantarum* (99.9%) and one as *L. acidophilus* with a low certainty of 85.6% (Table 2.5). The fifth strain Bp303.3.2 was not identified using the API 50 CHL strips as it had been previously identified as belonging to *Bifidobacterium* spp. The identity of Lr136.2.2 did not match the identity previously reported as *L. reuteri*. Previous work on bacterial identification using the API system has reported the incorrect identification of lactobacilli species as *L. acidophilus* (Klein *et al.,* 1998; Boyd *et al.,* 2005). In addition, Brolazo *et al.* (2011) found that *L. reuteri* detected using multiplex PCR, was not detected using the API system, suggesting *L. reuteri* is not on the API database. Although the API system is a rapid means of identifying large numbers of samples, this limited database, together with the inability to distinguish between closely related species, leaves molecular techniques to be the preferred method of identification.

For future confirmation of identity of isolates the reliability of the *Lactobacillus* specific primers, Lac 16S-f and Lac 16S-r, and the *Bifidobacterium*-specific primers, lm 26-f and lm 3-r (Table 2.1) were tested. Both primer sets showed species specificity, giving product sixes of approximately 350 bp and 1400 bp respectively, and were sequenced with their respective forward primers only (Table 2.5). Sequencing results confirmed the identity of the three *L. plantarum* strains, however, this method of identification correctly identified Lr136.2.2 as *L. reuteri*. Bp303.3.2 was also identitifed as *B. pseudolongum* subsp. *pseeudolongum.*

2.4.1.1. **16S rRNA gene PCR**

The extracted genomic DNA from the potential probiotic strains was used as a template for 16S rRNA gene PCR using the universal primers F27 and R5 (Table 2.1). The products obtained were approximately 1.5 kb and the nucleotide sequences were determined in both directions. Lp1.1.2.2, Lp6039.3 and Lp162.2 were identified as *Lactobacillus plantarum* with 100% similarity to sequenced *L. plantarum* (Accession numbers HQ286594.1, FJ763580.1, respectively; Table 2.5). Lr136.2.2 showed 99% similarity to *Lactobacillus reuteri*

(Accession number HM162424.1) and strain Bp303.3.2 was found to share 99% similarity to *Bifidobacterium pseudolongum* subsp. *pseudolongum* (Accession number AY174106.1).

Table 2.5. Preliminary identification of the five isolates

Strain	API 50 CHL	16S	Lac-specific 16S	Bif-specific 16S	ITS	*map*A
Lp1.1.2.2	*L. plantarum* (99.9%)	*L. plantarum* (100%) HQ286594.1	*L. plantarum* (98%) EF439684.1	Not amplified	*L. plantarum* (99%) AL935261.1	*L. plantarum* (100%) CP002222.1
Lp6039.3	*L. plantarum* (99.9%)	*L. plantarum* (100%) FJ763580.1	*L. plantarum* (98%) EF439684.1	Not amplified	*L. plantarum* (99%) AL935261.1	*L. plantarum* (100%) CP002222.1
Lp162.2	*L. plantarum* (99.9%)	*L. plantarum* (100%) FJ763580.1	*L. plantarum* (98%) EF439684.1	Not amplified	*L. plantarum* (99%) AB083120.1	*L. plantarum* (99%) CP002222.1
Lr136.2.2	*L. acidophilus* (85.6%)	*L. reuteri* (99%) HM162424.1	*L. reuteri* (99%) EF140740.1	Not amplified	*L. reuteri* (99%) EF412989.1	*L. reuteri* (99%) AP007281.1
Bp303.3.2	Not done	*B. pseudolongum* subsp. *pseudolongum* (99%) AY174106.1	Not amplified	*B. pseudolongum* subsp. *pseudolongum* (99%) GU361829.1	*B. pseudolongum* subsp. *pseudolongum* (99%) GU361829.1	Not amplified

In addition, the sequences obtained from the 16S rRNA genes were used for further analysis in the construction of bootstrap neighbour-joining phylogenetic trees (Fig. 2.2). Phylogenetic analysis was carried out separately for the *Lactobacillus* spp. (Fig. 2.2a), as it was for the *Bifidobacterium* spp. (Fig. 2.2b).

The analysis of the selected *Lactobacillus* spp. clustered as expected, Lp1.1.2.2, Lp6039.3 and Lp162.2 (all identified as *L. plantarum;* Table 2.5) grouped closely with *L. plantarum* type strain, and *L. paraplantarum* (Fig. 2.2a), suggesting the probiotic isolates are very closely related to the type strain, and fall into the *L. plantarum* group of *Lactobacillus* spp. Similarly, Lr136.2.2 (identified as *L. reuteri;* Table 2.5) also grouped closely to its respective type strain, but is not as closely related as the *L. plantarum* strains are to their type strain. Lr136.2.2 fell under the *L. reuteri* groups of *Lactobacillus* spp.

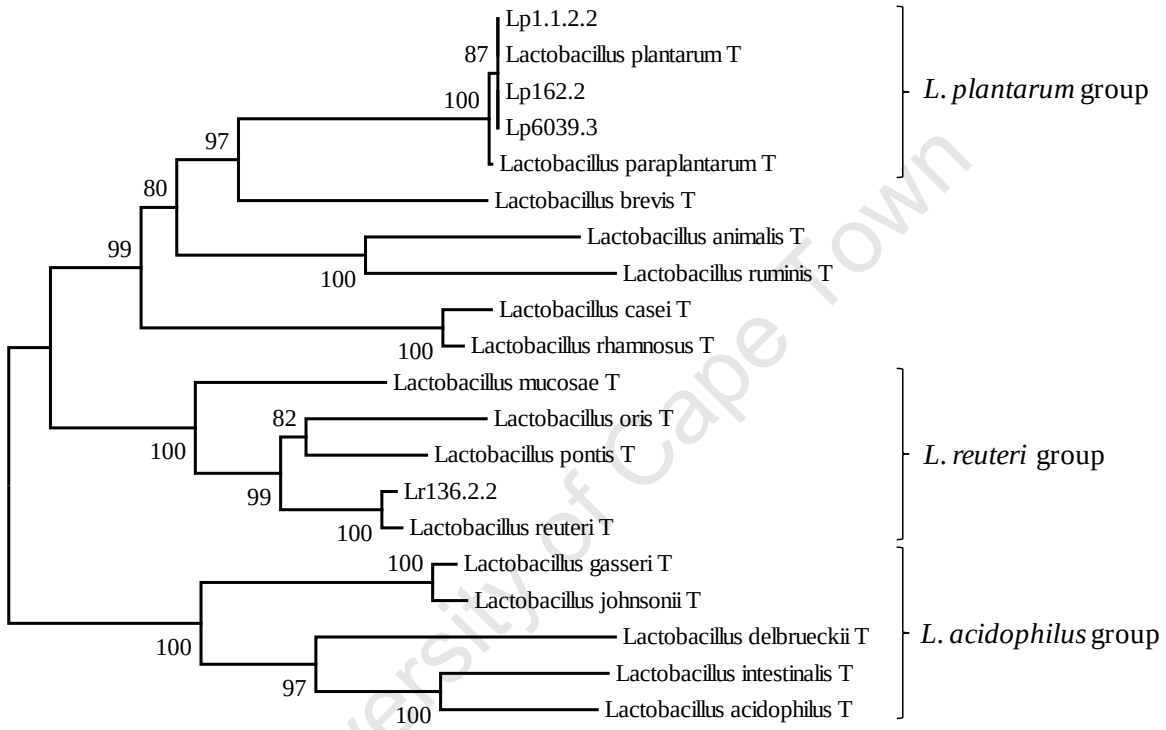

Figure 2.2a. Bootstrap consensus neighbour-joining phylogenetic tree using 16S rRNA gene sequences from the *Lactobacillus* species selected for characterisation as well as from type strains *L. plantarum* AJ965482, *L. paraplantarum* DSM10667, *L. brevis* M58810, *L. animalis* AB326350, *L. ruminis* AB326354, *L. casei* ATCC393, *L. rhamnosus* JCM1136, *L. mucosae* DSM13345, *L. oris* DSM4864, *L. pontis* LTH2587, *L. reuteri* L23507, *L. gasseri* ATCC33323, *L. johnsonii* ATCC33200, *L. delbrueckii* ATCC12315, *L. intestinalis* DSM6629 and *L. acidophilus* AY773947. Bootstrap values are shown at the nodes and represent 5000 iterations.

The *Bifidobacterium* spp. strain Bp303.3.2 isolated from ostriches was identified as *B. pseudolongum* subsp. *pseudolongum* (Table 2.5) and clusters close its type strain and closely related *B. pseudolongum* subsp. *globosum.* (Fig. 2.2b). Leblond-Bourget *et al.* (1995) reported that the V1, V2 and V3 regions of the 16S rRNA region of *Bifidobacterium* have a similarity of 82 to 99% and it has been recommended to amplify other regions such as the 16S-23S intergenic spacer regions for better identification (Felis *et al.*, 2001).

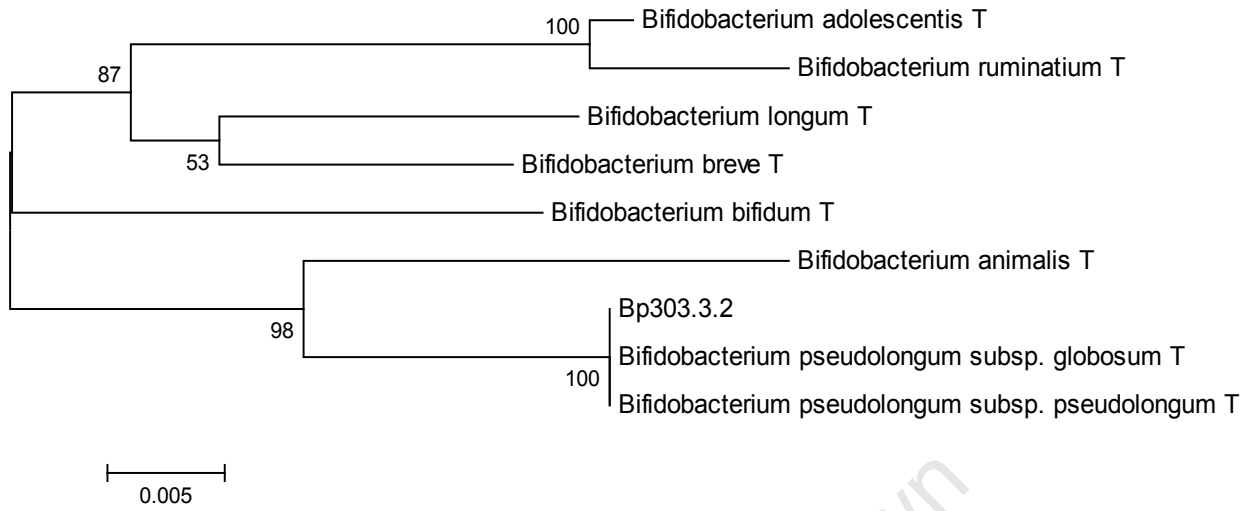

Figure 2.2b. Bootstrap consensus neighbour-joining phylogenetic tree using 16S rRNA gene sequence from the *Bifidobacterium* species Bp303.3.2, selected for characterisation, as well as from type strains *B. adolescentis* ATCC 15703, *B. ruminatium* JCM8222, *B. longum* ATCC15697, *B. breve* ATCC15700, *B. bifidum* KCTC3202, *B. animalis* AB050136, *B. pseudolongum* subsp. *globosum* JCM5820 and *B. pseudolongum* subsp. *pseudolongum* ATCC25526. Bootstrap values are shown at the nodes and represent 5000 iterations.

In order to discriminate between strains of the same species, less conserved regions like the 16S-23S intergenic spacer region (ITS) and the *mapA* genes were used to confirm the identities of the isolates. All the methods used here confirmed the identification of the strains.

Amplification of the 16S-23S intergenic spacer region (ITS) allowed a method to distinguish the probiotic strains from themselves and from a mixed microbial population using the fingerprint obtained. This is due to the hypervariable nature of the intergenic region compared to the 16S or 23S rRNA genes (Barry *et al.*, 1991). ITS-PCR of the five strains used in this study showed the typical fingerprint pattern, reported by Moreira *et al.* (2005), *Lactobacillus* having three bands, and *Bifidobacterium* having one (results not shown). The differing number of bands correspond to the short, medium and long spacer regions, are due to insertions of tRNA-Ala in the medium spacer regions, and tRNA-Ala and tRNA-Ile in the long spacer regions. Excision, amplification and sequencing of the small spacer region of the *Lactobacillus* strains allowed for confirmation of the identification of the strains found using the 16S rRNA gene (Table 2.5). The advantage of using ITS-PCR is its high discriminating power, for example, being able to differentiate between *L. casei* and *L. rhamnosus,* not

possible using 16S V2-V3 sequences (Tannock *et al.*, 1999). The phylogenetic analysis of the probiotic strains showed the intergenic spacer region of Lp162.2 to be most closely related to the *L. plantarum* type strain (Fig. 2.3a). The other *L. plantarum* strains of ostrich origin were also closely related to the type strain.

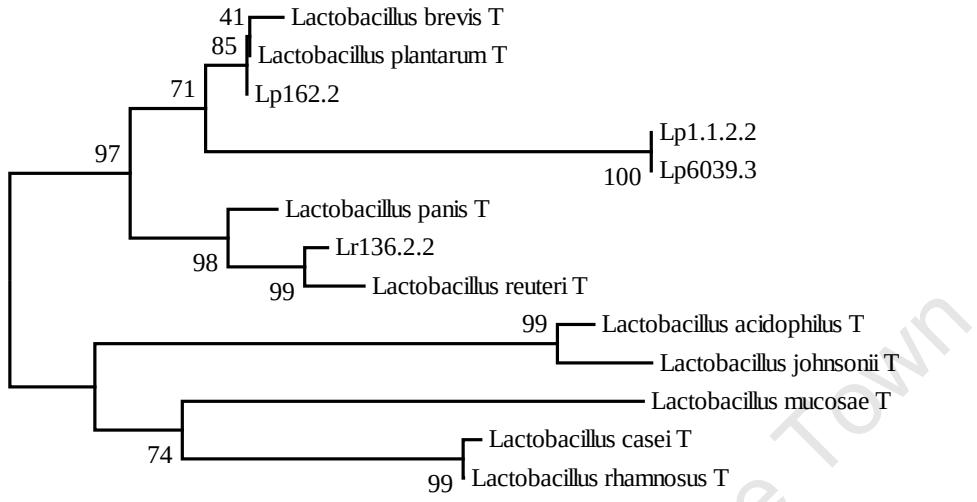

Figure 2.3a. Bootstrap consensus neighbour-joining phylogenetic tree using 16S-23S intergenic rRNA gene sequence from the *Lactobacillus* species selected for characterisation as well as from type strains *L. plantarum* AJ965482, *L. brevis* M58810, *L. casei* ATCC393, *L. rhamnosus* JCM1136, *L. mucosae* DSM13345, *L. panis* DSM6035, *L. reuteri* L23507, *L. johnsonii* ATCC33200 and *L. acidophilus* AY773947. Bootstrap values are shown at the nodes and represent 5000 iterations.

Similarly, the phylogenetic analysis of Bp303.3.2 showed it to be very similar to its type strain as seen in Fig. 2.3b.

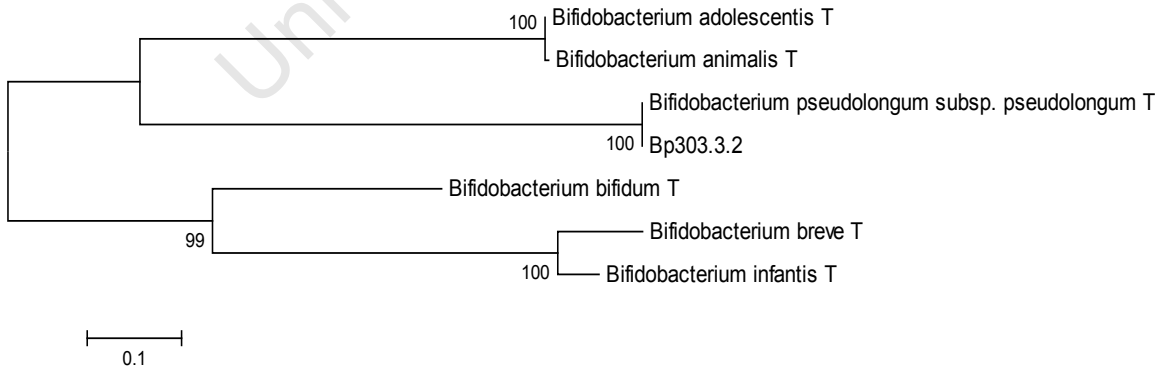

Figure 2.3b. Bootstrap consensus neighbour-joining phylogenetic tree using 16S-23S rRNA gene sequence from the *Bifidobacterium* species Bp303.3.2, selected for characterisation, as well as from type strains *B. adolescentis* ATCC 15703, *B. breve* ATCC15700, *B. bifidum* KCTC3202, *B. animalis* AB050136, *B. pseudolongum* subsp. *globosum* JCM5820 and *B. pseudolongum* subsp. *pseudolongum* ATCC25526. Bootstrap values are shown at the nodes and represent 5000 iterations.

Amplification of the *mapA* gene in the potential probiotic strains using degenerate primers gave a product of approximately 400bp for the four lactobacilli strains, but no product for the *Bifidobacterium* Bp303.3.2, suggesting that the sequence of this gene in *Bifidobacterium* spp. is very different to that of *Lactobacillus*, to which these primers were designed. The primers used were degenerate primers designed to the *mapA* sequences of *L. plantarum* 423 (Ramiah *et al.*, 2007) and *L. reuteri* NCIB 11951 (Miyoshi *et al.*, 2006). The multiple alignment of this gene (data not shown) showed the three *L. plantarum* strains to be identical in this region and Lr136.2.2 to have six mismatches when compared to the *L. reuteri* type strain. These sequences were therefore no use for the design of unique primers for accurate identification of the strains.

The *mapA* gene encodes a protein thought to enhance the adhesion of the bacteria to mucin in the gut (Rojas *et al.*, 2002). Adhesion to the mucosal layer is an important criteria in selecting probiotics (Kun Lee, 2009), and the detection of the *mapA* gene would give an indication as to the likelihood of the strain successfully adhering to the gut wall of the ostrich.

2.4.1.2. **RAPD**

RAPD analysis has been frequently used for molecular fingerprinting and allows rapid, specific, immediate identification of strains (Manan *et al.*, 2009). This technique allowed for detection of fed probiotics during the feeding trial. Optimisation of the method was needed as the PCR was performed on colonies which had been freshly isolated from faecal samples. Bead beating the colonies for 140 s and amplification using Kapa2G Robust *Taq* (Kapa Biosystems, R.S.A.) resulted in the clearest fingerprints (Fig. 2.4).

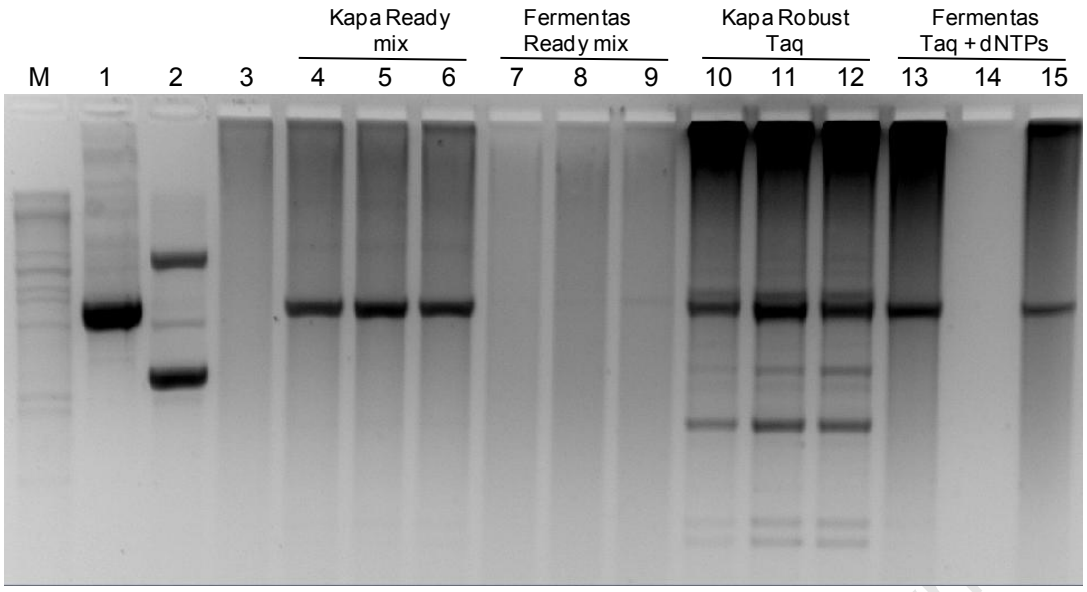

Figure 2.4. Optimisation of RAPD-PCR using different volumes of template and different master mixes using the same freshly isolated colony from a faecal sample, picked from an MRS agar plate. Lanes 4, 7, 10 and 13 used 0.5µl template; lanes 5, 8, 11 and 14 used 1 µl template and lanes 6, 9, 12 and 15 used 1.5 µl template. Lane: M, λ*Pst*I molecular weight marker; 1, OPL5 primer only control; 2, PL1 primer only control; 3, no template DNA control; 4-15, colony suspension in the above specified volumes, using the master mixes indicated above the respective lanes with primers OPL5 and PL1.

The sensitivity of the PCR was determined using genomic DNA of Lp6039.3 at amounts ranging from 20 ng to 200 ng using RAPD primers OPL05 and PL1 (Fig. 2.5). This was done to estimate at what level the strain would have to be present in the faeces to be detected using this method. Although 20 ng of DNA was detected, the best PCR yield was obtained using 100 ng DNA.

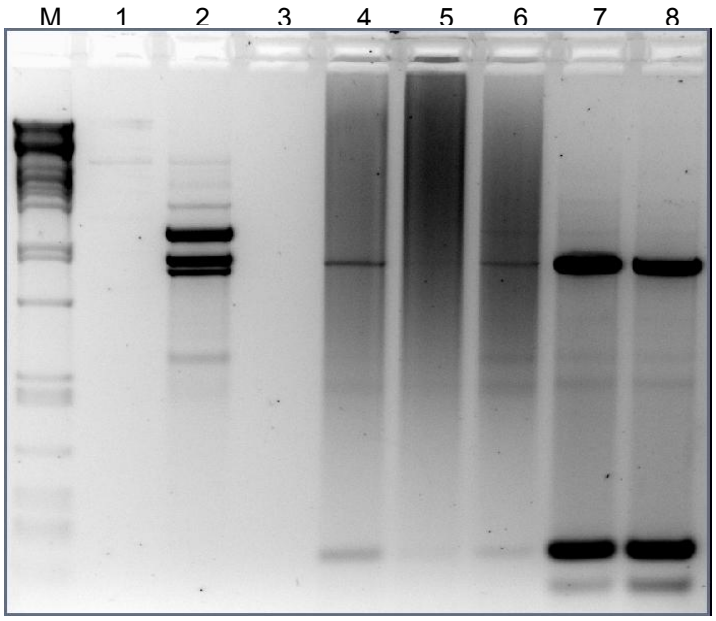

Figure 2.5. Sensitivity of RAPD-PCR using Kapa Robust Taq on different amounts of template DNA extracted from Lp6039.3. M. λ DNA digested with *Pst*I; Lane 1. Primer OPL05; Lane 2. Primer PL1; Lane 3. No template control; Lane 4. 20 ng DNA; Lane 5. 40 ng DNA; Lane 6. 60 ng DNA; Lane 7. 100 ng DNA; Lane 8. 200ng DNA.

Colonies of each probiotic were amplified with Kapa 2G Robust *Taq* (Kapa Biosystems, R.S.A.) using the optimised protocol and the patterns obtained can be seen in Fig. 2.6.

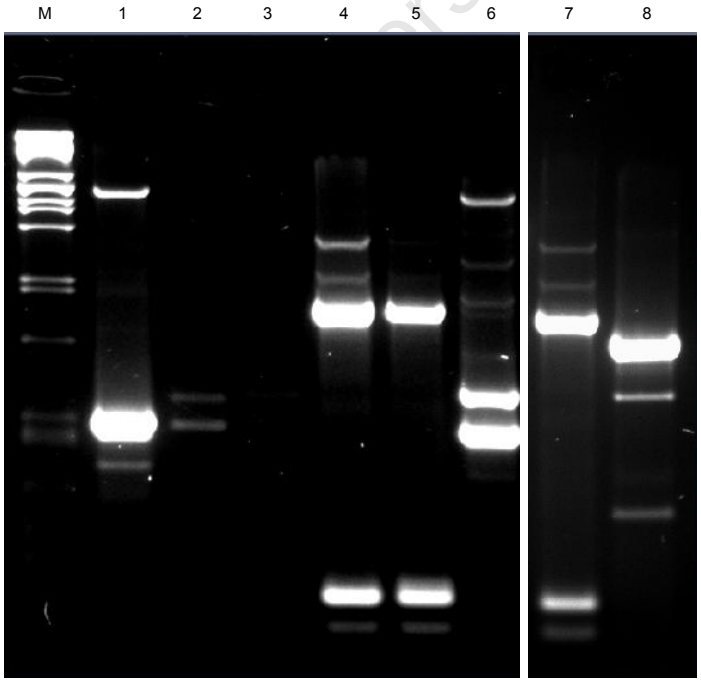

Figure 2.6. RAPD PCR amplification of DNA extracted from the five potential probiotic strains using Kapa Robust Taq. M. λ DNA digested with *Pst*I; Lane 1. Primer OPL05 with DNA obtained from Lp162.2.2; Lane 2. Primer PL1 with DNA obtained from Lp162.2.2; Lanes 3-8: Primers OPL05 and PL1 with: Lane 3. No template control; Lane 4. Lp162.2; Lane 5. Lp6039.3; Lane 6. Lr136.2.2; Lane 7. Lp1.1.2.2; Lane 8. Bp303.3.2.

The patterns obtained for the three *L. plantarum* strains, Lp162.2.2, Lp6039 and Lp1.1.2.2 are the same, as expected (although the top band in sample Lp6039 is much fainter than for Lp162.2.2 and Lp1.1.2.2; Fig. 2.6). *L. reuteri* and *B. pseudolongum* subsp. *pseudolongum* give unique fingerprints too. This confirms that this technique can discriminate between species but not between the same strains, and is a successful method for the differentiating between different *Lactobacillus* isolated from the ostriches.

2.4.2. Characterisation of isolates

2.4.2.1. Acid and bile tolerance assays

To confirm that the probiotic strains would survive the acid and bile levels encountered in the ostrich gut, *in vitro* tests were used to monitor the strains survival at different concentrations of bile and decreasing pH.

The growth of the five strains in both acid and bile showed similar trends. The three *L. plantarum* strains showed no decrease in growth in MRS at pH3 when compared to the control at pH 6.5 – 6.8. However, they did show reduced growth at pH 1 and 2 (Fig. 2.5). Increasing bile concentrations showed the same pattern as the decreasing pH, 2% and 3% bile inhibiting the growth of the *L. plantarum* strains slightly (Fig. 2.7). The *L. reuteri* and *B. pseudolongum* subsp. *pseudolongum* showed severely decreased growth in both acid and bile, and no growth was seen in the 6 hour time at pH1 and 3% bile. (Fig. 2.7 and 2.8). Acid and bile tolerance are considered intrinsic properties of LAB (Ouwehand *et al.*, 2002). *Bifidobacterium* spp. are considered to be less acid tolerant than some *Lactobacillus* spp. (Dunne *et al.*, 1999), however, the effect of bile on the *B. pseudolongum* in this study is surprising. Bile salt hydrolase (BSH) is reported to have been first isolated from *Bifidobacterium* spp., *L. acidophilus, L. gasseri, L. johnsonii* and some *L. plantarum* (Bergley *et al.*, 2004), and it would therefore be expected that the *B. pseudolongum* used here would be more tolerant to bile. However, Margolles *et al.* (2002) tested 19 *Bifidobacterium* strains and found the MICs to range from 0.125% to 2%. At 2% bile, the bifidobacteria in this study still showed some growth (Fig. 2.8). Stress tolerances to low pH and the presence of bile are reported to be strain specific and generalisations regarding the tolerances of species cannot be made (Bergley *et al.*, 2004). Strains obtained from harsh environments such as the

GIT have been shown to be more tolerant to acid and bile, and it is common to observe a "cross-resistance" to both of these conditions (Noriega *et al.*, 2004). It is believed that these tolerances can be either intrinsic or acquired (Margolles *et al.*, 2002; Noriega *et al.*, 2004). Margolles *et al.* (2002) obtained sodium-cholate-resistant *Bifidobacterium* strains by slowly exposing them to gradually increasing concentrations of this compound. Similarly, Collado and Sanz (2006) discovered a method of direct selection of acid-tolerant *Bifidobacterium* spp. by exposing human faecal samples to prolonged stressful conditions. These acid-resistant strains were also found to tolerate high concentrations of bile salt and NaCl. Given the strain-specific resistance profiles of bacteria and the incomparable results of *in-vitro* stress tests (Bergley *et al.*, 2004), all potential probiotics should be tested, and preferentially in conditions closely representing that of the conditions found in the GIT of the animal to be ingesting the strains.

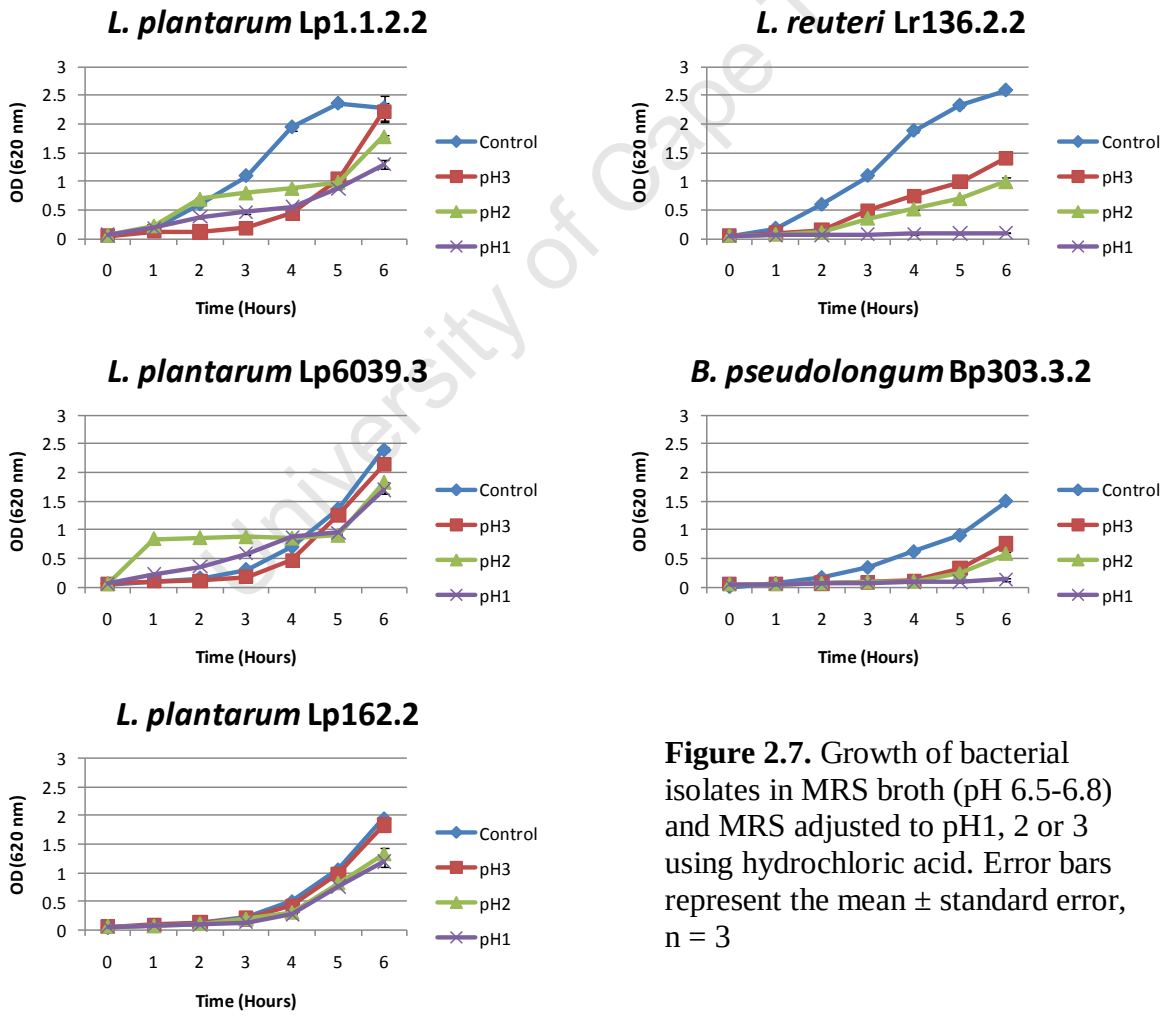

Figure 2.7. Growth of bacterial isolates in MRS broth (pH 6.5-6.8) and MRS adjusted to pH1, 2 or 3 using hydrochloric acid. Error bars represent the mean ± standard error, n = 3

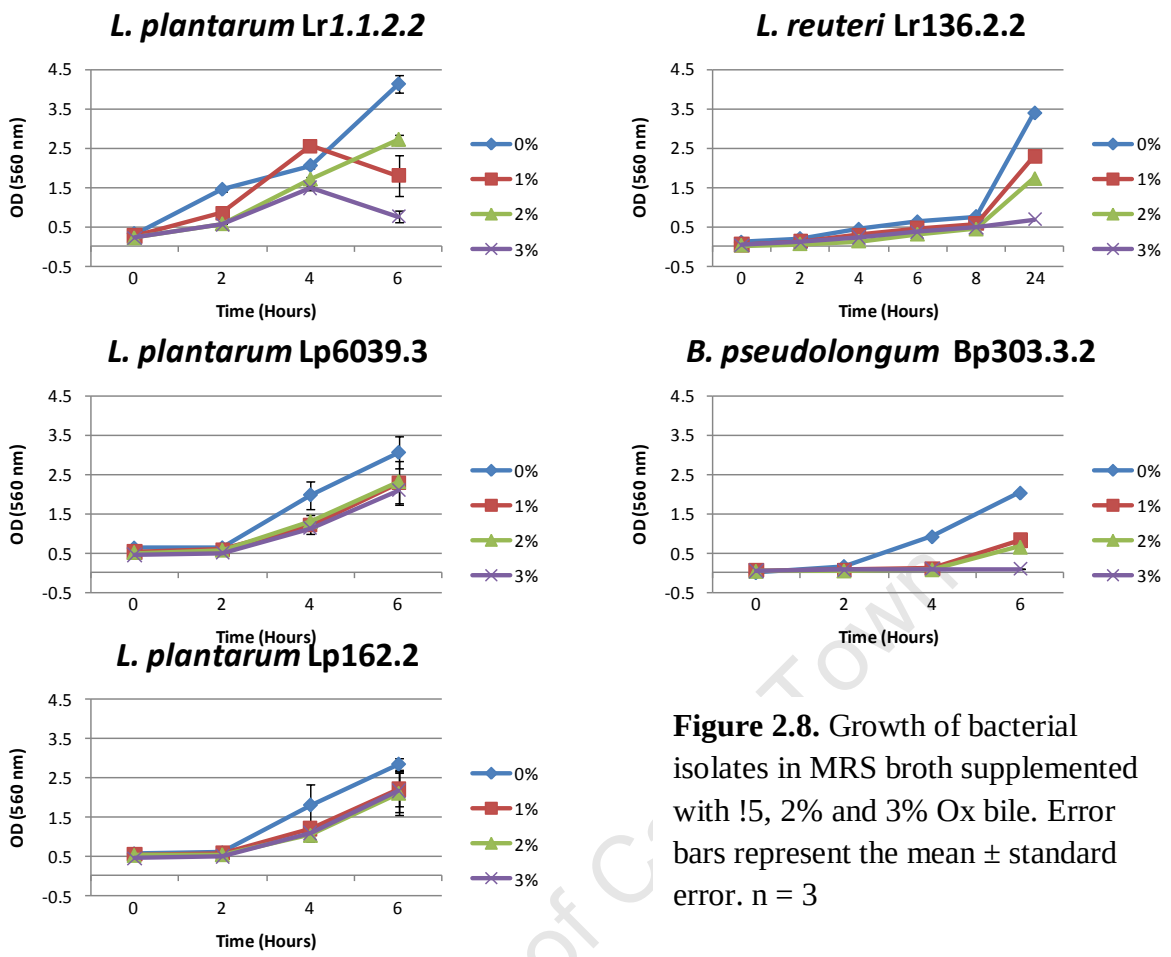

Figure 2.8. Growth of bacterial isolates in MRS broth supplemented with !5, 2% and 3% Ox bile. Error bars represent the mean ± standard error. n = 3

2.4.2.2. Measurement of aggregation properties between probiotic and pathogens

The ability of the strains to autoaggregate, as well as coaggregate with pathogenic bacteria was evaluated using the *in-vitro* measure of radioactivity developed by Collado *et al.* (2007a). Aggregation is considered to be an important property of probiotics, enabling them to achieve an adequate mass to manifest their beneficial properties (Collado *et al.*, 2007b). The ability to co-aggregate with other bacteria may offer probiotics an additional advantage over those which do not have this ability, allowing them to persist in the gut rather than being easily removed (Collado *et al.*, 2007b). Furthermore, the inhibitor-substances of bacteriocin-producing LAB, which aggregate with pathogens, may constitute an important host defence mechanism against infection (Spencer and Chesson, 1994). The results obtained for the auto- and coaggregation assays on the probiotic strains, using four potential pathogens are shown in

Table 2.6. The results are displayed as the autoaggregation of the probiotic strain, followed by the coaggregation results with each of the four pathogens tested

Table 2.6. Aggregation of probiotic strains with pathogens over 20 hours of incubation (%)

	Time in Hours				
	0	1	2	4	20
Lp1.1.2.2	0.0±0.0	4.6±0.4	22.9±3.5	21.5±4.9	57.4±8.0
+ *C.difficile*	0.0±0.0	7.4±1.3	19.5	39.6±5.6	61.3±3.5
+ *C.perfringens*	0.0±0.0	10.0±0.06	18.8	24.8±0.1	52.3±0.3
+ *E. coli*	0.0±0.0	3.8±0.2	10.1	17.6±0.6	53.0±0.5
+ *S. typhimurium*	0.0±0.0	3.9±0.1	9.2±0.2	15.2±0.05	49.2±0.2
Lp6039.3	0.0±0.0	5.0±0.6	18.4±2.3	21.7±4.7	58.2±6.9
+ *C.difficile*	0.0±0.0	5.3±0.4	14.9±0.8	27.8±0.6	60.5±0.4
+ *C.perfringens*	0.0±0.0	4.4±0.5	11.1±0.4	22.8±0.1	60.8±0.5
+ *E. coli*	0.0±0.0	2.9±0.2	9.8±0.3	22.8±0.6	59.7±0.4
+ *S. typhimurium*	0.0±0.0	0.0±0.0	4.5±0.2	14.1±0.5	55.2±0.5
Lp162.2	0.0±0.0	8.0±4.4	13.4±0.7	19.0±3.0	55.4±5.5
+ *C.difficile*	0.0±0.0	6.1±0.5	10.0±0.6	19.2±0.6	56.8±0.6
+ *C.perfringens*	0.0±0.0	7.5±0.3	11.7±0.3	23.3±0.4	57.1±0.6
+ *E. coli*	0.0±0.0	6.4±0.1	10.5±0.4	18.5±0.4	56.5±0.6
+ *S. typhimurium*	0.0±0.0	6.3±0.4	9.6±0.2	19.9±0.1	56.6±0.2
Lr136.2.2	0.0±0.0	4.6±0.1	13.1±1.4	21.4±5.5	60.3±7.8
+ *C.difficile*	0.0±0.0	7.4±1.3	19.5±0.1	39.6±5.6	61.3±3.5
+ *C.perfringens*	0.0±0.0	10.0±0.1	18.8±0.1	24.8±0.1	52.4±0.3
+ *E. coli*	0.0±0.0	3.8±0.2	10.1±0.2	17.6±0.6	53.0±0.5
+ *S. typhimurium*	0.0±0.0	3.9±0.1	9.2±0.2	15.2±0.0	49.2±0.2
Bp303.3.2	0.0±0.0	3.9±0.2	15.9±2.4	21.2±5.5	58.8±7.8
+ *C.difficile*	0.0±0.0	1.9±0.2	11.0±0.3	24.5±0.4	59.6±0.9
+ *C.perfringens*	0.0±0.0	3.1±0.2	11.1±0.5	22.1±0.2	52.6±0.5
+ *E. coli*	0.0±0.0	0.3±0.0	8.0±0.2	23.6±0.2	58.9±0.5
+ *S. typhimurium*	0.0±0.0	0.1±0.0	9.0±0.5	22.9±0.5	56.9±0.5
5 strain mix	0.0±0.0	5.2±1.1	16.6±2.3	25.6±2.6	64.0±3.3
+ *C.difficile*	0.0±0.0	3.5±0.3	13.9±0.3	25.4±1.2	54.3±0.8
+ *C.perfringens*	0.0±0.0	5.0±0.9	14.8±0.5	24.9±0.4	56.5±0.4
+ *E. coli*	0.0±0.0	1.0±0.1	7.8±0.4	17.9±0.4	53.2±0.5
+ *S. typhimurium*	0.0±0.0	0.3±0.2	6.0±0.4	15.7±0.1	48.8±0.5

All the probiotic strains showed the ability to autoaggregate and by four hours of incubation showed up to 21.7% aggregation. Probiotic properties are species-specific, and as this is the first study characterising probiotics of ostrich origin, there is no available literature for comparison. However, Collado *et al.* (2008) showed a commercial *L. plantarum* strain to autoaggregate up to 21.7% after 2 hr and 63.2% after 20 hr. This is similar to results observed in this study. The same work by Collado *et al.* (2008) showed much lower autoaggregation ability by *B. lactis*, a close relative of *B. pseudolongum* subsp. *pseudolongum*, aggregating to 28.8% by 20 hr. The bifidobacteria strain used here, Bp303.3.2, showed a higher autoaggregation by this time reaching 58.8%. The five strain probiotic mix showed similar aggregation abilities to the single strains and by 20 hours of incubation showed higher aggregation (64%) than any of the single strains. Each probiotic showed ability to coaggregate with the four tested pathogens after 20 hours of incubation. However, the percentage of coaggregation as well as the time required was demonstrated to be strain-specific (Table 2.6). This method of measuring aggregation using radioactive labels has been shown to be a reproducible and the most sensitive method when compared to using fluorescence and absorbance (Collado *et al.*, 2007b).

2.4.2.3. **Adhesion of the probiotic strains to chicken mucous and their ability to displace and inhibit pathogens**

Adhesion of probiotics to the mucosal surface of the gut is thought to be a prerequisite for the beneficial effects to be exerted, as it prolongs their transit in the gut. It is proposed that probiotics and their combinations colonise the gut and form a barrier which prevents adhesion of pathogens, mainly by competitive exclusion (Collado *et al.*, 2005, 2006, 2007c, 2008). Adhesion is a complex occurrence which may involve both a specific ligand receptor, and a non-specific mechanism (Vinderola *et al.*, 2003). Cell surface properties of probiotics, such as physiochemical and chemical makeup affect their ability to adhere (Boonaert and Rouxhet, 2000). The adhesion of each strain to mucous from different areas of the chicken gut was tested (Fig. 2.9). Probiotics are thought to be host-specific and therefore, although it was not ideal to use chicken mucous, no ostrich mucous was available at this time. Lp6039.3 adhesion showed similar values for all regions tested, whereas the other two *L. plantarum* strains, Lp1.1.2.2 and Lp162.2, as well as Lr136.2.2, showed slightly better adhesion to mucous from the large intestine than to that from the small intestine. All three *L. plantarum*

strains (Lp6039.3, Lp1.1.2.2 and Lp162.2) and the *L. reuteri* strain showed highest adhesion to colonic mucous, reaching 9.5, 11.5, 13.0 and 10.9% respectively (Fig. 2.9). Conversely, Bp303.3.2 showed higher adhesion percentage in the small intestine, reaching 13.5% to the mucous from the jejunum (Fig. 2.9). Very little literature is available on the distribution of microorganisms in the ostrich GIT, however, in the chicken model, Lactobacilli are found in their highest numbers in the caeca (Gabriel *et al.*, 2006). It would be expected that the strict anaerobes such as the *Bifidobacterium* species would be found further down in the gut in the more anaerobic environment and the facultative anaerobes to be in the less anaerobic environment.

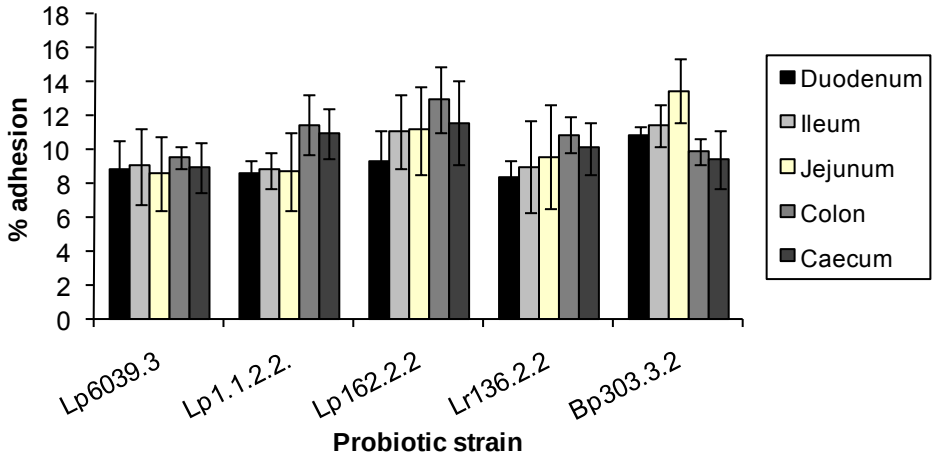

Figure 2.9. Adhesion of the five probiotic strains to chicken intestinal mucous preparations from five different intestinal areas. Results are expressed as the percentage of radioactivity recovered from immobilized mucous compared to radioactivity added to mucous (Error bars represent the mean ± standard error, n = 3).

The adhesion of four pathogens was tested against mucous from the same areas as used for the probiotic strains (Fig. 2.10).

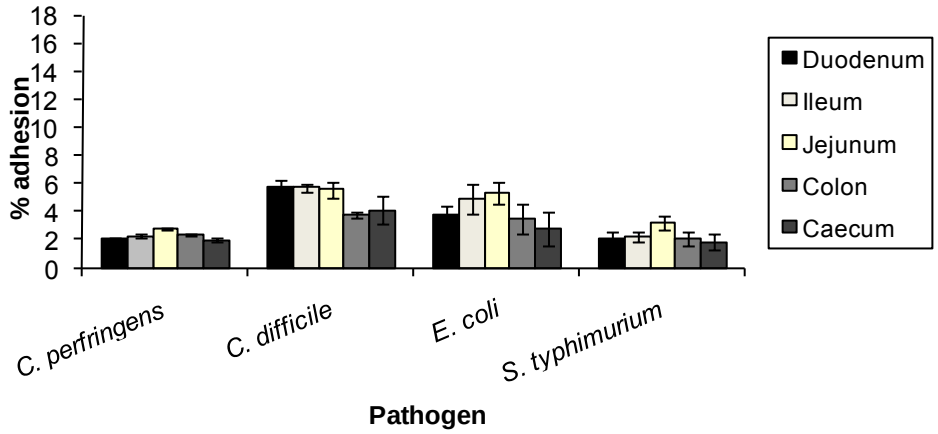

Figure 2.10. Adhesion of radiolabeled model pathogen strains *C. perfringens, C. difficile, E. coli* and *S. enterica* serovar Typhimurium to chicken intestinal mucous preparations from different intestinal sections. Results are expressed as the percentage of radioactivity recovered from immobilized mucous compared to radioactivity added to mucous. Error bars represent the mean ± standard error, n = 3).

In general, the pathogens adhered less successfully than the probiotic strains, ranging from 1.83% (*S. typhimurium* and caecum mucous) to 5.70% (*C. difficile* and duodenum mucous; Fig. 2.10). *C. perfringens* and *S. typhimurium* have the lowest ability to adhere to mucous from all areas of the gut, ranging from 1.98% in caecum to 2.72% in the jejunum, and 1.83% in caecum to 2.37% in jejunum, respectively. These results confirm work by Collado *et al.*, (2007c) where *C. perfringens,* and *S. typhimurium* were found to have similarly low adhesion abilities to pig intestinal mucous. On the other hand, *E. coli* and *C. difficile* differ from the previous two pathogens showing higher adhesion, again confirming work by Collado *et al.*, (2007c) which showed a significant increase in the ability of *E. coli* to adhere to mucous from various areas of the pig intestine and a slight increase in the ability of *C. difficile* to adhere. To eliminate the variable, jejunum mucous was chosen for further mechanism studies as pathogens adhered best to this mucous overall.

2.4.3. Adhesion mechanisms

2.4.3.1. Inhibition of pathogen adhesion to intestinal mucous

The adhesion of selected pathogens was significantly ($p < 0.05$) inhibited by all probiotic strains and their mixes in percentages ranging from 24.5% for the three strain mix against *S. typhimurium,* to 67.0% for Lr136.2.2 against *C. difficile* (Table 2.7A). In general, the probiotic combinations showed better abilities to inhibit the pathogen adhesion (Table 2.7A). *C. perfringens* was most effectively inhibited by the two strain mix (Lr136.2.2 and Lp162.2) which inhibited its adhesion by 63.29%, and least effectively by Lr136.2.2 on its own, which decreased its adhesion by 31.82%. *C. difficile* was successfully inhibited by all strains and their mixes, most effectively by Lr136.2.2 at 67.03%. *E. coli* was most effectively inhibited by the two strain mix at 58.84%, followed closely by Bp303.3.2 at 56.93%. It was least inhibited at 46.63% by the three strain mix. Finally, the five strain mix inhibited *S. typhimurium* 45.16% and the three strain mix by 24.48%. Bifidobacteria have been shown to inhibit a variety of pathogens (Fujiwara *et al,* 1999) and our work confirms this (Fig. 2.7A). These results show that probiotics which adhere well to mucous are not necessarily successful at pathogen inhibition and that inhibition is both probiotic and pathogen strain specific, confirming previous work (Collado *et al.,* 2005, 2006, 2007c).

2.4.3.2. Displacement of pathogens adhered to intestinal mucous

C. perfringens, E. coli and *S. typhimurium* were all significantly ($p < 0.05$) displaced by single probiotics, as well as the different probiotic mixes (Table 2.7B). In contrast, adhered *C. difficile* was displaced by the mixes but not by single probiotics. *C. perfringens* was more successfully displaced by the single strains than by the probiotic mixes, for example 72.67% by Lp162.2 compared to 48.55% by the five strain mix. The single strains appeared to enhance the adhesion of *C. difficile* significantly ($p < 0.05$), whereas it was significantly ($p < 0.05$) displaced by the mixes. *E. coli* was poorly, but significantly displaced by Lr136.2.2 and Lp1.1.2.2 at 5.90% and 9.65% respectively, but well displaced by all probiotic mixes, especially the three strain mix at 61.99%. *S. typhimurium* was significantly displaced by all strains and mixes, the least being 18.54% by Lp162.2 and 63.13% by the three strain mix. Probiotic strains in combination showed less ability to displace the pathogen adhesion than probiotic strain alone (Table 2.7B). The ability of the pathogens to be displaced inversely

differs with increasing ability of the pathogen to adhere to mucous. *C. difficile* showed the best adhesion to jejunum mucous, and the probiotics alone or as a mixture are unable to displace it. *E. coli* showed the next best adhesion with single probiotics offering very low, although significant displacement abilities of this pathogen. Again the mixes are more able to displace it. *S. typhimurium* and *C. perfringens* follow the same trend, showing weak mucous adhesion and easy displacement by probiotics.

2.4.3.3. **Competitive exclusion to intestinal mucous**

The single probiotic strains were able to inhibit adhesion ($p < 0.05$) of all the pathogens to the intestinal mucous except Lp6039.3 which could only inhibit *E. coli* (Table 2.7C). It was unable to compete for adhesion with *C. perfringens, C. difficile* and *S. typhimurium*. This may suggest that including this strain in the probiotic mix would have a detrimental effect on the ability to out-compete pathogens, however, the two and three strain combinations (not containing Lp6039.3) are also unable to out-compete the pathogens. The five strain probiotic mix was also unable to inhibit adhesion ($p < 0.05$) of any of the pathogens tested, in fact, all the different mixes appear to enhance pathogen adhesion. This may be due to the probiotics aggregating together and blocking the necessary cell membrane carbohydrate-binding adhesins on their cell surfaces (Malago and Koninkx, 2011), making them unavailable for blocking the carbohydrate moieties on the intestinal surface. The pathogens can then bind freely. *C. perfringens* and *S. typhimurium* were best inhibited by Lp162.2 (24.66% and 52.55%, respectively), and *C. difficile* and *E. coli* by Lr136.2.2 (31.22% and 31.06%, respectively). Probiotic combinations appear to increase the adhesion of pathogens to the mucous.

Table 2.7. Adhesion inhibition to intestinal chicken mucous of the tested pathogenic strains by the probiotics, alone and in various combinations. Results are shown as mean ± standard deviation. 2 strain mix: equal volumes of Lp1.1.2.2 and Bp303.3.2; 3 strain mix: equal volumes of Lp1.1.2.2, Lr136.2.2 and Bp303.3.2

[1] Adhesion of pathogens in the absence of a probiotic (control) was assigned a value of 0%

[2] Adhesion of preadhered pathogens following the addition of buffer without probiotics (control) was assigned a value of 0%

[3] Changes in the adhesion of pathogens in presence of probiotics applied simultaneously with pathogen. Control (pathogen adhesion) was assigned a value of 0%

A	**% adhesion inhibition[1]**								
Pathogen strain	**Probiotic strain tested**								**ANOVA**
	Lp1.1.2.2	**Lp6039.3**	**Lp162.2**	**Lr136.2.2**	**Bp303.3.2**	**5 mix**	**2 mix**	**3 mix**	**p-value**
C. perfringens	37.09 ± 10.27	37.97 ± 5.32	38.65 ± 2.75	31.82 ± 17.71	39.69 ± 12.19	47.04 ± 9.28	63.29 ± 2.22	38.11 ± 28.60	0.004
C. difficile	51.16 ± 6.04	51.72 ± 6.41	57.91 ± 7.86	67.03 ± 12.74	61.64 ± 0.91	61.98 ±5.77	41.47 ± 2.97	55.05 ± 7.38	0.0001
E. coli	51.65 ± 15.49	48.76 ± 2.88	53.42 ± 12.05	48.62 ± 5.02	56.93 ± 21.39	47.85 ± .070	58.84 ± 9.00	46.63 ± 16.36	0.001
S. typhimurium	39.86 ± 7.15	32.77 ± 2.74	36.37 ± 8.36	34.42 ± 13.76	41.23 ± 14.64	45.16 ± 2.22	43.19 ± 9.94	24.48 ± 6.31	0.005
B	**% pathogen displaced[2]**								
Pathogen	**Probiotic strain tested**								**ANOVA**
	Lp1.1.2.2	**Lp6039.3**	**Lp162.2**	**Lr136.2.2**	**Bp303.3.2**	**5 mix**	**2 mix**	**3 mix**	**p-value**
C. perfringens	71.21 ± 12.54	68.72 ± 14.73	72.67 ± 15.65	71.10 ± 18.94	70.62 ± 11.51	48.55 ± 4.24	56.58 ± 5.66	49.01 ± 9.17	0.0001
C. difficile	-74.11 ± 5.80	-101.88 ± 15.03	-52.62 ± 5.46	-89.32 ± 27.32	-73.87 ± 5.46	72.77 ± 12.91	63.24 ± 14.87	68.000 ± 16.46	0.0001
E. coli	9.65 ± 0.49	42.95 ± 21.27	14.05 ± 6.44	5.90 ± 4.81	19.22 ± 2.43	52.20 ± 10.12	58.38 ± 12.97	61.99 ± 17.59	0.0001
S. typhimurium	54.59 ± 19.77	52.35 ± 24.97	18.54 ± 2.65	49.03 ± 34.71	48.98 ± 31.75	45.17 ± 0.77	58.02 ± 3.26	63.13 ± 1.63	0.041

Table 2.7. continued

C	% reduction of adhesion of pathogen by competition[3]								
Pathogen	**Probiotic strain tested**								**ANOVA**
	Lp1.1.2.2	Lp6039.3	Lp162.2	Lr136.2.2	Bp303.3.2	5 mix	2 mix	3 mix	p-value
C. perfringens	9.10 ± 0.86	-61.50 ± 45.61	24.66 ± 4.18	15.89 ± 8.35	19.47 ± 7.98	-60.02 ± 38.24	-47.78 ± 63.89	-64.82 ± 2.03	0.026
C. difficile	14.98 ± 1.45	-19.97 ± 12.69	22.30 ± 5.23	31.22 ± 7.38	45.54 ± 3.64	-36.28 ± 5.15	-99.29 ± 52.64	-62.96 ± 3.70	0.0001
E. coli	23.44 ± 14.83	13.84 ± 7.36	21.18 ± 10.62	31.06 ± 5.68	43.10 ± 4.29	-27.33 ± 2.25	-134.76 ± 16.32	-46.01 ± 39.74	0.0001
S. typhimurium	32.12 ± 2.00	-17.00 ± 6.86	52.55 ± 3.50	33.48 ± 9.02	39.37 ± 19.11	-15.99 ± 5.34	-32.40 ± 0.20	-31.04 ± 24.53	0.006

2.4.3.4. **Antimicrobial action against selected pathogens**

The widespread ability of LAB and bifidobacteria to produce antimicrobial compounds such as organic acids, hydrogen peroxide and bacteriocins, infers an important biological role of these products (Versterlund, 2009). The activity of the strains against selected pathogens was tested by overlaying a colony of the probiotic with the indicator pathogen. Antimicrobial activity was indicated by a zone of inhibition and the area of this zone is represented in Fig. 2.11.

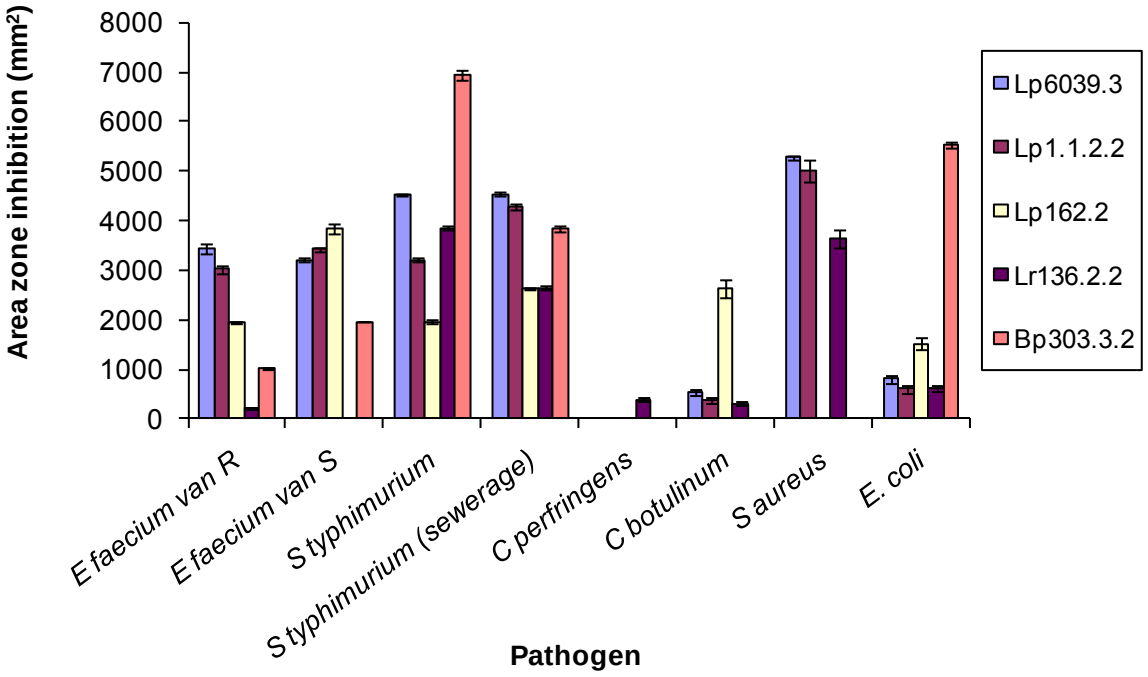

Figure 2.11. Antimicrobial properties of the probiotic strains measured by the area of the zone of inhibition produced against each indicator pathogen strain. Results shown as the mean zone of inhibition± standard error, n = 3.

All five strains showed high activity against *S. typhimurium,* a major pathogen in poultry (Gong *et al*., 2005), but much less activity against the *Clostridium* spp. particularly *C. perfringens* (Fig. 2.11). The three *L. plantarum* strains were active against all tested pathogens except *C. perfringens*, and *S. aureus* where Lp162.2 did not produce a zone of inhibition (Fig. 2.11). Lr136.2.2 was however, the only probiotic strain tested here to show antimicrobial activity against *C. perfringens*, considered to be one of the most important pathogens in ostrich farming. Bp303.3.2 showed antimicrobial activity against all pathogens except *C. perfringens, C. botulinum* and *S. aureus*. Its maximum activity was against *S. typhimurium,* followed by *E. coli*, both important ostrich pathogens.

Further studies on the antimicrobial agents produced here, indicated that the activity was not due to bacteriocins as protein fractions did not show zones of clearance when overlaid with the indicator strain (data not shown). The production of acid compounds was thought to be the active compound as when the supernatant of the respective probiotics was neutralised (pH 7), no antimicrobial activity was noted (data not shown). Treatment of these supernatants with catalase gave no change in activity and it was therefore concluded to not be due to hydrogen peroxide. In addition to the pH effect which lactic acid has on the cytoplasm of cells (Eschenbach *et al.,* 1989), the acid is reported to collapse the electrochemical proton gradient, causing bacteriostasis and finally death of susceptible bacteria (Eklund, 1989).

2.4.3.5. **Enzyme assays**

To test the ability of each strain to utilise particular substrates, each isolate was spotted in triplicate onto agar containing various substrates and zones of clearance were detected using the appropriate staining (Table 2.3). The results are shown in Table 2.8. All strains were able to utilise starch, cellulose and protein. None of the probiotics in this study were able to digest lipids or inulin. Lr136.2.2 was also unable to utilise phytic acid and Bp303.3.2 was unable to degrade xylan, phytic acid, pectin and had very limited ability to degrade cellulose (Table 2.8).

Table 2.8. The area of the zones of clearance produced by the isolates on BYG basal medium supplemented with specific substrates. The values are the average of three replicates.

	Isolates				
Enzymes	Zone of clearance (mm^2)				
	Lp1.1.2.2	Lp6039.3	Lp162.2	Lr136.2.2	Bp303.3.2
Amylase	15	18.3	12	11.5	42.3
Cellulase	11	17.5	8.3	5.3	1
Inulinase	0	0	0	0	0
Lipase	0	0	0	0	0
Pectinase	11.5	9	10.3	4.5	0
Phytase	5	9.5	11.3	0	0
Protease	21.3	25	5	4.3	11.3
Xylanase	8	7.5	5.3	9.5	0

The ability to hydrolyse phytic acid is considered important in animal farming as this acid, found naturally in plants and seeds, forms metal-phytate complexes (Oh *et al.*, 2004) which are indigestible to most animals and may lead to dietary deficiencies. Young farmed birds often suffer from soft bones and apparent rickets (Cooper and Gimbi, 1994) and hence

mineral availability is something which farmers need to address. Phytic acid plays a large role in lowering phosphorous absorption, as well as calcium, iron, zinc and magnesium (Oh *et al.*, 2003). Very few animals can degrade phytic acid naturally and this can lead to animals suffering from nutrient deficiencies (Marounek *et al.*, 2003). Phytic acid hydrolysis is best accomplished by the gut microbiota, as the addition of phytases to the feed is expensive and unstable (Oh *et al.*, 2004). The three *L. plantarum* strains included in the probiotic mix produced phytase (Table 2.8).

In addition to these enzyme assays, the API 50CHL strips also gave information on the enzymes produced by each isolate (Table 2.9). The API results agree with the results in Table 2.8 with respect to the inability of all strains to degrade inulin and showed all strains except Bp303.3.2 to be able to utilise cellobiose. The API strip showed only Bp303.3.2 to degrade starch, whereas the modified BYG plates gave positive results for all strains. This may indicate that the substrates used in these different tests vary slightly or that the modified agar technique is more sensitive to the APIs.

The probiotics tested here show a good range of substrate degradation which would be able to make more nutrients available for growth and so benefit the ostrich. The multistrain probiotic mixture should be more advantageous in this case with some strains being able to digest substrates other strains cannot.

Table 2.9. Selected API 50CHL results for relevant enzyme activities

Test	Active ingredient	Results				
		Lp1.1.2.2	Lp6039.3	Lp162.2	Lr136.2.2	Bp303.3.2
GLY	Glycerol	-	-	-	-	-
ERY	Erythritol	-	-	-	-	-
DARA	D-Arabinose	-	-	-	-	-
LARA	L-Arabinose	+	+	+	+	-
RIB	D-Ribose	+	+	+	+	+
DXYL	D-Xylose	-	-	-	-	-
LXYL	L-Xylose	-	-	-	-	-
ADO	D-Adonitol	-	-	-	-	-
GAL	D-Galactose	+	+	+	+	-
GLU	D-Glucose	+	+	+	+	+
FRU	D-Fructose	+	+	+	+	-
MNE	D-Mannose	+	+	+	+	-
SBE	L-Sorbose	-	-	-	-	-
RHA	L-Rhamnose	-	-	-	-	-
DUL	Dulcitol	-	-	-	-	-
INO	Inositol	-	-	-	-	-
MAN	D-Mannitol	+	+	+	+	-
SOR	D-Sorbitol	+	+	+	+	-
CEL	D-Cellobiose	+	+	+	+	-
MAL	D-Maltose	+	+	+	+	+
LAC	D-Lactose	+	+	+	+	+
SAC	D-Saccharose	+	+	+	+	+
INU	Inulin	-	-	-	-	-
RAF	D-Rafinose	+	+	+	+	-
AMD	Amidon (Starch)	-	-	-	-	+

2.4.3.6. **Antibiotic resistance profiles of the strains**

To determine the antibiotic resistance profiles of the five probiotic strains, commercially available antibiotic disks (Oxoid) were used and the results are shown in Fig 2.12. In addition, the well diffusion assay was used to test various concentration of tylosin and the highest concentration tested is shown in Fig. 2.12.

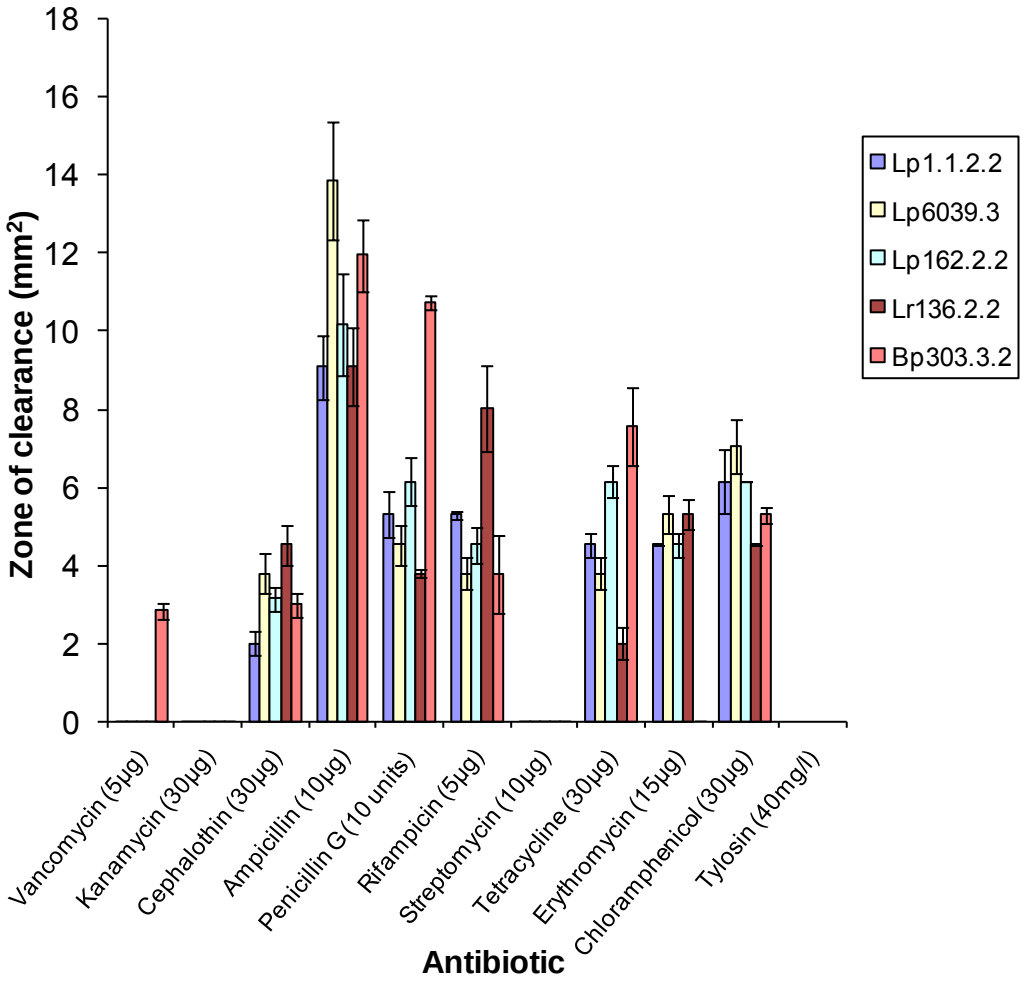

Figure 2.12. Antibiotic resistance profiles of 10 selected antibiotics. Results shown as the mean zone of inhibition ± standard error (n = 3).

Only Bp303.3.2 showed a slight sensitivity to vancomycin at 5µg (Fig. 2.12). All the strains were resistant to kanamycin and streptomycin and all strains except Bp303.3.2 were sensitive to erythromycin. All five strains were most sensitive to ampicillin. Bp303.3.2 showed high sensitivity to penicillin G and tetracycline, and Lr136.2.2 showed high sensitivity to rifampicin. The strains showed varying sensitivity to the remaining antibiotics tested here (Fig. 2.12). In addition, all five strains were shown to be resistant to tylosin at all concentrations tested (up to 40mg/litre, data not shown). In this study, tylosin was administered to the ostrich chicks at a 20mg/kg feed. It is therefore concluded that this concentration would not affect the probiotic strains.

LAB have been reported to be intrinsically resistant to many antibiotics due to their structure and physiology (Donohue, 2009). Temmerman *et al.* (2003) showed the majority of probiotics strains in their study were resistant to two or more antibiotics. Lactobacilli were

found to be resistant to kanamycin (81%), tetracycline (30%), erythromycin (12%) and and chloramphenicol (9%). Sullivan and Nord (2006) reported all isolated *L. plantarum* strains from bacteremic patients, to be resistant to vancomycin and Cannon *et al.* (2005) reported all isolated *L. plantarum* strains to be sensitive to erythromycin and ampicillin. The tetracycline resistance gene *tet*(W) has been isolated from *L. reuteri* (Kastner *et al.*, 2006). *Bifidobacterium* spp. have been reported to be sensitive to penicillin G and erythromycin (Kheadr *et al.,* 2006) and resistant to aminoglycosides (kanamycin and streptomycin) and vancomycin. Strain Bp303.3.2 (*Bifidobacterium pseudolongum* subsp. *pseudolongum*) was indeed sensitive to penicillin G, but resistant to erythromycin (Fig. 2.12). In addition Bp303.3.2 was resistant to kanamycin and streptomycin, but slightly sensitive to vancomycin. Mayrhofer *et al.* (2011) showed a *B. pseudolongum* subsp. *pseudolongum* of animal origin to be sensitive to chloramphenicol and resistant to kanamycin. This highlights the strain-specific resistance profiles, as Danielson *et al.* (2007) reported, showing great variation between species. The five gram positive, anaerobic probiotic strains used in this study were all resistant to kanamycin and streptomycin (Fig. 2.12). Gram positive anaerobes have been shown to be resistant to aminoglycosides (Davis, 1987) given their multiple cationic charges in their membrane rendering them impermeable to these antibiotics and this resistance is therefore likely to be intrinsic. Determining whether the resistance is intrinsic or acquired is important, since genes encoding for antibiotic resistance can be transferred between species in the same ecological niche (Kruse and Sorum, 1994). The antibiotic profile of potential probiotics must be determined before the product is commercialised to avoid introducing strains that carry transferable resistance genes.

2.4.3.7. **Thermotolerance assay**

The ability of the probiotics to survive exposure to heat during processing is necessary, depending on the technique to be used. Spray drying, for example, uses high temperatures and *in-vitro* examination of heat resistance would indicate the likelihood of the strains survival. The bacteria used here were in stationary phase as it has been previously reported that the stage of growth affects the heat resistance of the strain, and that stationary phase cells resulted in a higher survival rates after heat treatment (Teixeira *et al.,* 1994). The probiotics were exposed to 37°C, 50°C, 60°C and 80°C for 10 min. After incubation at 80°C, there were

too few colonies to count for all five strains tested. The results of each strains survival at the remaining test temperatures are shown in Figure 2.13.

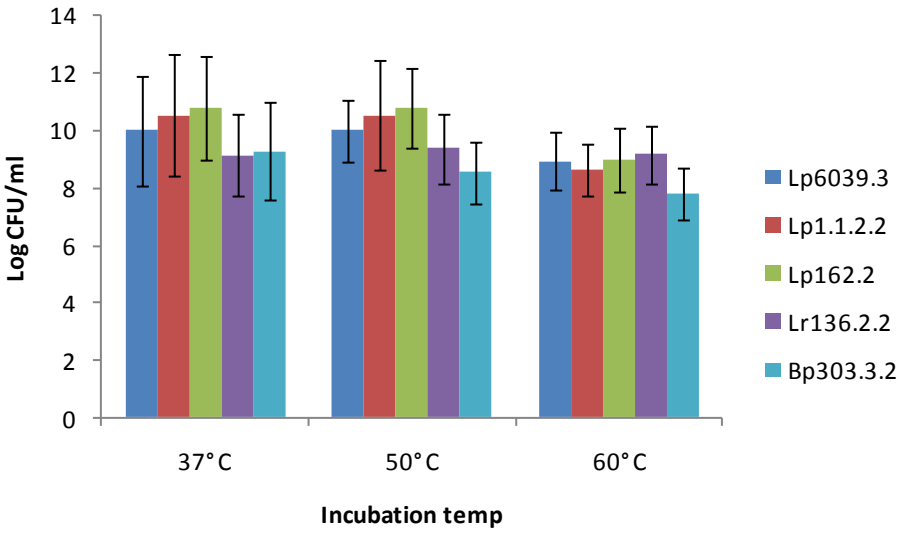

Figure 2.13. Survival of the selected probiotic strains after 10 min exposure to 37°C, 50°C and 60°C. Results shown as the mean ± standard error, n = 3.

The three *L. plantarum* strains survived equally well at 37°C and 50°C, however incubation at 60°C showed a decreased survival rate from approximately 10^{10} to 10^{9} CFU/ml (Fig. 2.13). *L. reuteri* Lr136.2.2 showed no difference in survival between the three incubation temperatures, maintaining a cell count of approximately 10^{9} CFU/ml. Nitisinprasert *et al.* (2000) isolated a thermotolerant *L. reuteri* strain from the chicken intestine, and other studies have isolated thermotolerant *L. paraplantarum* from the GIT of various animals (Sow *et al.*, 2005). The probiotic Bp303.3.2 showed higher temperature sensitivity, decreasing from 10^{9} CFU/ml at 37°C to 10^{8} CFU/ml at 50°C and 10^{7} at 60°C (Fig. 2.13). Lian *et al.* (2002) also showed a decrease in viability for *Bifidobacterium* spp. after spray drying (1 – 2 log/g dry mass reduction), but concluded that after the process, there was still an acceptable number of viable cells for probiotic use. All five strains still had an acceptable viability after exposure to 60°C, having cell counts of around 10^{8} CFU/ml, proving them suitable for processing up to 60°C. There are a variety of composite carrier matrices available which have been shown to improve the survival of bacterial strains under stressful conditions for example, using glycerol or skim milk as cryoprotectants (Pascual, *et al.*, 1999), and calcium alginate beads (Anal and Singh, 2007). Tests could be performed on the probiotic strains using these protectants to improve heat tolerance.

2.5. Conclusion

The beneficial effects of probiotics are well documented and include the ability of probiotics to adhere to the mucosa and outcomplete pathogens, aid in digestion by production of desirable enzymes and boost the immune system.

It is also well known that each strain and their combination should be tested *in-vitro* as probiotic effects are strain-specific. It is therefore vital to correctly identify and rigorously characterise each strain before commercialising the probiotic product. This chapter aimed to fully characterise five strains, previously isolated from ostrich faeces or the ostrich GIT, which were selected for their suitability for inclusion into ostrich chick feed with the intention of replacing the use of the growth-promoting antibiotic tylosin.

With confidence we can say that the five isolates were identified as: Lp1.1.2.2 – *L. plantarum,* Lp6039.3 – *L. plantarum,* Lp162.2 – *L. plantarum,* Lr136.2.2 – *L. reuteri* and Bp303.3.2 – *B. pseudolongum* subsp. *pseudolongum.*

The five isolates were shown to successfully adhere to the chicken mucous, a factor considered critical for the beneficial effects to be exerted. The single strains, as well as all combinations tested could prevent adhesion of four pathogens, *C. difficile, C. perfringens, E. coli* and *S. typhimurium,* tested here. The single strains were unable to displace pathogenic *C. difficile,* but were able to displace the other three pathogens, whereas the mixes could displace all four pathogens. The single strains were able to out-compete the pathogens once they had adhered to the mucous, except Lp6039.3 which could not out-compete *C. perfringens, C. difficile* and *S. typhimurium.* Again, a strain-specific, combination-specific pattern is observed.

The functionality of the five probiotic strains was evident. All five probiotics produced antimicrobial compounds against the potential pathogens *S. typhimurium* and *E. coli.* Only Lr136.2.2 was active against *C. perfringens,* considered the main pathogen in ostrich farming. However, when looking at the adhesion mechanisms above, *C. perfringens* was successfully inhibited, displaced and out-completed by all strains (except Lp6039.3) and mixtures so the administration of these probiotics should be beneficial to the ostrich chicks.

Given the importance of cellulose as a component of ostrich feed, the production of cellulase by all strains is highly beneficial. Although Bp303.3.2 produced low levels of this enzyme, the combination of all five strains would produce it in reasonable amounts. Amylase and protease were produced by all five strains in different amounts and pectinase and xylanase were produced by all except Bp303.3.2. These additional enzymes are thought to contribute towards increasing digestion and therefore improving the feed conversion ratios in animals.

The identification of the strains placed them in the GRAS (generally regarded as safe) category of species used for probiotics. The characterisation of these strains showed an overall beneficial profile, but highlighted the strain-specific nature of probiotics, highlighting the need of thorough identification and characterisation before each potential strain is used in feeding trials. The five strains all showed a resistance to tylosin, an antibiotic commonly used in animal husbandry, concluding that it did not alter the viability of these probiotics in this feeding trial. The enhancement of properties such as the enzyme production by a multi-strain mix is obviously beneficial. However, the effect on adhesion needs to be determined when used as a multi-strain mix, as this may alter the probiotic effects beneficially or detrimentally compared to single strains.

The next chapter explores the scale-up production and method of delivery of the five strain combination in feeding trials to ostrich chicks. The effect of the probiotics on their faecal and mucosal bacterial populations was monitored using culture-dependent techniques.

CHAPTER 3

The effect of dietary supplementation with *Lactobacillus plantarum, Lactobacillus reuteri* and *Bifidobacterium pseudolongum* subsp. *pseudolongum* on the faecal and gut microbial populations using culture-dependent techniques

Contents

3.1 **Summary**.....75
3.2 **Introduction**.....76
3.2.1 Aims and objectives of this study.....80
3.3 **Materials and Methods**.....80
3.3.1 Bacterial strains and growth conditions.....80
3.3.2 Development of method of delivery of probiotic strains to ostrich chicks.....81
3.3.3 RAPD.....83
3.3.4 Pilot-scale ostrich feeding trial.....83
3.3.5 Large-scale feeding trial.....84
3.4 **Results and Discussion**.....87
3.4.1 Development of method of delivery of probiotic strains to ostrich chicks.....87
3.4.2 Pilot feeding trial (Carnarvon, Karoo).....90
3.4.3 Feeding trial (Elsenburg).....91
3.4.3.1 RAPD analysis of faecal samples.....91
3.4.3 Ostrich chick growth and mortality rates.....93
3.4.3.4 Microbiological analysis of ostrich chick faecal samples.....98
3.4.3.5 Microbiological analysis of ostrich chick mucosal samples.....103
3.5 **Conclusions**.....109

3.1 Summary

Lactobacillus plantarum, Lactobacillus reuteri and *Bifidobacterium pseudolongum* subsp. *pseudolongum* were investigated in a feeding trial on ostrich chicks. The five strain mixture was incorporated into calcium alginate encapsulated beads, which improved their viability. The chicks were fed this probiotic mix alone, or in combination with the antibiotic, tylosin, in order to elucidate and compare the effect of probiotics on the growth rate and health of the chicks. No significant difference was observed between treatments for growth rate or feed conversion ratio, however the mortality rate in the probiotic-fed group was noticeably higher than that of the groups receiving tylosin. These birds were the only groups to have *C. perfringens* in the duodenal mucosal scrapings. The changes in the culturable microbiota were observed using selective growth media for enumeration of total anaerobes, lactic acid bacteria, *Bifidobacterium* spp., *Clostridium* spp., *Salmonella* spp. and *Enterobacteriaceae.* Overall, the faecal samples did not show many significant differences in the culturable bacteria between treatments, however, as expected, the majority of bacterial counts increased significantly over time for each treatment. At week two, treatment E and ET showed higher counts for LAB and *Bifidobacterium* spp. showing the effect of receiving probiotics on these bacterial groups. Treatment E also showed higher coliform counts as well as *C. perfringens* counts at week two, and after this time point the chicks in this treatment died. The prevalence of this pathogen decreased over time in treatments receiving tylosin, indicating the effectiveness of this antibiotic against this pathogen. The mucosal samples showed increasing trends for total bacteria, LAB and *Enterobacteriaceae,* while *C. perfringens* disappeared by the second slaughter point for all groups except in the colon and caecum of treatment ET. Treatments C and CT showed higher LAB, total anaerobes and coliform counts than E and ET at slaughter one. Interestingly, only treatment E showed the presence of *C. perfringens* in all areas of the gut tested. *C. perfringens* is considered to have played a role in the high mortality rates of these chicks, especially those only receiving probiotics.

3.2 Introduction

Agricultural animal farming for human consumption has a main objective: to get the animal to slaughter age as fast and as economically as possible. Ostrich farming is not only expensive, but also suffers mortality rates of up to 74.8% in the first three months of life (Cloete *et al.,* 2001). This mortality is due to stress and pathogenic invasion such as fading chick syndrome and enteritis caused by *C. perfringens* (Shivaprasad, 1993; Huchzermeyer, 2002). Naturally, ostrich chicks ingest the parent's faeces as a means of obtaining a healthy microbiota which would protect them against these invasions by providing the initial inoculum of beneficial bacteria to develop their gut microbiota (Cooper *et al.,* 2010), and chicks in open pastures seldom get enteritis (Huchzermeyer, 2002). In intensive ostrich farming, the environment into which the chicks are born does not allow for this and pathogenic bacteria colonise the gut freely. There is therefore, widespread use of antibiotics, such as tylosin, commonly used in animal farming for its prophylactic properties as well as for its growth promoting effects (Collier *et al.,* 2003). This antibiotic is used in South Africa, particularly in the ostrich farming industry due to the high mortality rates of farmed ostrich chicks. In addition, the cost involved in rearing an ostrich chick to the slaughter age of nine months, is high when compared to the feed costs alone (Mike Jarvis, personal communication). It has been suggested that the slaughter age of the ostriches could be brought down to seven months for economic reasons. However, in this case, the birds need to reach slaughter weight in less time and an alternative to the use of growth promoting antibiotics would need to be found.

Tylosin is a macrolide antibiotic with broad spectrum activity against Gram positive anaerobes, as well as some Gram negatives (Poole *et al.,* 2003). Like other macrolides, tylosin has a bacteriostatic effect on susceptible organisms, inhibiting protein synthesis through binding to the 50S subunit of the bacterial ribosome. It is commonly used as a growth promoter at concentrations between 10-40 mg/kg of feed and as medication at concentrations between 20-50 mg/kg of feed (Tertius Brand, personal communication). In this study it was administered daily in the feed at a concentration of 20 mg/kg of feed. Enteric *E. coli* and *Salmonella* spp. are intrinsically resistant to tylosin (Shryock *et al.,* 1998), therefore, in this study it would be primarily targeting pathogenic *C. perfringens*. When used for promoting growth, its effect on the microbiota is not fully understood (Poole *et al.,* 2003). The use of tylosin at subinhibitory levels

is known to reduce susceptible bacteria and exhibit benefits to the host including inhibition of bacterial virulence and enhancement of immune system (Shryock *et al.,* 1998). There is limited information on the composition of gastrointestinal microbiota of humans and animals, however, both culture-dependent and -independent methods have demonstrated that most gastrointestinal bacteria are Gram positive anaerobes (Gueimonde and de los Reyes-Gavilán, 2009). However, this study focused on the effect of subinhibitory concentrations of tylosin on the ostrich microbiota. The overuse of antibiotics in intensive animal husbandry may lead to the long-term development of drug resistant human pathogens (Fey *et al.,* 2000) and has lead the European Union to ban the growth promoting use of antibiotics in animal farming. Due to these concerns, there is a significant interest in the development of an alternative and sustainable solution to improving growth and health of farmed animal species.

Microbial cultures have shown potential as a replacement to the prophylactic and growth promoting use of antibiotics, due to associated benefits such as antimicrobial production and competition with pathogens for adhesion sites (Casey *et al.,* 2007; Margolles *et al.,* 2009). Probiotics have shown great promise in this scope, being used in humans (Collado, 2009) as well as farm and domesticated animals (Lim and Tan, 2009). Previous research has shown probiotics to be most effective when isolated from the host they are to be used in (Rinkinen *et al.,* 2003) and therefore, in this study, five potential probiotic strains were isolated from wild and farmed ostrich faeces and gut scrapings. Three *Lactobacillus plantarum,* one *Lactobacillus reuteri* and one *Bifidobacterium pseudolongum* subsp. p*seudolongum,* showed potential in initial probiotic screening assays (Chapter 2). *Lactobacillus* spp. and *Bifidobacterium* spp. are constituents of most animal intestinal microbiota and are some of the most commonly used probiotic strains due to their proposed benefits (Margolles *et al.,* 2009). These benefits include increasing body weight gain and egg quality in layer chickens (Nahashon *et al.,* 1994) and increasing body weight gain and survival rates of piglets (Abe *et al.,* 1995). However, considerable strain-to-strain variation is seen among these species and it is therefore important to screen each potential probiotic before inclusion in a dietary supplement.

Prebiotics are nondigestible food ingredients which selectively stimulate the proliferation of resident beneficial bacteria in the gut, especially *Bifidobacterium,* by 10-100 fold (Boehm and Stahl, 2003). When administered together with a probiotic, the prebiotic supplement is referred

to as a synbiotic. There is increasing scientific evidence of synbiotics aiding young animals to increase their growth performance (Patterson and Burkholder, 2003; Fleige, 2007). Some prebiotics also offer protection against cell injury during processing like freeze drying (Crittenden, 2009). In this study, raftilose ®P95, a commercial fructooligosaccharide (FOS) product containing a mixture of oligofructose, glucose, fructose and sucrose, was used as both a prebiotic as well as a cryoprotectant. FOS has been shown to have good prebiotic properties in numerous human feeding trials (Boehm and Stahl, 2003; Bouhnik *et al.*, 2006) and has been shown to elicit a response in the bifidobacteria and lactobacilli populations in luminal and mucosal samples (Suzuki *et al.*, 2006).

For the probiotics to have the desired effect, they have to be delivered to their site of action in sufficient quantities (Lee, 2009). The dosage administered is dependent on several factors:

- Type of probiotic used
    - Lactobacilli, bifidobacteria, yeast, and/ or combination,
    - Each needs to be separately studied,
- Daily dose
    - As yet, there is not a systematic study on the effective dosage required for specific applications,
    - Needs to be sufficient, but not excessive as this can lead to side effects such as diarrhea,
- Daily frequency of administration
    - Probiotics which do not adhere well to the mucosa, or are degraded by the harsh GIT conditions may have to be administered more frequently than more successful probiotics,
- Timing of administration
    - There is no information on this, but it is thought that between meals is optimal for exposure of the strains to receptor sites as food may dilute the probiotic,
    - Administration in water is thought allow more contact with mucosal receptor sites,

- Duration of administration
    - This depends on the disorder being treated,
- Method of delivery
    - Appears to have minimal effect on probiotic efficacy,
- Viability
    - Depends on the mechanism of the probiotic effect,
    - Each probiotic/combination needs to be evaluated respectively.

Probiotic treatment has had positive reports (Jin *et al.,* 1998b; Kabir *et al.,* 2004; Gil De Los Santos *et al.,* 2005) as well as negative effects in broilers (Watkins and Kratzer, 1984; Priyankarage *et al.,* 2003). However, it is difficult to compare studies of probiotics as their efficacy depends on many factors, including the environmental and stress status of the animal (Patterson and Burkholder, 2003). In a previous investigation, a five strain probiotic combination was found to be effective at increasing growth performance of chickens, as well as improving digestive tract function at a concentration 10^8 cfu/kg feed (Mountzouris *et al.,* 2010). However, they suggested a probiotic concentration of $>10^9$ cfu/kg of feed for beneficial modulation of the chicken caecal microbiota. Given the sensitivities of ostrich chicks, the probiotic mix in this study was administered at 10^8 cfu/kg feed. Mountzouris *et al.* (2007) also demonstrated that a five-bacterial species competitive exclusion probiotic product for broilers was effective for promoting growth and modulating caecal microbiota composition and metabolic activity. In addition, the same probiotic mix was effective at reducing *Salmonella* Enteritidis in *Salmonella* Enteritidis-challenged broilers (Mountzouris *et al.,* 2009). Furthermore, Mountzouris *et al.* (2011) found that feeding broilers probiotic mixes at concentrations of 10^9 or 10^{10} cfu/kg of feed resulted in a beneficially altered caecal microbiota.

Although probiotics have been incorporated into fermented food products like yoghurt for decades (Capela *et al.,* 2005), including them into a wider range of food and maintaining their viability over a longer shelf life is a challenge. The five probiotics used in this study are considered to be strict anaerobes, and therefore protection from oxygen was critical for securing their viability following long- to medium-term storage. The temperature of the ostrich pens was maintained at 30ºC, and therefore, this is another factor which needed to be considered as the

probiotics were given to the ostriches with the food in the morning and left in the pens during the day. As mentioned above, it is thought to be optimal to administer the probiotics in the water so as not to get any interference from food particles. In this case, however, had the probiotics been mixed with the drinking water, and left all day, it was suspected they would sink to the bottom of the drinking tank. The different strategies tested here for successful administration of probiotics to the chicks included freeze drying the probiotic combination in skim milk and raftilose®P95, encapsulation of the probiotic mix in calcium alginate beads, and immersing these beads into cocoa butter, and finally vacuum impregnation into the ostrich feed.

Culture-dependent techniques such as plating on selective agar have been used traditionally for enumeration of bacterial groups. This method is cheap, easy and quick and offers high taxonomical discrimination (Vaz-Moreira *et al.*, 2011). It also gives information on viability of the strains which culture-independent methods do not. This is particularly useful in this study where it is important to know the effect of the production processes on the viability of the probiotics that are to be tested.

3.2.1 Aims and objectives of this study

The main objective of this study was to formulate a probiotic mix that could successfully replace the use of growth promoting tylosin and prevent invasion by common pathogens. These factors are critical to the long-term growth and success of the currently-depressed local ostrich farming industry. In this chapter we investigate different modes of probiotic delivery to the chicks and carry out a pilot feeding trial on a commercial ostrich farm with the aims of monitoring the effects of probiotics and tylosin on the mortality rates and feed conversion ratios of the chicks. Once this feeding trial had shown no detrimental effects for the probiotic mix (Juste-Poinepen, 2008; du Toit, unpublished), a larger feeding trial was carried out at Elsenberg Agricultural Centre (Stellenbosch, South Africa).

3.3 Materials and Methods

3.3.1 Bacterial strains and growth conditions

Five selected strains of ostrich origin (Chapter 2) were included in the probiotic mix: *L. plantarum* 6039.3 (Lp6039.3), *L. plantarum* 1.1.2.2 (Lp1.1.2.2), *L. plantarum* 162.2 (Lp162.2),

L. reuteri 136.2.2 (Lr136.2.2) and *B. pseudolongum* subsp. *pseudolongum* 303.3.2 (Bp303.3.2). All bacterial strains were cultured anaerobically, as described in Chapter 2.

For enumeration of bacterial populations in faecal samples, the following selective media and conditions were used: De Man Rogosa Sharpe (MRS) agar (Merck) according to De Man *et al*. (1960), supplemented with 0.05% cysteine hydrochloride (Sigma Aldrich) and 15g/l bacteriological agar (Merck) for total lactic acid bacteria, Brain Heart Infusion (BHI) agar (Bacto) supplemented with 0.05% cysteine hydrochloride (Sigma Aldrich) and 15g/l bacteriological agar (Merck) for total anaerobes, Beerens agar ((Beerens, 1990) containing in g/l: Columbia agar (Oxoid) 39.0; glucose 5.0; cysteine hydrochloride 0.5; agar 1.5; propionic acid 5.0 ml) for total *Bifidobacterium*, Tryptose Sulphite Cycloserine (TSC) agar (46g/l Perfringens agar base (Oxoid), 2 vials/l TSC supplement (Oxiod)) for isolation of *Clostridium perfringens*, Salmonella Shigella (SS) agar (Merck) for detection of *Salmonella* and *Shigella*, and McConkey agar (Merck) for detection of *Enterobacteriaceae*. MRS, BHI, Beerens and TSC were incubated anaerobically at 37°C for 48 h, and the SS and McConkey were incubated aerobically at 37°C for 24 h. Cell numbers were recorded after incubating the plates for the desired period and viability recorded as log colony forming units (cfu)/g faeces.

3.3.2 Development of method of delivery of probiotic strains to ostrich chicks

3.3.2.1 Freeze drying of probiotics for addition to ostrich feed

A single colony of each of the probiotic strains was inoculated separately into 5 ml MRS broth supplemented with 0.05% cysteine (MRS-Cys) and cultured under anaerobic conditions for 18 h. This culture was checked for purity using a Gram stain and 1 ml of this was inoculated into 500 ml MRS-Cys broth and grown as above. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C (Beckman Coulter J2-21 M/E), washed twice in 1x PBS and resuspended in 100 ml 5% (w/v) skim milk, 5% (w/v) raftilose ®P95. The strains were freeze dried separately to test the viability of the single strains after processing, as well as mixed together in equal volumes before being freeze dried to ensure overall survival of the mix. The suspensions were shelled in an ethanol bath in a drying flask for 2 h and then freeze dried (Balzers-Pirani gauge TiG 030) at 10 mbar for approximately 48 h. The freeze dried product was transferred to a sterile Sterilin tube and maintained at 4°C in a dessicator. Cell viability of the freeze dried probiotics was measured using the plate count method. Briefly, 0.1 g of the freeze dried probiotic product

was suspended in 1x PBS and was serially diluted in 1x PBS and 100 µl of the suspension was spread onto MRS-Cys solid growth media. The cell numbers were determined after incubating the plates at 37°C for 48 h under anaerobic conditions and viability determined as cfu/g of freeze dried product.

3.3.2.2 Calcium alginate encapsulation of culture

Bacterial strains were grown in 500 ml MRS-Cys for 18 h under anaerobic conditions. The cells were collected by centrifugation at 10,000 rpm for 10 min, washed twice in 1x PBS and resuspended in 50 ml sterile 5% milk, 5% raftilose. The five suspensions were combined and added to 250 ml 3.6% sodium alginate and mixed thoroughly. This mixture was poured into a sterile customized funnel and dripped through a blue eppendorf tip into 0.1M $CaCl_2$ with stirring. The alginate beads, approximately 2-3mm in diameter, were left to harden in this solution for 45 min, and then transferred to 0.85% sterile saline solution for a further 20 min. The beads were finally rinsed in sterile water for 10 min, air dried and stored at 4°C. To test viability of encapsulated probiotics, three alginate beads were resuspended in 1.5 ml 0.1 M sodium citrate (pH 6.0) using a Mini-Beadbeater-1 (Biospec Products, Oklahoma, U.S.A) for 1 min. This step released the bacteria from the alginate by sequestering the calcium ions which cause the alginate to gel. The viable probiotic cells were quantified by spread plating on MRS agar with anaerobic incubation at 37°C for 48 h. Different bead sizes were tested using a yellow eppendorf tip, resulting in beads of approximately 1 mm in diameter, a cut-tip blue eppendorf tip, giving beads of about 4 mm diameter, and also beads were allowed to drop directly out of the nozzle of the funnel (4 mm wide) giving beads approximately 4-5mm in diameter.

3.3.2.3 Cocoa butter encapsulation

Bacterial strains were grown as previously described (Chapter 2) and encapsulated in alginate as described above (section 3.3.2.2). Commercially available cocoa butter was heated to 100°C and allowed to cool to approximately 40°C in a water bath. Alginate encapsulated probiotic beads (1 mm in diameter) were added to a final concentration of approximately 10^9 cfu/ml cocoa butter and mixed, while the temperature was maintained at 40°C. This mixture was poured into sterile plastic petri dishes, hardened at 4°C, cut into 1 cm^3 blocks and stored at 4°C. Viability of cells encapsulated in this manner was determined by plate counts on MRS agar. A block of cocoa butter containing encapsulated probiotics was diluted in 1x PBS with 0.1% Tween 80 and was

incubated at 40°C for 15 min, prior to plating on MRS solid media, to aid in the release of encapsulated probiotic cells from the cocoa butter mixture and was incubated for 48 h at 37°C in an anaerobic chamber.

3.3.2.4 Incorporation of probiotics into ostrich feed by vacuum impregnation

The strains were grown as previously described (section 3.3.1). The cells were collected by centrifugation (10,000 rpm for 5 min), washed twice in 1x PBS, and resuspended in 5 ml of a sterile solution of 5% skim milk and 5% raftilose ®P95. This solution was impregnated into the ostrich feed using vacuum impregnation technology to a final concentration of approximately 1 x 10^8 culturable cells/g feed. 50 g ostrich feed was placed in a glass jar sealed with a rubber seal and clamp. A vacuum pump drew a vacuum of 80 kPa inside the glass jar and 5 ml of the bacterial suspension was drawn into the vacuum jar and thoroughly mixed by shaking the jar. During this intake of bacterial suspension, the pressure inside the vessel was decreased to 50 kPA where it was maintained for 5 min. Thereafter the vacuum was slowly decreased to atmospheric pressure and the impregnated ostrich feed was emptied onto clean Whatman 3MM paper to dry for 18 h at 22°C. To test the viability of the probiotics after impregnation, 1 g of feed was added to 10 ml sterile water and left overnight at 4°C. Serial dilutions of this were made and spread-plated, in triplicate, onto MRS agar and the cell numbers calculated after incubation for 48 h at 37°C as cfu/g of feed. The impregnated feed was stored at 4°C.

3.3.3 RAPD

Colonies used for RAPD analysis were cultured from faecal samples from ostrich chicks in both feeding trials. Ten colonies were randomly selected from MRS-Cys agar and suspended in 1 ml sterile water containing 0.1 mm zirconia/silica beads. This was subsequently bead beaten at 4,800 oscillations/min for 140 s. This was left at 4ºC for 10 min and the supernatant used as PCR template, using primers OPL5 and PL1 (Chapter 2). RAPD PCR reactions and conditions employed are as described in Chapter 2.

3.3.4 Pilot-scale ostrich feeding trial

A pilot feeding trial was carried out at Tier Vlei commercial ostrich farm in Carnarvon, South Africa, with a similar setup to the main feeding trial described below. The five strain probiotic

mix was used, as described below and administered as calcium alginate encapsulated beads. As this feeding trial was carried out on a commercial farm, the antibiotics oxytetrachloride and colimix were administered on an "as and when needed" basis, dependent on the development of illness at a concentration recommended by the veterinarian. The five probiotic strains used were found to be resistant to both of these antibiotics. Faecal samples were cultured on MRS, BHI and Beerens agars and 10 colonies from MRS agar were selected for analysis using RAPD. BHI plates were incubated both aerobically and anaerobically, and randomly selected colonies were gram stained and tested for catalase (Maehly and Chance, 1954) activity. The ostrich chicks were weighed in crates in batches of ten on a large-scale scale and the weights recorded every two weeks, to reduce the stress of handling the chicks, and feed conversion ratios were calculated as described below (section 3.3.5.1). Total DNA was extracted from faecal samples using ZR Fecal DNA Kit (Zymo Research, C.A., U.S.A.) according to the manufacturers' protocol and the diversity of total bacteria, *Lactobacillus* and *Bifidobacterium* was analysed using DGGE (data not shown).

3.3.5 Large-scale feeding trial

3.3.5.1 Administration and doses of probiotics

This trial was carried out at Elsenburg Agricultural Centre (Stellenbosch, South Africa). Three day old ostrich chicks from Oudtshoorn and Darling (South Africa) were weighed, tagged and randomly allocated to probiotic (E) or control (C; no probiotic treatment) pens (n=17). Each group was done in triplicate (three pens for probiotic treatment and three pens for control groups) and the pens were arranged so that similar treatments were not situated in neighbouring pens (Fig. 3.1). Another set of pens were allocated for probiotic and control groups also receiving the antibiotic tylosin (ET and CT) at a concentration of 20 mg/kg of feed daily. All chicks had unlimited access to food and water. The pelleted feed was formulated and mixed by Elsenburg Agricultural Centre and consisted of (w/w): 50% maize meal, 10% lucern meal, 20% soya oilcake meal, 13% full fat soya meal, 1% oil, 2% feedlime, 1% monocalciumphosphate, 0.5% salt and 0.5% vitamin and mineral mixture. The pens had cement floors overlaid with rubber matting and were separated by low concrete walls. The ambient temperature was kept between 25-30ºC and the chicks had controlled access to the outdoors from two weeks of age. These outdoor areas were also contained so the chicks could not mix with members from other

treatments. One alginate bead containing the probiotic mixture was fed to each chick in the treatment groups, and this was counted as day one of the trial. The chicks were weighed separately in a crate on an industrial sized scale on day one and weekly hereafter, and the average weights per treatment were calculated. This was done more frequently than in the pilot feeding trial as weighing every two weeks was found to be too broad a time difference. On day seven another alginate bead was fed to each chick. The average viability of one bead was calculated to be 1.84 x 10^8 cfu/bead. In order to ensure that from day 14 each chick received at least 1 x 10^8 bacteria, 500 mg of alginate beads were mixed in with the normal ostrich feed daily. The control group received normal feed pellets. Treatment ceased after nine weeks. Weekly, the average mass of food eaten by the ostrich chicks was divided by the average weight gain of each treatment to get the feed conversion ratio. Mortality rates were recorded and birds that died were sent to a veterinarian for postmortem analysis. At weeks two and five a total of 12 ostrich chicks (one from each pen) were euthanized by severing of the spinal cord and the digestive tract dissected. Mucosal scrapings from the duodenum, colon and caecum were immediately put into 1 x PBS and frozen at -20°C.

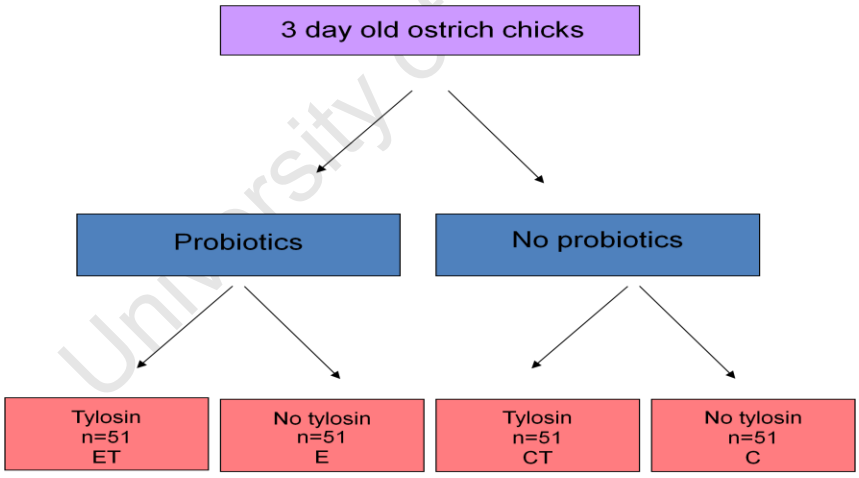

Figure 3.1 The layout of the feeding trial showing the numbers of ostrich chicks per feeding treatment. The treatments were divided into three (*n*=17) and the pens were arranged so as not to have the same treatments adjacent to each other. Treatments: E, probiotic mix; ET, probiotic mix + tylosin; C, control; CT, tylosin.

3.3.5.2 Faecal sample collection

Anaerobic travel swabs (In vitro diagnosticum, Oxoid) were used to collect excreta on day zero since the quantity of faeces generated by five day old ostrich chicks is not sufficient to be collected. From day seven onwards, six faecal samples were collected in sterile faecal collection tubes (Lasec, South Africa) and three of these were swabbed with the anaerobic travel swabs for anaerobic storage and later analysis of anaerobic bacteria. Samples were rapidly transported to the laboratory and samples were immediately frozen at -80°C until analysed or fixed in paraformaldehyde and frozen for later FISH analysis (Chapter 5). The travel swabs were used for selective culturing, as described below.

3.3.5.3 Microbiological analysis of ostrich chick faecal samples using culture techniques

Faecal matter from anaerobic travel swabs was suspended in 1 ml sterile pre-reduced PBS under anaerobic conditions. Five faecal samples were pooled per pen and plated in duplicate onto the various selective agar under the conditions described above in section 3.3.1.

3.3.5.4 Microbiological analysis of ostrich chick mucosal samples using culture techniques

Homogenised scrapings from the colon, caecum and duodenum were serially diluted in 0.9 ml sterile pre-reduced 1x PBS under anaerobic conditions and spread plated onto the selective agars as described above.

3.3.5.5 Statistical analysis

The effects of the addition of probiotic and tylosin to the ostrich diet on the culturable numbers of chosen bacterial groups were analysed using the two-way analyses of variance (ANOVA). Stata statistical program (version 11.0) was used to perform *post hoc* Kruskal-Wallis analysis. Statistically significant differences were established at critical levels of $p<0.05$ and $p<0.1$ as indicated.

3.4 Results and Discussion

3.4.1 Development of method of delivery of probiotic strains to ostrich chicks

The relative survival of probiotic bacteria during processing, storage and passage through the gastrointestinal tract may limit their usefulness in food applications (Lahtinen *et al.,* 2007). However, several attempts have been made to increase survival of probiotic microorganisms using encapsulation techniques. Of these, freeze drying, lipid-based encapsulation, feed impregnation and alginate encapsulation were investigated in this study.

3.4.1.1 Freeze drying

Water activity is an important factor to consider when wanting to extend the shelf-life of a quiescent, dried probiotic product (Crittenden, 2009). As water activity and moisture levels increase, survival of the probiotic decreases. Drying of microorganisms is one of the most common methods of preservation (Carvalho *et al.,* 2004) and is frequently used for the preservation of large quantities of probiotic bacteria (Carvalho *et al.,* 2004; Capela *et al.,* 2005; Bolla *et al.,* 2011; Salgado *et al.,* 2011). In this study, all five probiotic strains maintained a high degree of viability after being freeze dried and processed separately (data not shown), as well as when they were freeze dried together in a skim milk and raftilose ®P95 solution (Fig. 3.2). Probiotic bacteria differ in their ability to survive freeze-drying, perhaps due to differences in surface area of the microorganism, variations in cell wall and membrane composition, sensitivity to process oxygen and temperature exposure, and/or water activity and changes in solute concentration (Carvalho *et al.,* 2004). Cryoprotectants are often used during freeze drying processes to improve a bacteria's ability to survive this treatment and increase its shelf life, and include sugars, such as lactose or sucrose, monosodium glutamate and ascorbate in a milk or water base (Bigret and Mäyrä-Mäkinen, 2004). These can be added to the growth media or to the mix to be freeze dried. The protective effects provided by carbohydrates during freeze-drying is well documented and is thought to be due to their ability to raise the glass-phase transition temperature, enabling the cells to reach the glassy phase without nucleating intracellular ice (Fowler and Toner, 2005). Alternatively, prebiotics such as fructooligosaccharides (FOS) have been shown to improve viability of *Bifidobacterium* spp. in skim milk when included in the growth media and was reported to increase the shelf-life by 55.7% after 4 weeks of refrigerated

storage (Shin *et al.*, 2000). Raftilose ®P95 was found to increase the viability of a mix of freeze dried probiotic strains, including *L. rhamnosus, L. casei, L. acidophilus* and *B. infantis*, over four weeks of storage at 4ºC (Capela *et al.*, 2005). Skim milk proteins have also been shown to prevent cellular injury by stabilizing cell membrane constituents thus making it an effective cryoprotectant (Castro *et al.*, 1995). In addition, milk proteins may form a protective layer on the surface of cell wall proteins, while the calcium in milk enhances survival following dehydration (King and Su, 1993).

Although the freeze dried probiotic mix displayed good viability after 25 days of storage at 4ºC (Fig. 3.2), this method was not used as the mode of probiotic preparation and delivery in these ostrich feeding trials. The reasons for not making use of freeze dried probiotics for this feeding trial included the small processing capacity of freeze drying probiotic cultures, together with difficulties in on site application of the dried final product. Although freeze dried probiotic strains can be incorporated into dry feed pellets (Timmerman *et al.*, 2006) it is time consuming and expensive as the probiotics need to be co-extruded and prepared with formulated feed.

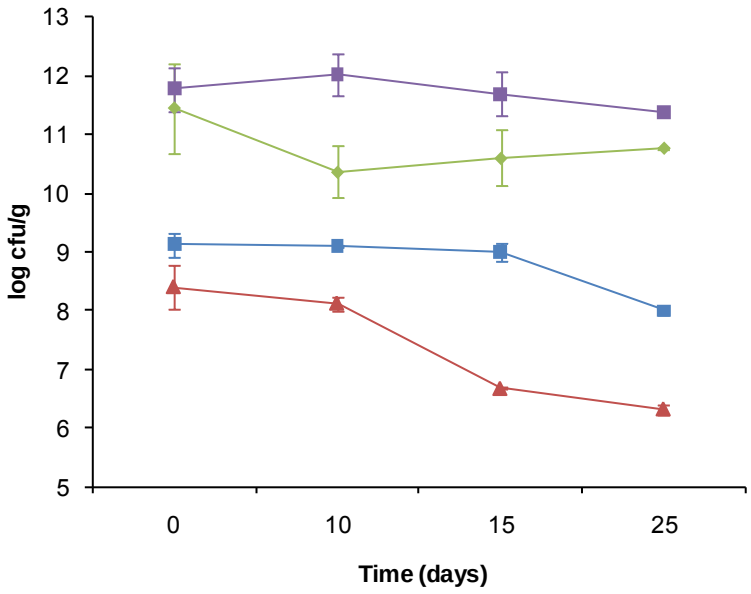

Figure 3.2 Viability of the five strain probiotic mix over 25 days after freeze drying in 5% skim milk and 5% raftilose ®P95 (♦), encapsulation in calcium alginate (■), vacuum impregnation into the feed pellets (▲) and entrapping the calcium alginate beads into cocoa butter (■). All variations were stored at 4ºC. Error bars represent the mean ± standard error, n = 3.

3.4.1.2 Calcium alginate encapsulation

Another more widely used method of immobilization of probiotics is entrapment in calcium alginate beads (Sheu and Marshall, 1993; Sultana *et al.,* 2000; Krasaekoopt *et al.,* 2004). In this study, this method maintains the initial viability of the probiotic mix over 25 days at 4ºC (Fig. 3.2). Furthermore, this method is relatively simple, low cost and non-toxic. It involves packaging the cells into a shell material, such as sodium alginate, which offers them protection from unfavorable environments, but permits diffusion of nutrients into and out of the matrix and, thereby enables the probiotic cells to maintain long-term viability (Talwalkar and Kailasapathy, 2004).

Encapsulation has been shown to reduce cell injury or cell loss (Rao *et al.,* 1989) and to stabilise cells, enhancing their viability and stability in fermented milks and in the gastrointestinal tract (Kailasapathy, 2003). It has been shown to increase the survival of lactobacilli in frozen food-stuffs (Sheu and Marshall, 1993), in unfavorable conditions such as gastric juice (pH 1.2; Ross *et al.,* 2008), and to slow the decline in viable *L. acidophilus* and *Bifidobacterium* spp. cells during storage in yoghurt, when compared to free cells (Sultana *et al.,* 2000). In comparison to freeze dried probiotics, the shelf life is enhanced as it is when freeze dried, but storage at 4ºC is not necessary (Capela *et al.,* 2006). Of the delivery mechanisms studied here, calcium alginate encapsulation of the probiotic mix was the best alternative to overcome the problem of poor stability, survival in the GIT as well as practical issues around delivery to chicks, and was the method used in this feeding trial.

3.4.1.3 Cocoa butter encapsulation

The cocoa butter encapsulated calcium alginate beads provided a good matrix for protecting the probiotic beads, maintaining the viability of the strains sufficiently over 25 days (Fig. 3.2). Encapsulation of cells in cocoa butter has been shown to increase culturability of two *Bifidobacterium longum* strains during storage and to extend the viability of the bacteria as compared to non-encapsulated strains (Lahtinen *et al.,* 2007). The improved culturability observed with cocoa butter encapsulated strains is thought to be due to protection offered by the lipid matrix against storage stress such as blocking exposure of cells to water and associated stress factors such as H^+ ions. It is thought that using cocoa butter to protect bacteria against such

stresses is matrix encapsulation, rather than 'true' encapsulation as some cells are attached to the surface of the lipid particles and are thus exposed to the stress factors. Some amount of bacterial shedding of cells from the matrix may also occur. In this study, the probiotic strains were first encapsulated in calcium alginate beads after being mixed with the cryoprotectants, skim milk and raftilose ®P95, and then embedded in the cocoa butter, thus preventing surface exposure of some bacteria to the stress factors. They also had the protection of the cryoprotectants, known to avoid damage caused during processing and further extend shelf life (Fowler and Toner, 2005). Due to the low melting temperature (approximately 37°C) of cocoa butter, the probiotic microorganisms entrapped in this matrix would be released at human body temperature (Lahtinen *et al.*, 2007), and similarly in this case at ostrich body temperature. Although the cocoa butter was a good option for maintaining probiotic viability, it was not a viable option in this case due to its low melting temperature and the high temperatures experienced on the ostrich farms, sometimes reaching temperatures above 45°C. Handling of the cocoa butter cubes was also complicated.

3.4.1.4 Vacuum impregnation

Vacuum impregnation of fruit and vegetables has been shown to be a viable method for incorporation of functional foods such as probiotics into food matrices, providing new product categories and new commercial opportunities (Alzamora *et al.*, 2005; Huddy, 2010). In this study, vacuum impregnation of the probiotic mix into ostrich feed was a successful means of incorporating the probiotics into food, the viability of the strains remaining above 10^6 cfu/g feed over 25 days storage at 4ºC (Fig. 3.2). However, after day 10, a substantial decrease was seen in viability. For this reason, as well as the suitability of the calcium alginate beads, this method was not used to administer the probiotics to the chicks.

3.4.2 Pilot feeding trial (Carnarvon, Karoo)

The pilot feeding trial in Carnarvon was carried out to test the delivery, safety and success of the five strain probiotic mix. Delivery of the probiotic mix via the calcium alginate bead proved successful and easy. Total anaerobes and lactic acid bacteria increased in the probiotic treated birds compared to the controls (data not shown). Although DGGE analysis of faecal samples using universal, *Lactobacillus*- and *Bifidobacterium*-specific primers showed no significant differences in diversity (Shannon-Wiener index; data not shown), feed conversion ratios

improved, growth rates increased and mortality rates decreased, for birds receiving the probiotic mix.

The pilot feeding trial was carried out on a commercial ostrich farm and it was therefore difficult to interfere with the normal running of the farm including dosage of antibiotics (oxytetrachloride and colimix) and a commercially available probiotic mix (Protexin). However, the outcome of this feeding trial showed that chicks receiving the five strain probiotic mix increased their weight gain, improved their feed conversion ratios and decreased the mortality rates when compared to the control group (Juste-Poinepen, 2008; du Toit, unpublished). Therefore, the mix was chosen for further assessment in the following feeding trial, carried out on an experimental research farm, under conditions which were more controlled.

3.4.3 Feeding trial (Elsenburg)

3.4.3.1 RAPD analysis of faecal samples

RAPD analysis was evaluated here as a possible method for detecting the fed bacterial strains in the faecal samples. The pathogens obtained for the five probiotic strains are shown in Chapter 2. Faecal samples were cultured on MRS agar and, 10 colonies were selected and amplified with *Lactobacillus*-specific RAPD primers (Fig. 3.3). The pattern, indicating the presence of the included *L. plantarum* strains, is seen in the probiotic group (lanes 9, 11, 12 and 15, 17, 19, 20, 22, 31 and 33) and not in the control group at the same time point (Fig. 3.4). The RAPD fingerprints for the probiotic fed group show a more diverse range of patterns in comparison to the control group, which mainly shows only one or two bands per lane. With the maturing gut, the microbial diversity in the gut changes (Barnes *et al.*, 1972), and accompanying this, a change in RAPD fingerprints obtained would be expected. However, a more intense and diverse range would be expected for the probiotic group as found by Fuentes *et al.* (2008), who reported an increased diversity and presence of certain *Lactobacillus* in the gut and faecal samples of mice receiving *L. casei* and *L. plantarum.*

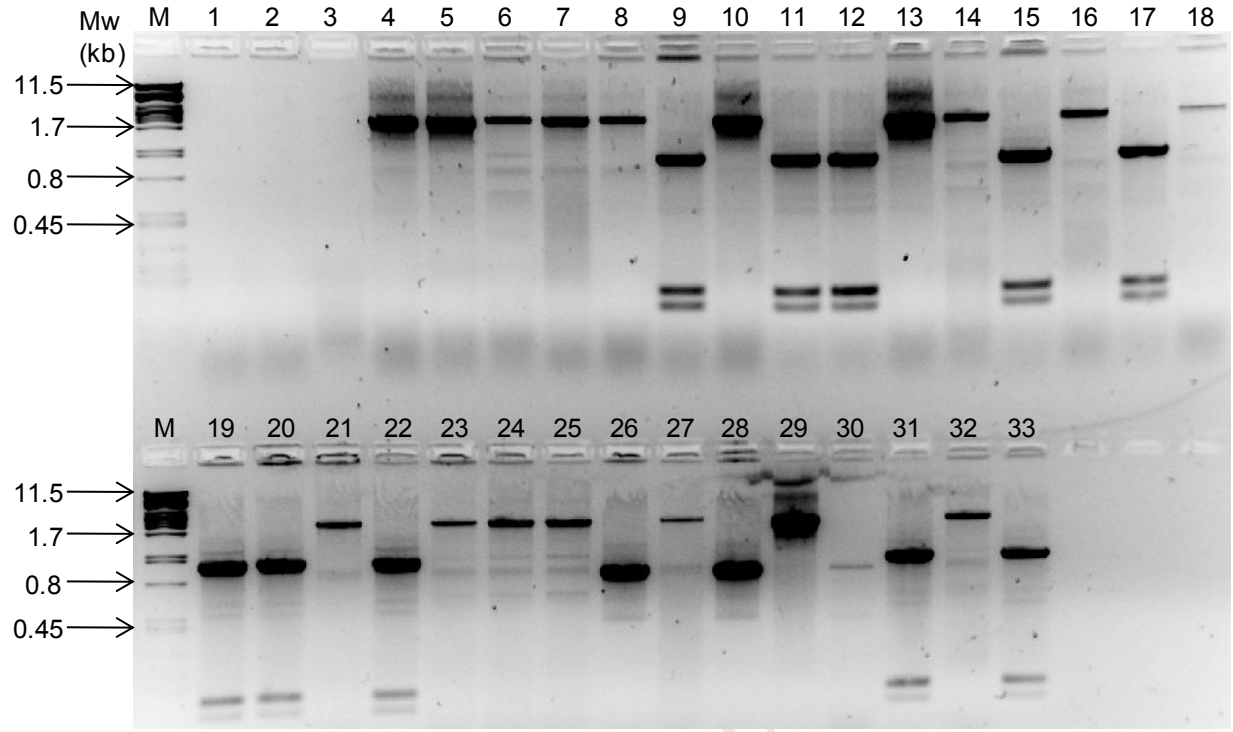

Figure 3.3 RAPD analysis of colonies picked from MRS agar obtained from faecal matter of ostrich chicks receiving the probiotic mix at week two of the feeding trial, amplified with *Lactobacillus*-specific primers OPL-05 and PL1. Lanes: M, λ-DNA digested with *Pst*I; 1, OPL-05 primer only; 2, PL1 primer only; 3, no DNA control; 4-13, probiotic treatment pen 1; 14-23, probiotic treatment pen 2; 24-33, probiotic pen 3.

RAPD PCR enabled rapid genotyping of the microbial biodiversity found in ostrich faecal samples and gave a positive identification of the fed *L. plantarum* strains, however, for an initial screen for the presence of the administered probiotic mix, based on fingerprints, it has been reported to have low resolution (Martín-Platero *et al.*, 2009). In addition, this method provides limited information on the ostrich gut microbiota as only those culturable on MRS were studied. For these reasons, this method was not continued in this study.

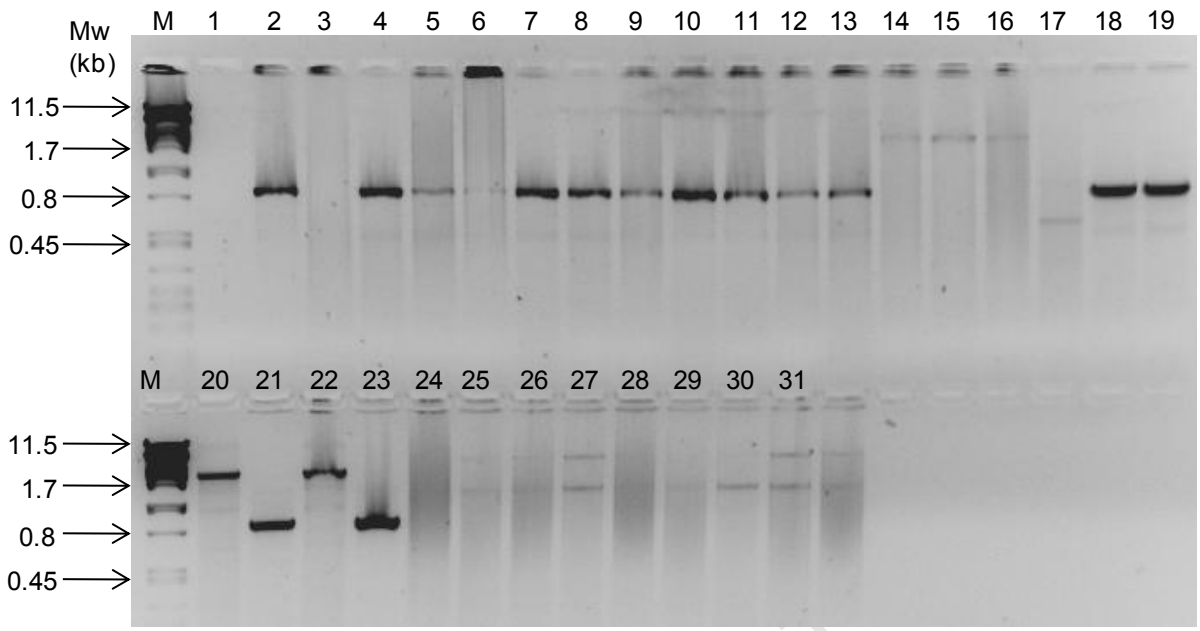

Figure 3.4 RAPD analysis of colonies picked from MRS agar obtained from faecal matter of ostrich chicks in the control group at week two of the feeding trial, amplified with *Lactobacillus*-specific primers OPL-05 and PL1. Lanes: M, λ-DNA digested with *Pst*I; 1, no DNA control; 2-11, control group pen 1; 12-21, control group pen 2; 22-31, probiotic pen 3; last lane, overflow from lane 31.

3.4.3 Ostrich chick growth and mortality rates

During the feeding trial, the ostrich chick's weights were recorded (Fig. 3.5), as well as FCR (Fig. 3.6) and mortality rates (Table 3.1). On a weekly basis, each chick was weighed separately and the average weight for each treatment calculated to establish whether the probiotics had enhanced the growth rate of ostrich chicks in comparison to the control group and to the tylosin-treated chicks. Improved growth rate in poultry, following dietary probiotic supplementation, is a commonly reported effect (Nahashon *et al.*, 1994; Abe *et al.*, 1995; Jin *et al.*, 1998a; Jin *et al.*, 1998b; Timmerman *et al.*, 2006; Thanh *et al.*, 2009; Mountzouris *et al.*, 2010; Mountzouris *et al.*, 2011; Thu *et al.*, 2011). In the pilot trial, this specific probiotic mix was shown to significantly ($p<0.05$) increase the weight gain of ostrich chicks in comparison to birds fed a control diet (data not shown). In the large-scale feeding trial at Elsenberg, the weights of the chicks did not show any differences (Fig. 3.5) until week three where treatment C showed a higher, but not statistically different mean weight than the remaining three treatments, which were all comparable.

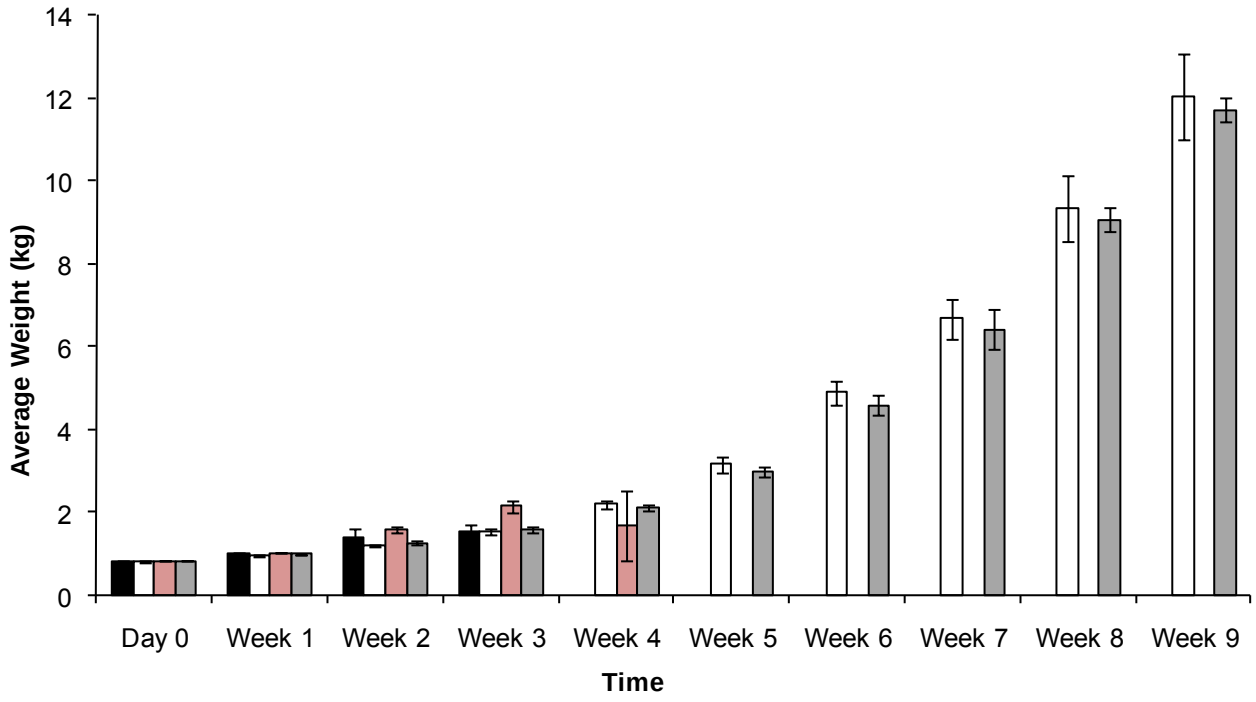

Figure 3.5 Average weights of ostrich chicks in each treatment group E (■), ET (□), C (■) and CT (■). Error bars represent the mean ± standard error (n=51).

By week four, the control bird's weights were lower than that of the ostriches receiving the treatments ET and CT, and group E had all died. This suggests that the probiotic mix was not as effective as we had anticipated at increasing ostrich growth rates at an early stage, as previously reported (data not shown), and that the antibiotic tylosin may initially retard ostrich chick growth. Unfortunately, the majority of control group, C died after four weeks, leaving too few chicks for statistical analysis for comparison of weight gain. Groups ET and CT increased from week four at a comparable rate reaching approximately 12 kg by week nine. Mushi *et al.* (1998) reported weight loss in the first seven days after hatching due to the use of nutrients from the egg yolk, followed by an average weight gain of 1.3 kg per week for ostrich chicks not receiving any feed supplements under intensive farming methods up to 12 weeks. In this study, the chicks did not show such a rapid weight gain until week five, where the birds receiving treatments ET and CT displayed overall weight gains of 1.3 and 1.2 kg/week, respectively. This suggests that tylosin is not an effective weight enhancer in ostrich chicks, given that the ostrich chicks' weight gain reported by Mushi *et al.* (1998) was higher despite not receiving tylosin. Glatz and Miao (2008) report that the body weight of an ostrich chick increases 11-fold from day three until day

72. This study shows the body weight increasing in accordance with this for treatments ET and CT.

A feed conversion ratio is a measure of an animal's efficiency in converting feed into body mass, and is defined as the amount of feed consumed to produce one kg of weight gain (de Verdal *et al.*, 2011). The lower the FCR, the more efficiently the animal is converting the food eaten into weight gained. Initially, treatment E had the lowest FCR, however the four treatments did not differ significantly ($p>0.05$) at week one (Fig. 3.6). The control group decreased more rapidly to below treatment E by week two and maintained the lowest FCR until week four, at the ideal value of 1.4 (www.ostrichesonline.com). From week four onwards, treatments ET and CT maintained similarly low FCRs, reaching 1.4 by week six, and by week eight, treatment ET had an FCR of 0.96 and CT 1.45. No significant differences between FCR's were observed between these four treatments. Kreibich and Sommer (1995) reported ostrich chicks under six months old to have a FCR of 1.4 to 1.6, which is similar to the reported ideal ostrich FCR of 1.5 (www.ostrichesonline.com). Mountzouris *et al.* (2007) showed that antibiotic treatment of broiler chickens significantly reduced their FCR compared to the control, where as probiotic treatment reduced the FCR but not significantly when compared to the control. The average FCR of commercially raised broilers up to two months of age is reported to be approximately 2 (Williams, 1994; Mountzouris *et al.*, 2007; FAO, 2010), showing that ostriches have a better FCR than commercially raised poultry. Tylosin treated poultry chicks showed very efficient FCRs and, although the use of growth promoting antibiotics is banned, tylosin is a well documented growth promoter (Collier *et al.*, 2003). It is not possible to draw any significant conclusions on the effect the probiotic mix had on the FCR of ostrich chicks as treatment E died after week two, and the FCR of treatment ET is very similar to that of CT.

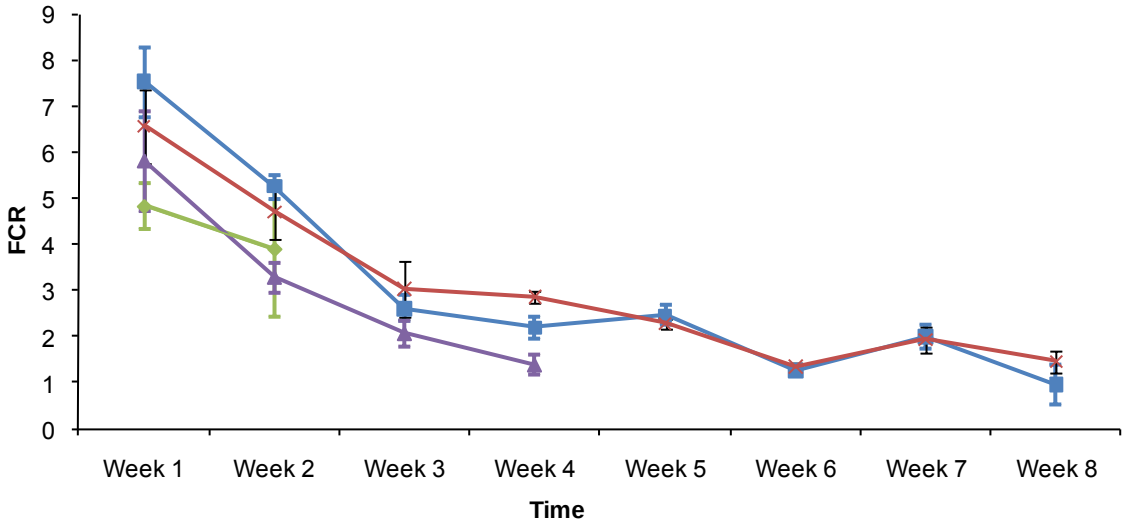

Figure 3.6 Feed conversion ratio of ostrich chicks in treatment groups E (♦), ET (■), C (▲) and CT (x). Error bars represent the mean ± standard error (*n* = 51).

The mortality rates of ostrich chicks receiving treatments E and C followed similar patterns, as did ET and CT (Table 3.1). This suggests that tylosin has a profound effect on reducing ostrich chick mortality rates, and is evident during the first week of the trial, where treatments E and C had mortality rates of 33 and 31%, respectively, in comparison to treatments ET and CT with mortality rates of 16 and 22%, respectively (Table 3.1). The mortality rate within treatments E and C increased comparably, although the rate was faster for treatment E than for treatments C, reaching approximately 92% in week four in comparison to 61% for treatment C. At this time point, both treatments ET and CT showed mortality rates of 39.2%. Treatment E and C did not show 100% mortality rate, as birds taken for slaughters were not considered for this data. From week four onwards, mortality rates for treatment C increased rapidly, while treatment ET and CT gradually increased and by week nine 51% of treatment ET had died while 45% of treatment CT had died. This shows that the probiotic treatment alone has a negative effect on the survival of the chicks, while the combination of probiotics and tylosin shows similar results to group CT. Post mortem analysis of most of the deceased birds indicated severe colitis.

Table 3.1. Cumulative percentage mortality of ostrich chicks in each treatment group throughout the feeding trial.

Time	Treatment			
	E	ET	C	CT
During week1	33.3	15.7	31.4	21.6
week 2	47.1	23.5	43.1	25.5
week 3	60.8	31.4	54.9	29.4
week 4	92.2	39.2	60.8	39.2
week 5	-	47.1	88.2	41.2
week 6	-	49.0	92.2	41.2
week 7	-	49.0	-	43.1
week 8	-	51.0	-	43.1
week 9	-	51.0	-	45.1

Probiotics are thought to reduce host mortality rates via competitive exclusion of pathogenic microorganisms, thereby preventing the colonization and proliferation of enteric pathogens within the host's digestive tract (Timmerman *et al.*, 2006). However, inconsistent mortality rates were observed in trials when broiler chickens were fed *Lactobacillus* preparations (Watkins and Kratzer, 1984; Jin *et al.*, 1998a, b and 2000; Zulkifli *et al.*, 2000). Ostrich farming suffers from particularly high mortality rates, not only from pathogens, but as a result of handling and transportation associated stresses, starvation, leg problems, navel and yolk sac infections, and poor ventilation in the pens which can result in high ammonium levels which influences the health of chicks (Glatz and Miao, 2008). Glatz and Miao (2008) also suggest that low digestive enzyme activity in young ostrich chicks may contribute to high mortality rates. Probiotic microorganisms are also considered to contribute to the health of the host by production of supplemental digestive enzymes (Ziaei-Nejad *et al.*, 2006). The five probiotic strains used in this study were shown to produce zones of clearing on carboxy-methyl-cellulose solid media (cellulase test) and xylan solid media (xylanase test; Chapter 2), as well as a positive test for cellobiose using the API strip (Chapter 2). Furthermore, diet plays an important role in the health of young ostrich chicks. At six weeks old an ostrich chick is able to digest 66 and 38% of dietary hemicelluloses and cellulose, respectively (Swart *et al.*, 1993a). However, at three weeks old

young ostrich chicks can only utilize 6.5% of ingested fibre (Angel, 1993) and high fibre feeds can cause intestinal obstructions. The diet fed in this study contained high fibre, with 50% maize meal and 10% lucerne meal. Given the low usage of fibre at this age, this may have contributed to the high mortality rates observed here, including treatments ET and CT. In addition, Sato *et al.* (1994) found obstructions such as maize, lucerne or hay, in the proventriculus and gizzard to be the cause of death in ostrich chicks in their study. Suffocation due to over-stocking in the pens is yet another common reason for mortality. In Zimbabwe, the space requirement for day old chicks is 0.16 m^2/chick increasing by 10% per week (Hallam, 1992). The pens used in this study were 4 m^2 and contained 17 chicks per pen (0.24 m^2/chick). Although this is adequate initially, the pens remained the same throughout the feeding trial, and by week two the pens would have been smaller than the recommended size. Overcrowding of young ostrich chicks can lead to stress and physical abnormalities, such as leg deformities. During the course of the feeding trials the chicks were handled by humans regularly, initially when being fed the probiotic encapsulated bead, and thereafter when being weighed. This has been reported to cause stress and result in high mortalities in ostrich chicks (Hicks, 1992).

3.4.3.4 Microbiological analysis of ostrich chick faecal samples

In this study, conventional microbiological techniques, using selective solid growth media, were used to analyse the microbial composition of faecal (Fig. 3.7 and Table 3.2) and gut mucosal samples (Table 3.3). Fuller (1989) described probiotics to beneficially improve the host's microbial intestinal balance. Ingesting probiotics should result in the development of a healthy microbiota which excludes pathogens (Pascual *et al.*, 1999) and favours the colonisation by beneficial bacteria, generally enhancing the gut health. In this analysis, $p < 0.05$ was used for statistical significance, but as the sample size here was so small (n = 9), the confidence interval was increased to $p < 0.1$.

MRS agar favours the growth of lactic acid bacteria, and the presence of sodium acetate in this medium suppresses the growth of competing bacteria. When comparing the LAB counts between the treatments at given time points, weeks two and nine showed significant differences ($p = 0.06$ and 0.1, respectively; Table 3.2a). The graphical display (Figure 3.7A) shows treatment E and ET having higher LAB counts than C and CT at week two, and at week nine, ET being higher

than CT. When comparing the treatments over time, a significant difference is observed for treatments C, ET and CT (p = 0.07, 0.03 and 0.02, respectively; Table 3.2b), and perhaps this is due to the decrease for these treatments from week two to week four. Hereafter, treatments ET and CT gradually increase again. This trend is interesting as all three treatments show this decrease in LAB by week four, just after the death of all chicks in treatment E. This could indicate that the LAB population was affected by an event such as diarrhea, for example, which was detrimental to the chicks in E but the other three treatments were able to survive. Previous work by Collier *et al.* (2003) showed tylosin to increase the lactobacilli counts significantly; however this is only seen at weeks two and nine, although the probiotic mix contains two different species of *Lactobacillus* which would be expected to increase their numbers in the ostrich gut. The combination of probiotics and tylosin does however show a higher final LAB count than that of the chicks only receiving tylosin. Mountzouris *et al.* (2007) found that feeding a probiotic mix including two *Lactobacillus* strains and a *Bifidobacterium* to broiler chickens increased the numbers of lactobacilli, bifidobacteria and Gram positive cocci compared to the control and antibiotic treated birds. It is important to note here that the five probiotics used in this study were shown to tolerate tylosin at the level used in the feeding trial (Chapter 2).

BHI agar is an enriched non-selective media used for cultivation of most anaerobic bacteria and other fastidious organisms. The total anaerobe counts found in the four treatments showed significant differences between them at week four (p = 0.1; Table 3.2a) and week seven (p = 0.08), and visual analysis of the graph (Fig. 3.7B) indicates that treatment ET has higher numbers of anaerobes than C and CT at both time points, indicating a role of the probiotics. Due to the lack of specificity with culturing on selective agar, it is unknown whether these anaerobes are beneficial or potential pathogens. Treatment CT is the only group showing a significant difference over time (p = 0.07; Table 3.2b) and this is possibly due to the dip seen in anaerobes at week four and seven. Again, the overview of the data trends show treatment E increasing while the other three remain constant in the first two weeks, again suggesting the probiotic mix alone increases the total anaerobes initially. Hereafter, treatment ET shows the highest prevalence of this bacterial group. Tylosin appears not to have an effect on the total anaerobes. In the development of the microbiota, it is expected to see an increase of anaerobic bacteria as the gut matures (Mead and Adams, 1975).

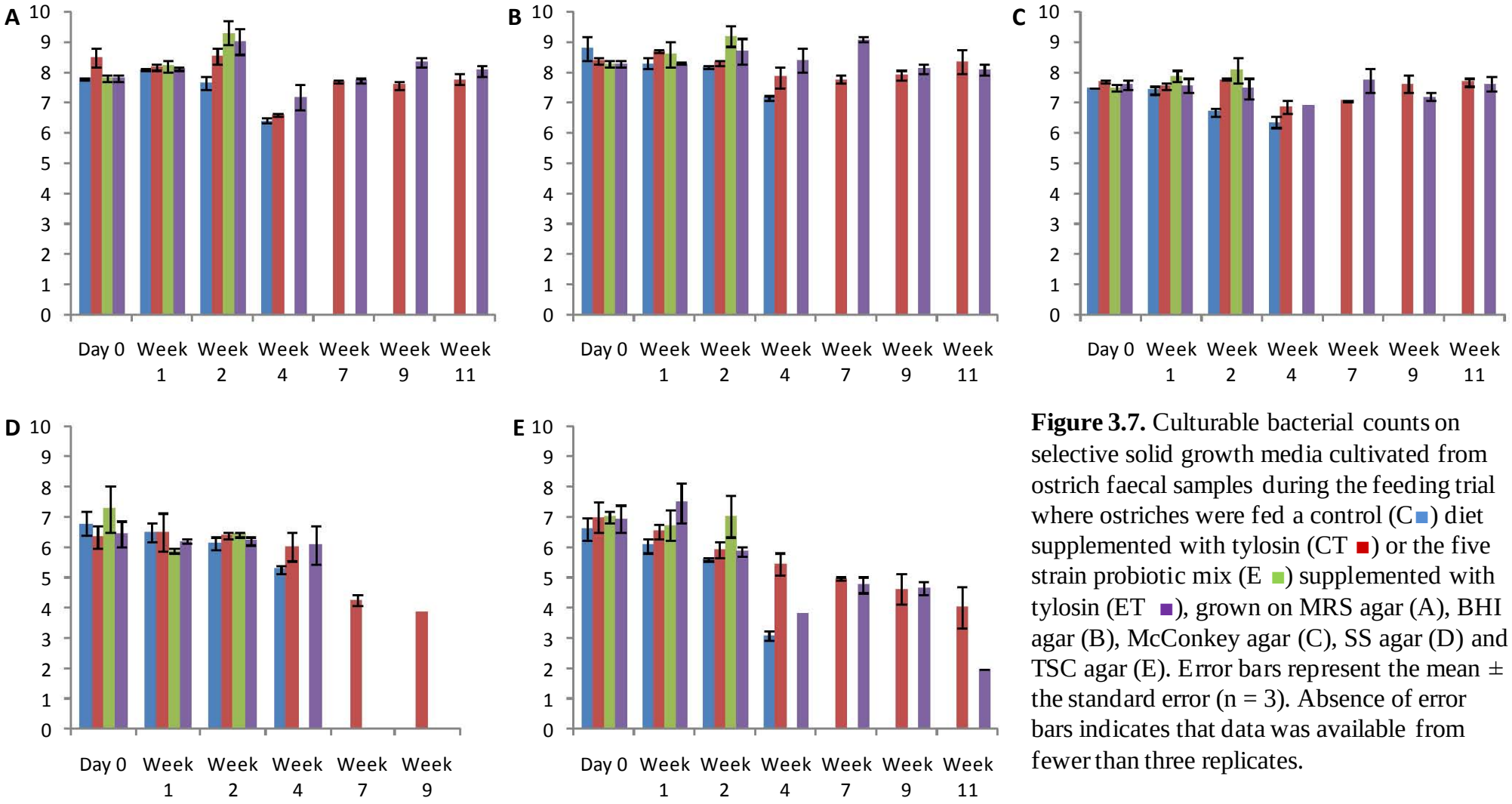

Figure 3.7. Culturable bacterial counts on selective solid growth media cultivated from ostrich faecal samples during the feeding trial where ostriches were fed a control (C■) diet supplemented with tylosin (CT ■) or the five strain probiotic mix (E ■) supplemented with tylosin (ET ■), grown on MRS agar (A), BHI agar (B), McConkey agar (C), SS agar (D) and TSC agar (E). Error bars represent the mean ± the standard error (n = 3). Absence of error bars indicates that data was available from fewer than three replicates.

This is not seen here but may be due to the methods of enumeration used. Although the faecal samples were transferred rapidly to the anaerobic travel swabs, they were exposed to the air before being collected. This may have an effect on the culturable numbers of anaerobes.

Table 3.2. *P*-values obtained by performing the Kruskal-Wallis statistical test on the plate counts obtained on the selective growth media used for (a) between treatments at a certain time point and (b) over time (11 weeks) for each treatment.

a

	Agar				
Time	MRS	BHI	McConkeys	SS	TSC
Day 0	0.22	0.88	0.46	0.67	0.76
Week 1	0.74	0.25	0.46	0.43	0.50
Week 2	0.06	0.22	0.05	0.20	0.76
Week 4	0.48	0.10	0.17	0.67	0.12
Week 7	0.32	0.08	0.56	N/A	0.37
Week 9	0.10	0.46	0.95	N/A	0.28
Week 11	0.21	0.87	0.28	N/A	0.38

b

	Agar				
Treatment	MRS	BHI	McConkeys	SS	TSC
E	0.59	0.19	0.08	0.30	0.59
ET	0.03	0.47	0.98	0.60	0.03
C	0.07	0.16	0.08	0.08	0.10
CT	0.02	0.07	0.07	0.25	0.01

McConkey's agar contains bile salts and crystal violet dye to inhibit the growth of most gram positive bacteria, but selecting for *E. coli, Enterobacter* and *Klebsiella*. This group of bacteria showed a significant difference at week two between the treatments (Table 3.2a), the control group C being significantly lower (p = 0.05) than the other three treatments (Fig. 3.7C). Treatments E and C (p = 0.08; Table 3.2b) and CT (p = 0.07) differed over time in prevalence of *Enterobacteriaceae.* Treatment E showed an increase in prevalence of this bacterial group, and C and CT a decrease. Both treatments ET and CT do not fluctuate much over the trial suggesting that tylosin and probiotic treatment do not affect this bacterial population. Adhesion studies with this probiotic mix showed it to be able to inhibit and displace *E. coli* but unable to out compete it (Chapter 2). Shryock *et al.* (1998) reported an intrinsic resistance of both *E. coli* and *Salmonella* spp. to tylosin, which appears to be the case in this study too.

SS agar is used for the selection of pathogenic enteric bacilli, especially *Salmonella. Salmonella* spp. did not differ significantly between treatments (Table 3.2a), however, treatment C changed significantly over time (p = 0.08; Table 3.2b), decreasing steadily (Fig. 3.7D). All treatments remained stable up to week two, however, at week four, the three remaining treatments showed a reduced number of *Salmonella* spp. and by week seven it was found to be below the limit of detection in treatments ET and C, indicating that it was present below the detection limit. Tylosin

alone (CT) is not effective as this treatment still has *Salmonella* spp. present at week nine, although there appears to be a technical problem at week seven and nine as this bacterial group was only detected on one of the duplicate agar plates, hence the absence of error bars, and the other two groups were not represented at all (Figure 3.7D), again suggesting it was present below the limit of detection. An intrinsic resistance of *Salmonella* spp. to tylosin was reported by Shryock *et al.* (1998). Again the adhesion data reported in Chapter 2 shows the probiotic mix to be able to inhibit and displace *Salmonella typhimurium* but unable to out compete it.

TSC media is used for the quantification of *C. perfringens* and is characterised by the development of black colonies due to the reaction of hydrogen sulphide produced by *C. perfringen*s, and the ferric salt present in the media. The numbers of *C. perfringens* did not show any significant differences between treatments. However, over time, treatments ET and CT differed significantly (p = 0.03 and 0.01, respectively) as did treatment C (p = 0.1), all groups decreasing over time, except treatment C which showed a peak at week nine (Fig. 3.7E). This agrees with previous work by Gérard *et al.* (2008) where *Clostridium* spp. titres decreased over time in broiler chickens supplemented with probiotics and those not supplemented. *C. perfringens* appears to be more constantly controlled when antibiotics are ingested and by week 11, the combination of probiotic and tylosin (ET) seems more effective at lowering this pathogens count than treatment CT. The postmortem performed on chicks from all treatments which died were reported to show intestinal lesions similar to those caused by *C. perfringens*. When comparing groups C and CT it can be seen the CT counts for *C. perfringens* are always higher than the C group, except for week 9, showing that tylosin is not as effective against *C. perfringens* as previously reported (Collier *et al.*, 2003; Martel *et al.*, 2004). This is not the trend seen in group ET over 11 weeks. The counts for ET decreased by 10^5 cfu/g, again confirming the effectiveness of the five strain probiotic mix and tylosin combination against this pathogen. Adhesion studies (Chapter 2) showed that the single probiotics as well as the five strain combination were able to inhibit and displace *C. perfringens*, explaining the decrease in prevalence of this pathogen by competitive exclusion in these cultured bacterial studies.

The data obtained from the Beerens agar plates was not included in this study due to uneven growth and contamination. This was unfavorable as the abundance of bifidobacteria are often used as biomarkers for the efficacy of pre and probiotics (Apajalahti *et al.,* 2006) and in this study would have given useful information on the effectiveness of *B. pseudolongum* subsp. *pseudolongum* as a probiotic for use in ostrich chicks. The lack of data from Beerens agar plates may have been due to medium composition and the fact that some bifidobacteria are more difficult to culture than others (Martinaeu, 1999).

The chicks in treatment E died after week two. The selected culturing of faecal samples from these chicks did not expose any significant indications as to the cause of death. At week two, treatment E shows the highest numbers of all three pathogenic groups, although these differences are not significant. Treatment CT showed similar numbers to E on SS agar at week two. The postmortems indicated death was likely due to *C. perfringens* lesions in the guts of these chicks, but perhaps it was a combination of reasons, given that the chicks in this treatment suffered continually from diarrhea, and that *Salmonella* spp. and *E. coli* are important contributors to this disorder. The diarrhea would cause weakness and general bad health of these chicks, and together with the *C. perfringens*, may have caused the death of the chicks in this treatment.

The production of antimicrobial compounds by lactobacilli active against pathogens such as *E. coli, Clostridium* and *Salmonella* spp. is well documented (Naaber *et al.*, 2004, Coconnier-Polter *et al.*, 2005, Bernbom *et al.*, 2006). The cumulative antimicrobial effect of the strains used in this probiotic mix showed strong activity against *S. typhimurium* and *E. coli*, but weak activity against *C. perfringens* indicator strains (see Chapter 2). This data was obtained testing each isolate separately. In combination these five strains may act differently against these pathogens *in vivo.* Treatment ET did successfully reduce the *C. perfringens* counts on TSC agar compared to CT, and perhaps this is due to production of antimicrobials by the probiotic mix.

3.4.3.5 Microbiological analysis of ostrich chick mucosal samples

To compare the culturable counts of the chosen bacterial groups found in the faecal samples and gut mucosal samples, gut scrapings from three different intestinal areas were cultured on selected

agar as above. **S**tatistical tests showed any significant changes in the certain areas of the gut for each bacterial group over time.

MRS agar detected LAB in all sections of the gut tested (Table 3.3). Some of these groups of bacteria are facultative anaerobes and are therefore expected to be present in all sections of the gut. Between the two slaughter times, significant increases were noted in the caecum of treatment ET ($p = 0.05$), in the colon of treatment C ($p = 0.04$) and in the duodenum of treatment CT ($p = 0.001$; Table 3.3). The probiotics fed in this study were shown to adhere well to all sections of the gut mucosa (Chapter 2) and would have been expected to cause an increase in LAB in all sections of the gut tested here.

Table 3.3. Culturable bacterial counts on selective solid growth media, (MRS, BHI, McConkeys and TSC) cultivated from ostrich gut mucosal samples during the feeding trial where ostriches were fed a control (C) diet supplemented with tylosin (CT) or the 5 strain probiotic mix (E) supplemented with tylosin (ET), in three different gut locations: duodenum (duo), colon (col) and caecum (caec). Statistical analysis between the two slaughter points (slaughter 1 (S1) at week 2; slaughter 2 (S2) at week 5) displayed any significant differences between the two time points ($p<0.05$).

Treatment/gut region	MRS			BHI			TSC			McConkeys		
	S1	S2	p value	S1	S2	p value	S1	S2	p value	S1	S2	p value
Eduo	5.92			4.51			4.04			0.00		
Ecol	5.61			4.73			3.72			5.23		
Ecaec	5.10			5.08			4.19			5.99		
ET duo	5.13	7.64	**0.33**	5.79	7.24	**0.01**	0.00	0.00		0.00	7.56	
ETcol	5.00	7.40	**0.16**	5.37	7.20	**0.001**	0.00	3.26		6.13	7.26	**0.03**
ETcaec	4.98	7.18	**0.05**	5.03	7.25	**0.02**	3.78	3.63	**0.43**	6.16	7.18	**0.05**
Cduo	7.13	7.42	**0.46**	7.54	7.14	**0.01**	0.00	0.00		6.40	7.36	**0.08**
Ccol	7.38	7.62	**0.04**	6.51	7.63	**0.001**	4.51	0.00		7.74	7.61	**0.19**
Ccaec	7.08	7.49	**0.28**	6.64	7.70	**0.02**	0.00	0.00		8.23	7.44	**0.03**
CTduo	6.55	7.22	**0.001**	6.27	6.51	**0.23**	0.00	0.00		6.19	6.55	**0.04**
CTcol	7.39	7.51	**0.41**	7.39	7.52	**0.15**	5.53	0.00		7.45	7.55	**0.62**
CTcaec	7.18	7.54	**0.30**	7.13	7.57	**0.02**	4.46	0.00		3.19	7.50	**0.001**

As expected, the total anaerobe counts show significant increases for most regions of the gut between the two slaughters (Table 3.3). In treatment ET and C, this was evident in all gut regions (duodenum $p = 0.01$; colon $p = 0.001$; caecum $p = 0.02$) in both cases, and in the caecum of treatment CT ($p = 0.02$). This decrease was expected in most animals as there are more anaerobes found in the distal gut than in the duodenum, where facultative anaerobes would be

more abundant (Mead and Adams, 1975). It may also be due to the velocity of digesta flow being higher in the duodenum than in the distal gut and the commensal anaerobes being "washed-out" (Tannock, 1995). The probiotics fed in this study were shown to adhere well to mucous from all sections of the gut mucosa (Chapter 2) suggesting a reason why treatment ET showed significant increases for anaerobes in all sections of the gut, compared to treatment C which showed a decrease in the duodenum and treatment CT which showed no significant change in the duodenum.

The culturable counts on McConkeys agar show significant increases for treatment ET in all sections of the gut tested, treatment C in the duodenum, and treatment CT in the duodenum and caecum (Table 3.3). Treatment C showed a significant decrease in the caecum as expected as the caecum would harbour a higher prevalence of strict anaerobes and the duodenum more facultative anaerobes (Mead and Adams, 1975). The increases of coliforms seen for treatments ET and CT in the colon and caecum is surprising. The digest flow rate in the caecum and colon is slower than in the duodenum, and it could therefore be possible that these increases are observed because the culturable facultative microbes found on McConkeys agar were being passed though the colon and caecum, not actually resident there. However, Mead and Adams (1975) reported facultative anaerobes to predominate in the caeca of chickens for the first two to three days post-hatching, and to be present throughout the life of the bird. Barnes *et al.* (1972) reported high level of enterococci, lactobacilli and coliforms in the caeca of chickens.

At slaughter one, TSC agar showed *C. perfringens* to be present in all regions of the gut in treatment E, but was only present in the colon in treatment C and CT, and in the caecum in treatment ET and CT (Table 3.3). Only treatment E showed the presence of this pathogen in the duodenum. This may be significant as by the second slaughter, chicks in treatment E had died and the pathogen was only detected in treatment ET, in the colon and caecum where it had increased to 10^3 cfu/g mucosa. This implies again that the probiotic mix encouraged the colonisation of the gut by this pathogen. It also shows tylosin's role in *C. perfringens* control, confirming a bacteriostatic rather than bactericidal mode of action on susceptible organisms.

Interestingly, there was a lack of growth on SS agar for the mucosal scrapings at both slaughter times (week two and week five), indicating that *Salmonella* spp. were not likely to be involved in the mortality seen in this trial.

In the chicken model, the alimentary canal is reported to be sterile on hatching, but is rapidly colonised by bacteria from the mother and the surrounding environment (Amit-Romach *et al.*, 2004). Previous studies have shown a high prevalence of anaerobic bacteria capable of decomposing uric acid in the caecal microbiota of broiler chicks within a few hours of hatching (Mead and Adams, 1975). During the first four days post-hatch, streptococci and enterobacteria colonise the small intestine and caecum. After the first week, lactobacilli predominate in the small intestine, and the caecum is colonized mainly by anaerobes such as *Bacteroides*, with lower numbers of facultative aerobes (Lev and Briggs, 1956; Mead and Adams, 1975). An adult chicken's caecal microbial community takes 30 days to mature, being dominated by obligate anaerobes such as bifidobacteria and bacteroides (Barnes *et al.*, 1972). The culture-dependent approach to investigate microbial diversity adopted in this chapter, did not allow for species-specific enumeration of the ostrich chick microbiota, but it can be seen that LAB, anaerobes and *E. coli* are present in substantial numbers throughout the gut of the ostrich chick by week five (slaughter time two; Table 3.3).

The comparison between culturable bacteria from faecal and gut mucosal scrapings from three different areas of the ostrich GIT

To highlight the differences observed in culturable bacteria between the faecal samples and gut scrapings of different areas of the ostrich GIT, the bacterial counts obtained on the various selective media at week two, four, five and seven were compared (Table 3.4). Week two and five are the time points for slaughter one and two, respectively. As there were no faecal counts available at week five, week four and seven faecal counts were used for the comparison.

At the first slaughter point (week two), the faecal counts for all bacterial groups tested were higher than those found in any region of the gut mucosal scrapings, except on McConkeys agar where the colonic and caecal scrapings from treatment C were higher than the counts in the

faecal samples (Table 3.4). By the second slaughter (week five), the coliform numbers on McConkeys showed a similar prevalence between faecal and mucosal samples.

Although the numbers of LAB were higher in the faecal samples at week two for all the groups, by the second slaughter (week five), total LAB counts for faecal and gut samples were similar, indicating that the microbiota had stabilised by this time. No *C. perfringens* were detected in treatment C and CT mucosal samples, although it was present in the faecal samples. Treatment ET showed similar counts for this pathogen between faecal and mucosal samples, but none were detected in the duodenum, as mentioned previously. The faecal samples showed a high abundance of *Salmonella* species at week two (approximately 10^6 cfu/g faecal sample; Fig. 3.7D), but no growth was observed on SS agar for any of the gut mucosal samples at either of the slaughter points. By week seven, only treatment CT still showed the presence of this pathogen in the faecal samples. This could suggest that *Salmonella* was not able to colonise the gut by adhering to the mucosa. Instead, it was passed out in the faeces, suggesting that it was inhibited or out-competed for binding sites in the gut by the probiotics and commensal bacteria.

Differences between the culturable counts in the faecal samples compared to the mucosal samples could be due to the adhesion properties of the bacterial groups tested, some adhering less well than others, and being easily washed off (Zhu *et al.,* 2002). Zhu *et al.* (2002) also report a consistent difference in microscopic examination of mucosal scrapings and caecal contents, the latter having higher bacterial counts. Zoetendal *et al.* (2002) have reported similar findings describing significant differences between bacterial communities recovered from human faeces as compared to mucosa-associated communities. Other studies have shown that there are no significant differences in the detected bacterial numbers in faeces and mucosal samples (Wallace, 2008). Another possible explanation for the difference is the difficulty associated with culturing of anaerobic bacteria. The exposure of the faecal samples to oxygen before culturing would assumedly have an effect on the viable and culturable anaerobes detected. Corthier *et al.* (1996) reported that faecal samples exposed to the air for one hour resulted in killing of the dominant anaerobic bacteria present.

Table 3.4. The comparison between ostrich faecal and gut mucosal samples culturable bacterial counts on selective solid growth media, (MRS, BHI, TSC, McConkeys and SS) during the feeding trial where ostriches were fed a control (C) diet supplemented with tylosin (CT) or the 5 strain probiotic mix (E) supplemented with tylosin (ET), in three different gut locations: duodenum (duo), colon (col) and caecum (caec). The faecal counts closest to the slaughter one (at week two) and slaughter two (at week five) time points were compared (week two, week four and week seven).

Treatment/region	MRS				BHI				TSC				McConkeys				SS			
	W2	W4	W5	W7	W2	W4	W5	W7	W2	W4	W5	W7	W2	W4	W5	W7	W2	W4	W5	W7
	S1		S2		S1		S2		S1		S2		S1		S2		S1		S2	
E faecal	9.30				9.19				7.03				8.08				6.43			
Eduo	5.92				4.51				4.04				0.00							
Ecol	5.61				4.73				3.72				5.23							
Ecaec	5.10				5.08				4.19				5.99							
ET faecal	9.04	7.19		7.74	8.73	8.42		9.08	5.85	3.82		4.76	7.49	6.91		7.73	6.22	6.07		
ET duo	5.13		7.64		5.79		7.24		0.00		0.00		0.00		7.56					
ETcol	5.00		7.40		5.37		7.20		0.00		3.26		6.13		7.26					
ETcaec	4.98		7.18		5.03		7.25		3.78		3.63		6.16		7.18					
C faecal	7.64	6.40		8.03	8.20	7.17		7.97	5.61	3.08		4.67	6.69	6.36		6.36	6.13	5.28		
Cduo	7.13		7.42		7.54		7.14		0.00		0.00		6.40		7.36					
Ccol	7.38		7.62		6.51		7.63		4.51		0.00		7.74		7.61					
Ccaec	7.08		7.49		6.64		7.70		0.00		0.00		8.23		7.44					
CT faecal	8.56	6.60		7.71	8.33	7.85		7.78	5.93	5.44		5.00	7.78	6.88		7.06	6.41	6.02		4.26
CTduo	6.55		7.22		6.27		6.51		0.00		0.00		6.19		6.55					
CTcol	7.39		7.51		7.39		7.52		5.53		0.00		7.45		7.55					
CTcaec	7.18		7.54		7.13		7.57		4.46		0.00		3.19		7.50					

3.5 Conclusions

Delivery of the mixture of probiotic strains to the ostrich chicks by encapsulation in calcium alginate beads was shown to be quickest, easiest and most effective means of maintaining cell viability during processing and storage. Furthermore, the ease of administering the beads to the chicks offered another distinct advantage over the other methods investigated in this study.

Probiotic supplementation had no significant effects on weight gain and/or feed conversion ratios, and the previously observed growth promoting properties of tylosin were not observed in this study due to the lack of a control comparison. In addition, the mortality rates for ostriches fed only the probiotic-supplemented diet were highest, in comparison to those receiving the control diets, which suggest that a component of the probiotic mix or the five strain combination may have encouraged pathogenesis in the ostriches. These birds were the only group to have *C. perfringens* in the duodenal mucosal scrapings. Adhesion studies on this mixture of putative probiotic strains showed that it was able to inhibit and displace the pathogens, *C. perfringens*, *C. difficile*, *E. coli* and *S. typhimurium*, but was not able to out compete them for adhesion sites in the mucosal membrane in the gut. This suggests that one of the above-mentioned pathogens was present in the feed or ostrich pens from the start of the trial. As the pens were open to the environment, it was impossible to control the presence of pathogens in the pens. Although the pens were regularly washed, it was impossible to prevent the introduction of a pathogen from the outside via rodents, or the spread of a pathogen already present in some of the faeces throughout the whole pen. In addition, the pens receiving probiotics were adjacent to those not receiving probiotics, all in the same shed, and therefore the possibility of the spread of probiotics by aerosols or workers cannot be eliminated. A feeding trial using different combinations of the probiotic strains or the use of single strains would confirm whether this five strain combination was encouraging pathogenesis in the ostrich gut.

The microbiota of the ostrich chick's faecal and gut samples were analysed using a culture-dependent approach in this chapter. At week two the faecal samples showed treatments E and ET to have significantly higher LAB counts, as expected as they were receiving probiotic supplementation MRS allows for the growth of potential pathogens such as *Staphylococcus* and one of the manufacturers of MRS have also described moderate growth of *E. coli* on MRS

(www.condolabs.com). Week four showed treatment C to harbour significantly fewer anaerobes than ET and CT. After this time point, treatment C died, suggesting this reduction in anaerobes was linked to a pathogen present. It is interesting to note that the pathogen, *C. perfringens* decreases significantly for treatments CT and ET over time, indicating that the bacteriostatic effect of tylosin was sufficient to prevent mortality in these groups. The post-mortems showed *C. perfringens* lesions to be prevalent and therefore this pathogen could have been responsible for the death of these chicks. However, there was a clear effect on the *C. perfringens* population on the different areas of the gut, being found by slaughter two in the anaerobic environments of the caecum and the colon, only in treatment ET. The bacteriostatic effect of tylosin on this pathogen was clearly seen in the caecum of chicks in treatment ET where the levels of the pathogen did not change. Faecal samples showed consistently higher counts for *C. perfringens* than the mucosal counts, indicating that this pathogen is being constantly passed through the gut of the chicks and that it appears to be resistant to tylosin.

The method of bacteriological culture is biased, since many selective culture media are not absolutely selective. Furthermore, these media do not equally support the growth of the different species comprising a population, and not all bacteria are presently cultiviable (Penders *et al.*, 2006). Non-culturable organisms include those for which the specific growth requirements are not available; slow-growing organisms out-competed in the presence of fast-growing microorganisms and injured organisms, which cannot withstand the stressful conditions imposed by laboratory-based cultivation (Vaz-Moreira *et al.*, 2011). Although culture-dependent techniques have been commonly used to study the gut microbiota of various animals (Pieper *et al.*, 2006; Mountzouris *et al.*, 2007; Rehman *et al.*, 2007; Vaz-Moreira *et al.*, 2011), ideally both culture-dependent and culture-independent methods should be used together for comparable analyses. In the following chapters, culture-independent, molecular based techniques, such as DGGE (Chapter 4) and FISH (Chapter 5), are used to supplement the data obtained in this chapter.

CHAPTER 4

Molecular analysis of the microbial diversity of the ostrich gastrointestinal tract using denaturing gradient gel electrophoresis

Contents

4.1. **Summary** 112

4.2. **Introduction** 112

4.2.1. Aims and objectives of this study 114

4.3. **Materials and Methods** 114

4.3.1. Faecal sample collection and DNA extraction 114

4.3.2. DGGE analysis of bacterial diversity 115

4.3.3. Excision of DNA fragments and sequence analysis 117

4.3.4. Cluster analysis 117

4.3.5. Shannon-Wiener diversity index 118

4.4. **Results and Discussion** 118

4.4.1. DNA extraction and PCR amplification 118

4.4.2. Choice of PCR primer pairs and the generation of reference ladders 118

4.4.3. DGGE analysis of PCR products generated from ostrich chick faecal samples . 120

4.4.4. DGGE analysis of total bacteria, *Bifidobacterium* and *Lactobacillus* diversity in ostrich chicks 121

4.4.5. Cluster analysis 122

4.4.6. Band richness 131

4.4.7. Statistical analysis of microbial diversity using the Shannon-Wiener index (H') 132

4.4.8. Sequence analysis 136

4.5. **Conclusion** 148

4.1. Summary

The ostrich farming industry is plagued by high chick mortality rates up to three months of age. A healthy gut microbiota is thought to play an important role in the competitive exclusion (CE) of pathogens, and an in-depth understanding of the microbial diversity in the intestines of newly-hatched chicks, as well as the effect of in-feed probiotics, may lead to the development of possible CE candidates to reduce pathogen invasion of the gastrointestinal tract. Since culture-dependent microbial techniques have their drawbacks, DGGE based molecular analysis was undertaken of the total bacteria and *Bifidobacterium* and *Lactobacillus* populations in faecal samples from ostrich chicks under three months old. Newly-hatched chicks were fed a control diet (C) or a diet supplemented with the antibiotic tylosin (CT), probiotics (E) or probiotics and tylosin (ET). The microbial community diversities present in faecal samples from the ostrich chicks in each group were analysed by calculating the Shannon-Wiener diversity index and major bands were excised from the DGGEs and their nucleotide sequence determined. Feeding of probiotics alone had no effect on the total bacteria or *Lactobacillus* spp. populations, but clustering of probiotic treated chicks was seen with regard to the *Bifidobacterium* spp. populations. When probiotics were fed together with tylosin, no changes were seen in the diversity of the total bacterial population or the *Bifidobacterium* spp., however, a significant decrease in diversity of the *Lactobacillus* spp. population was observed. The early death of treatment E may have been due to the strain and dose combination of the probiotics used or the immature status of newly-hatched chicks' GIT. No bands correlated to the *C. perfringens* included in the reference ladder of the DGGE representing total bacteria althoughthe death of both group E and the control group was thought to be due to *C. perfringens* due to the lesions found in the gut during the post-mortem. Tylosin was able to maintain the chicks in a healthy state up until the end of the feeding trial at nine weeks.

4.2. Introduction

Although culture based techniques, such as those used in the previous chapter, provide the first insight into the complexity of the animal GIT, these methods only allow for detection of culturable bacteria, they are time consuming and the results are dependent on the selectivity of the culture medium used (Collado and Sanz, 2007b). Molecular techniques based on

nucleic acid sequence similarities, such as denaturing gradient gel electrophoresis (DGGE) and fluorescent *in-situ* hybridisation (FISH), overcome some of these limitations and are useful in the detection of uncultivated bacteria (Harmsen *et al.*, 2000). Even the richest culture medium is not able to support the growth of the majority of microbes isolated from the GIT of humans and animals due to exclusive growth requirements, resulting in a gross underestimation of microbial diversity and numbers (Apajalahti and Kettunen, 2006).

DGGE is a method which has been widely used in mutation analysis, and has also been successfully used to provide a rapid survey of microbial communities. It is based on the separation of PCR products according to the melting behaviour of the different amplicons (Nikolausz *et al.*, 2005). Fischer and Lerman (1980) suggested that the separation of these DNA fragments was not dependent on the length of the fragment, but that the DNA molecule partially dissociates due to the increasing concentration of denaturants (urea and formamide) at high temperatures (ie the melting behaviour is sequence dependent). This partially-melted DNA fragment is now retarded in its movements through the polyacrylamide matrix. They also suggested that a double stranded DNA molecule migrates through the polyacrylamide gel until its least stable region is melted, indicating that base-pair changes in the melting domain significantly alter the overall mobility of the molecule. In addition, a 40 base pair GC-rich "clamp" is included into one of the two primer sequences, which is then incorporated into each amplicon generated during the PCR. This clamp avoids single-stranded DNA strands migrating out of the gel by preventing total denaturation of the DNA molecule and allows optimal resolution of fragments in the denaturing gradient (Myers *et al.*, 1985; Muyzer *et al.*, 1993).

In PCR-DGGE, primers are used to amplify target DNA fragments (usually the 16S rRNA genes) from a mixed template (faecal matter in this case) resulting in amplicons of the same size, but containing different sequences. The benefits of using the rRNA genes include that they are present in the genome of all organisms and have a highly conserved structure, comprised of both conserved and variable sequence regions. The conserved regions provide a base for primer design and annealing, and the variable regions provide a possibility for molecular separation using DGGE. In this study, universal, *Lactobacillus*- and *Bifidobacterium*-specific primers were used. The use of these specific primers gives an indication of the *Bifidobacterium* and *Lactobacillus* species present in the ostrich gut. These lactic acid bacteria are thought to be important indicators of gut health and are thought to beneficially affect the host (Holzapfel and Schillinger, 2002). Universal primers targeting the

variable V3 region of the 16S rRNA genes of all eubacteria, Univ-341-f and Univ-515-r, were used to get an overall view of all the different species of bacteria present in the ostrich GIT. This is extremely useful in getting a profile of bacteria present, including new and unusual species. Muyzer *et al.*, (1993) estimated that any target DNA present at 1% or more of the total bacterial pool can be detected. To improve the specificity and yield, one can use genus- and group-specific primers to isolate and characterise a certain group present. To detect the lactobacilli present in the faecal samples, the *Lactobacillus* species-specific primer, Lab-0677-r, also targeting the V3 region of the 16S rRNA gene was used in combination with Univ-341-f. The *Bifidobacterium* were detected using *Bifidobacterium* species-specific primers, Bif-164-f and Bif-662-r. These primers target the V2 and V4 variable regions, respectively (Langendijk *et al.*, 1995) and have been shown to be specific for the genus *Bifidobacterium* (Satokari *et al.*, 2001). These PCR products are then separated in a sequence dependent manner by DGGE on a chemical denaturing gel (usually formamide and urea) resulting in a fingerprint, representative of the various species present in the sample. Muyzer *et al.* (1993) were first to apply PCR-DGGE to the field of microbial ecology, pioneering the use of rRNA genes for characterisation of a microbial community.

4.2.1. Aims and objectives of this study

The aims of this chapter are to evaluate the diversity of the microbiota in the faeces of ostrich chicks, examined in chapter three, using universal primers, *Bifidobacterium* and *Lactobacillus* group-specific primers and evaluating the effect of the administration of probiotics and/or the antibiotic tylosin on these communities and their diversity. In addition, the detection of the probiotic in the faeces of the test group would indicate successful passage though the GIT, surviving the harsh conditions and maintaining the integrity of their nucleic acid so as to be detected by DGGE.

4.3. Materials and Methods

4.3.1. Faecal sample collection and DNA extraction

Fresh faecal samples were collected in sterile containers and kept on ice while on route to the laboratory where they were immediately transferred to a freezer at -20 ºC. Total genomic DNA was extracted from frozen faecal samples using the Zymo Genomic Extraction Kit

(Inqaba Biotec) according to the manufacturers' protocol with the exception that 5 samples per pen were combined to get a better description of the diversity (Zhou *et al.*, 2007) and faecal samples were bead beaten for 140 sec (as described in Chapter 2) prior to extracting the total DNA. For the reference ladder, DNA was extracted from the probiotic strains and type strains, as described above. The PCR products obtained from the DNA extracted from the faecal samples of birds from each pen were run separately for statistical analysis of the diversity indices. For comparison between treatments, the pooled samples from each of three pens were combined to give one lane representing a treatment group (see feeding trial layout in chapter 3).

4.3.2. DGGE analysis of bacterial diversity

The DNA was subjected to two rounds of PCR targeting the 16S rRNA gene using the following primer sets (Table 4.1; 4.2):

- Total bacteria: Univ-341-f and Univ-515-r, followed by Univ-341-f-GC and Univ-515-r
- *Lactobacillus* specific: Univ-341-f and Lab-0677-r, followed by Univ-341GC-f and Univ 515-r
- *Bifidobacterium* specific: Bif 164-f and Bif 662-r, followed by Univ-341-f-GC and Univ 515-r

The PCR reaction mix (50 µl) contained: 25 µl of 2 x Fermentas master mix, 12.5 pmol of each primer, bovine serum albumen (0.6 µg/µl final concentration), 200 ng of genomic DNA (first round) or 1 µl first round PCR product (second round) and nuclease free sterile water (Fermentas).

Table 4.1. Primers used in the nested PCR reactions for analysis with DGGE

Primer name	Sequence (5' – 3')	Reference
Univ-341-f	cct acg gga ggc agc ag	Muyzer *et al.*, 1993
Univ-341-f-GC	cgc ccg ccg cgc gcg gcg ggc ggg gcg ggggcacgg ggg g-tcc tac ggg agg cag cag	Muyzer *et al.*, 1993
Univ-515-r	atc gta tta ccg cgg ctg ctg gca	Lane, 1991
Lab-0677-r	cac cgc tac aca tgg ag	Heilig *et al.*, 2002
Bif-164-f	ggg tgg taa tgc cgg atg	Langendijk *et al.*, 1995
Bif-662-r	ccc acc gtt aca ccg gga	Langendijk *et al.*, 1995

Table 4.2. The expected product size and annealing temperatures of the primer combinations used

Primer combination						Expected product size (bp)
		30 cycles				
Univ-341-f and Univ-515-r	96°C 4 min	96°C 40 sec	55°C 30 sec	72°C 45 min	72°C 7 min	174
		30 cycles				
Univ-341-f and Lab-0677	96°C 4 min	96°C 40 sec	55°C 30 sec	72°C 45 sec	72°C 7 min	352
		35 cycles				
Bif-164-f and Bif-662-r	96°C 5 min	96°C 30 sec	63°C 20 sec	68°C 40 sec	62°C 20 sec 68°C 7 min	510
		30 cycles				
Univ-341-f-GC and Univ-515-r	96°C 4 min	96°C 40 sec	55°C 30 sec	72°C 45 sec	72°C 7 min	311

The product of the second PCR was analysed by DGGE using the DCode Universal Mutation Detection system (BioRad, California), and separated on polyacrylamide gels containing 8% (vol/vol) polyacrylamide (37.5:1 acrylamide-bisacrylamide) and 50 X TAE buffer (2M Tris Base, 1M Acetic acid, 50mM EDTA, pH8, and water). A denaturing gradient was formed with deionised formamide (Sigma) and urea (Sigma-Aldrich). A 100% denaturant was defined as 7M urea and 40% v/v deionized formamide. Gels were poured into a 160 x 160 x 1 mm parallel gel sandwich using a manual gradient former (Bio-Rad, CA). A denaturing gradient of 45 – 65% was used for the separation of total bacteria using the universal primers, 45 – 55 % for *Lactobacillus* spp. using the Lac primers and a gradient of 55 – 65% was used for the separation of *Bifidobacterium* spp. For analysis, 10 µl of each PCR product was loaded and electrophoresis was carried out for 18 h in 1 X TAE buffer. A pre-run voltage of 180V was maintained for 10 min and hereafter the voltage was kept constant at 62V as was the temperature, at 60ºC. Gels were stained in ethidium bromide as per manufacturer's instructions (Bio-Rad) and viewed and photographed the using Gel Doc system (BioRad, CA). This procedure was carried out at least twice to ensure reproducible results.

4.3.3. Excision of DNA fragments and sequence analysis

Dominant DNA fragments were excised as described by Omar and Ampe (2000) with some modifications. Briefly, after taking the photograph, the gels were restained with ethidium bromide for 30 min and destained for 5 min in 25 ml of 1x TAE. DNA bands were cut out aseptically using surgical blades, added to sterile nuclease free water, and left to elute at 4°C for 18h.

DNA obtained from excised DGGE fragments was amplified with primers Univ-341-f-GC and Univ-515-r as described previously. The PCR products were subjected to DGGE again as described above, to ascertain the position and purity of the bands. Thereafter, the DNA from the excised band was reamplified using Uni-431-f and Univ-515-r and purified using the Biospin PCR purification kit (Bioflux) and the concentrations of purified PCR products were determined spectrophotometrically using a Nanodrop instrument (Nanodrop Technologies). The pure PCR products were ligated into the pTZ57R/T vector (Fermentas) and transformed into *E. coli* DH5α. Colony PCR was used to confirm the presence of the correct construct and one to three of these transformed *E. coli* cells were sequenced using the M13-f primer.

Plasmid extraction and determination of the nucleotide sequence data was done by Macrogen Inc., Seoul, Korea. Further sequence analysis and editing was carried out using the DNAman software programme (Lynnon Biosoft, version 4.13). The closest match of the partial 16S rRNA gene was determined by searching the GenBank and NCBI databases using the BLAST algorithm (Altschul *et al*., 1997). A similarity of 97% or more to the 16S rRNA gene sequences of type strains was used as a basis for identification.

4.3.4. Cluster analysis

The relationship of the DGGE banding patterns of the two population groups was determined by a clustering algorithm using Quantity-One (Biorad, CA). This programme assesses distances migrated by each band through the gel and compares lanes using a pairwise comparison to calculate a Dice similarity coefficient between each pair based on the number of bands common to both lanes. Dendrograms were constructed using an unweighted pair group method with arithmetic averages (UPGMA).

The images were analysed for the number of bands per samples (absence versus presence) and this served as a measure of apparent bacterial richness.

4.3.5. Shannon-Wiener diversity index

The microbial diversity of the faecal samples was determined by calculating the Shannon-Wiener diversity index, H' (Ledder *et al*., 2007). This was calculated using the following formula:

$$H' = - \sum (P_{i)} (\log_e P_i)$$

where P_i is the importance probability of the bands in the gel lane. The importance probability P_i was calculated as

$$P_i = n_i / N$$

where n_i is the height of a band peak and N is the sum of all band peaks in the densitometric curve. Since the data was not normally distributed, nonparametric analysis was done manually using a Mann-Whitney U test (when comparing two cases) or the Kruskal-Wallis one way analysis test (when comparing more than three cases). $p \leq 0.05$ was interpreted as being statistically significant.

4.4. Results and Discussion

4.4.1. DNA extraction and PCR amplification

The Zymo Genomic Extraction Kit (Inqaba Biotec) together with bead-beating resulted in high DNA yields, ranging from 200- 500ng/µl from 200mg starting material. Inhibitors from the faecal material as well as the DNA extraction kit present in the PCR are thought to interfere with the amplification process. To avoid this, BSA was added to the PCR reaction mix as it has previously shown to improve the PCR product yield and specificity. BSA stabilises the Taq DNA polymerase as well as interacts with any inhibitory compounds (Nagai *et al.,* 1998). The quality of the DGGE gels of ostrich faecal matter was found to not be as good as those obtained from human subjects (Reid, personal communication), perhaps reflecting the higher levels of PCR inhibitors due to the plant material in the ostrich feed.

4.4.2. Choice of PCR primer pairs and the generation of reference ladders

Total bacteria in the faecal samples were detected using the Univ-341-f and Univ-515-r primer pair, and a denaturing gradient of 45-65% was found to be optimal for these primers

in this study. The type strains used (*L. plantarum* ATCC 700211^T; *L. reuteri* ATCC 23272^T; and *B. pseudolongum* subsp. *pseudolongum* ATCC 25526^T) were found to migrate to the same position as the respective probiotic strains (Fig. 4.1A) and therefore, only the probiotic strains were included in the reference ladder here. In addition, *Clostridium perfringens* ATCC 13124^T was added (Fig 4.1A) as it was considered to be one of the main pathogens involved in ostrich chick mortality in this study and including it in the reference ladder would allow for rapid visual confirmation of its presence in the faecal samples. Three *L. plantarum* strains were used in the probiotic mix (Lp6039.3, Lp1.1.2.2 and Lp162.2), and, as expected, migrated to the same position on the denaturing gel. The other two probiotic strains, Lr136.2.2 (*L. reuteri*) and Bp303.3.2 (*B. pseudolongum* subsp. *pseudolongum*), as well as *C. perfringens*, showed good separation and were used in combination as the reference ladder (Fig 4.1A).

The primer combination Univ-341-f and Lab-0677-r detected all *Lactobacillus* reference strains used here (Fig. 4.1B). Previous work by Magwira (2008) showed that using a denaturing gradient of 45-55% resulted in the best separation of the highest number of *Lactobacillus* species using these primers.

The reference ladder used with these primers included the three *L. plantarum* strains and the *L. reuteri* included in the probiotic mix, as well as *L. brevis* ATCC 14869^T; *L. gasseri* ATCC 33323^T; and *L. pentosus* ATCC 8041^T. The latter two strains were found to migrate to the same position as *L. plantarum* but *L. gasseri* migrated to a specific position, as did *L. reuteri.*

The reference ladder used with the *Bifidobacterium*-specific primers used here, Bif-164-f and Bif-662-r included *B. adolescentis* ATCC 15703^T, *B. animalis* subsp. *lactis* DSM 10140^T, *B. longum* NCIMB 702259^T, *B. pseudolongum* subsp. *pseudolongum* ATCC 25526^T, (Fig. 4.1C). Kullin (2010) found that a denaturing gradient of 55-65% was best for separation of species using these primers. All of these type strains co-migrated with the exception of *B. longum*, which migrated to a specific position above the rest. Previous authors have shown this set of primers to be specific to a broad range of species from the genus *Bifidobacterium* (Langendijk *et al.*, 1995; Kok *et al.*, 1996; Satokari *et al.*, 2001). However, the strains used to test the specificity of these primers have only been of human origin. The specificity has been shown to be the case here too with all sequences being returned as belonging to this genus as well as the primers selectively amplifying *Bifidobacterium* only.

4.4.3. DGGE analysis of PCR products generated from ostrich chick faecal samples

The DGGE gels were run with two intentions. The first was to analyse the banding similarities using the programme Quantity-One (Bio-Rad, CA) to show the clustering of the different treatments. In this case, only two treatments per gel could be compared in a single gel. For this analysis, the faecal samples from the three different pens were pooled (ie 15 faecal samples combined per lane). The second aim was to compare the microbial diversity in treatment groups statistically, by keeping the three pens per treatment separate so as to get three Shannon-Wiener diversity (H') values per group and be able to verify any significant differences observed between them. For this, five faecal samples from each pen were pooled to minimise the degree of variation between the samples (Zhou *et al.*, 2007). This was a preliminary study, focusing on the major bacterial groups and any differences associated with them over the course of the feeding trial.

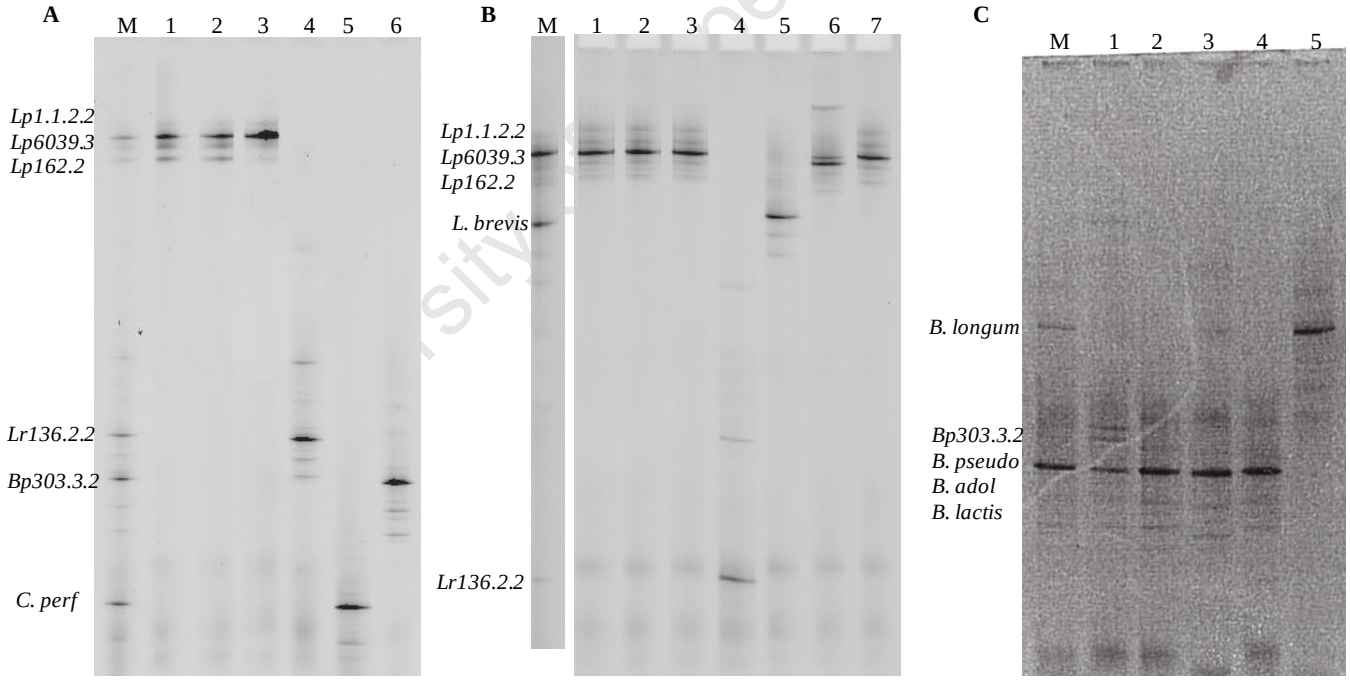

Figure 4.1. DGGE analysis of 16S rRNA gene PCR products from genomic DNA of probiotic and type strains, 1 X TAE, 62V for 18 hours and a constant temperature of 60ºC. A. Universal primers, a gradient of 45-65%, lanes: M, reference ladder of the five probiotic strains and *C. perfringens*; 1, Lp1.1.2.2; 2, Lp6039.3; 3, Lp162.2; 4, Lr136.2.2; 5, Bp303.3.2; 6, *C. perfringens* ATCC 13124. B. *Lactobacillus*-specific primers, a gradient of 45-55%, lanes: M, reference ladder of the four *Lactobacillus* probiotic strains and *L. brevis* ATCC14869^{T}; 1, Lp1.1.2.2; 2, Lp6039.3; 3, Lp162.2; 4, Lr136.2.2; 5, *L. brevis* ATCC 14869^{T}; 6, *L. gasseri* ATCC 33323^{T}; 7, *L. pentosus* ATCC 8041^{T}. C. *Bifidobacterium*-specific primers, a gradient of 55-65%, Lanes: M, reference ladder of the five *Bifidobacterium* strains; 1, Bp303.3.2; 2, *B. pseudolongum* subsp. p*seudolongum* ATCC 25526^{T}; 3, *B. adolescentis* ATCC 15703^{T}; 4, *B. lactis* DSM 10140^{T}; 5, *B. longum* NCIMB 702259^{T}. DGGE analysis of PCR products generated from ostrich chick faecal samples

4.4.4. DGGE analysis of total bacteria, *Bifidobacterium* and *Lactobacillus* diversity in ostrich chicks

In order to compare microbial diversity between the treatments, faecal samples from the three replicate pens were combined, resulting in one lane per treatment per time point being analysed per gel. As expected, variation in band frequency as well as intensity was observed both between treatments and between time points of a single treatment.

The primers used in this study showed good sensitivity and specificity, detecting all the respective strains used (Fig. 4.1). Although the reference ladder could be compared to the unknown bands in the faecal samples in a visual examination of DGGE profiles (Tannock, 2002), co-migration of different species to the same banding position in the gel is common (Green *et al.,* 2010). It is therefore more useful and reliable to excise the bands of interest and sequence them directly to accurately profile a microbial community. Moreover, the great bacterial richness observed using the universal primers, makes this method of visual analysis difficult. In addition to the dominant bands seen, some less dominant bands were also present. These may represent bacterial species present in fewer numbers or may highlight one of the problems associated with DGGE: PCR bias where some species are preferentially amplified over others (Ishii and Fukui, 2001). There were also some indistinct bands observed, which can lead to an overestimation of diversity. These could be due to the high diversity of bacteria in the faecal samples which are present in varying proportions, artifactual bands due to formation of secondary product during the PCR reaction (Janse *et al.,* 2004), sequence heterogeneities of rRNA genes (Nübel *et al.,* 1996) or heteroduplexes which generally remain high up in the gel due to their lower melting temperatures (Satokari *et al.,* 2001).

In our studies, examples of co-migration of different species are given below. In the *Lactobacillus*-specific DGGE of faecal samples, *Weissella confuse* (band six), *Weissella paramesenteroides* (band nine) and *L. plantarum* (band 14), migrated to the same positions as reference strains *L. plantarum, L. brevis* and *L. reuteri,* respectively (Fig. 4.3A and C). Interestingly, the *Bifidobacterium*-specific DGGE of faecal samples did show some correspondence. *B. adolescentis* (band one) and *B. pseudolongum* subsp. *pseudolongum* (band 14) aligned to the cluster in the reference ladder containing *B. pseudolongum* subsp. *pseudolongum, B. adolescentis* and *B. animalis* subsp. *lactis* (Fig. 4.4A and D). However, bands which would be expected to migrate to certain positions based on their sequencing

identity, do not. For example, although bands nine and 14 were both identified as *B. pseudolongum* subsp. *pseudolongum*, and form a prominent band throughout treatments ET and CT, they do not migrate to the position corresponding to the type strain in the reference ladder.

DGGE of faecal samples from ostrich chicks from all treatments showed a consistent microbial diversity throughout, with major bands occurring in all or most of the samples run on a gel. Nevertheless, unique minor banding patterns were observed between treatments and time points. The universal and *Lactobacillus* populations appear to be quite diverse when comparing the number of bands; however, the *Bifidobacterium* group seems less diverse with fewer than four species in most cases.

4.4.5. Cluster analysis

To examine if there was any relationship between the observed banding patterns and the treatments given to the chicks, the faecal samples were analysed and displayed as dendrograms created with Quantity-One (Fig. 4.2, 4.3 and 4.4). For this, the DGGE gels were run with one lane per treatment (15 faecal samples combined).

4.4.5.1. Universal primers for total bacteria

The effect of feeding the five strain probiotic mix on the ostrich microbiota was evaluated by comparing group E (receiving the probiotics) to the control group C (Fig. 4.2A). The initial time points of both treatments cluster together, as expected, as do the week one and two time points of treatment E. This indicates that the probiotics resulted in a distinct difference in total bacteria in the first two weeks compared to the control group. Interestingly, treatment C showed more intense bands than E, for example band 23 (*Anaerostipes butyraticus*), band 24 (*Turicibacter sanguinis*) and band 26 (*Akkermansia muciniphila*, section 3.4.8.1). *T. sanguinis* is a possible pathogen and it is clearly less present in treatment E than C. Due to the early death of chicks in these treatments, it was not possible to analyse these two groups further.

The effect of tylosin on the microbial diversity of group fed probiotics was also tested by comparing the probiotic group E to the group fed probiotics and tylosin, ET (Fig. 4.2B). Again the initial time points for these two treatments cluster together as expected and

grouping according to treatment is seen hereafter. This suggests that the probiotic mix alters the microbial community and that tylosin causes a further alteration. The probiotic strains were found to tolerate the level of tylosin administered to the chicks for its growth promoting properties (Chapter 3). Band 11, excised from a lane representing treatment E, was identified as the pathogen *Klebsiella pneumoniae* and does not appear to be present in treatment ET, perhaps due to its sensitivity to tylosin. An indication of the probiotics effect on *E. coli* is visible, with band nine (identified as *E. coli* KO11, 100%) present in E day zero and ET day zero fading out over the next two time points for both treatments. As an intrinsic resistance of *E. coli* to tylosin has been reported (Suchodolski *et al.*, 2009), it can be assumed this disappearance of this pathogen is due to the probiotic mix. Indeed, the adhesion assays in chapter two showed the probiotic mix to be capable of inhibiting and displacing *E. coli.* Tanikawa *et al.* (2010) reported an interesting difference between the times occupied in the caeca of two *E. coli* strains, highlighting the transition state of the microbiota. No bands corresponding to the *C. perfringens* included in the reference ladder were observed for any treatments (Fig. 4.2).

Tylosin's effect on the diversity of the control group was tested (Fig. 4.2C). The initial time points cluster together, and hereafter no clustering according to treatment is seen. Week one clusters together for both treatments, as do week two, suggesting tylosin does not affect the autochthonous microbiota. Interestingly, most bands are more intense in treatment C than CT, perhaps indicating an effect of tylosin in reducing the quantity of certain bacteria in the gut. This is interesting as Collier *et al.*, (2003a) showed a significant decrease in total bacteria in the ileum of pigs treated with tylosin after two weeks, but that by week four, the tylosin-treated total bacteria counts did not differ from the controls. They suggested this was due to the replacement of tylosin-susceptible strains with tylosin-resistant strains. Perhaps this would have been the case in this study too. Knothe (1977) showed that extended subtherapeautic use of tylosin resulted in tylosin-resistance and cross-resistance to other macrolides, lincosamidines and streptogramines. Bands two and three (*Clostridium clostridioforme* and *Lachnospiraceae*, respectively) are present in both treatments and increase gradually over time, the later time points showing them both to be more prevalent than at day zero.

When comparing the effect of probiotics on groups treated with tylosin over a longer time period (ET vs CT; Fig. 4.2D), no clustering according to treatment is seen. However, grouping according to time is observed, with the early colonisers clustering together and the

later colonisers (week four to week nine) clustering together. Both treatments show band 18 and 21 (both identified as *Akkermansia muciniphila*) present initially abundantly, but fading out after week two, suggesting sensitivity to tylosin. In contrast, bands 19 and 22 (*Roseburia intestinalis* and *Bacteroides dorei*, respectively) seem to be established in both treatments after two weeks, and may represent the development of the normal microbiota. The lack of clustering according to treatment indicates that the probiotics do not have an effect on groups treated with tylosin and that both treatments develop similar microbiota with time. Collier *et al.* (2003a) also found that the use of tylosin resulted in similar pig ileal microbiota between individuals throughout tylosin treatment and by day 21, tylosin treated pigs were indistinguishable from the control pigs in terms of microbial homogeneity.

Klarin *et al.* (2008) showed that feeding *L. plantarum* 299v together with an antibiotic, to patients with antibiotic-associated diarrhoea and *Clostridium difficile*-associated disease, decreased colonisation by *C. difficile* compared to the control group only receiving the antibiotic. The combination also increased the proportion of lactobacilli compared to the control group which showed a decrease for this taxon. Other enteric bacteria increased similarly in both groups. They also found that the control group showed a more varied diversity than in the treatment group, but that this variety included potential pathogens. In this current study, the diversity of eubacteria in ostrich chicks seemed to mature at the same rate in both treatments, indicated by the same common bands. The relatively low number of bands could indicate that a number of species are falling below the 1% threshold (10^{12} bacteria/g). An alternative possibility for the low number of bands is that the diversity of total bacteria in faecal samples is so high; the primers may have been too dilute to detect them all.

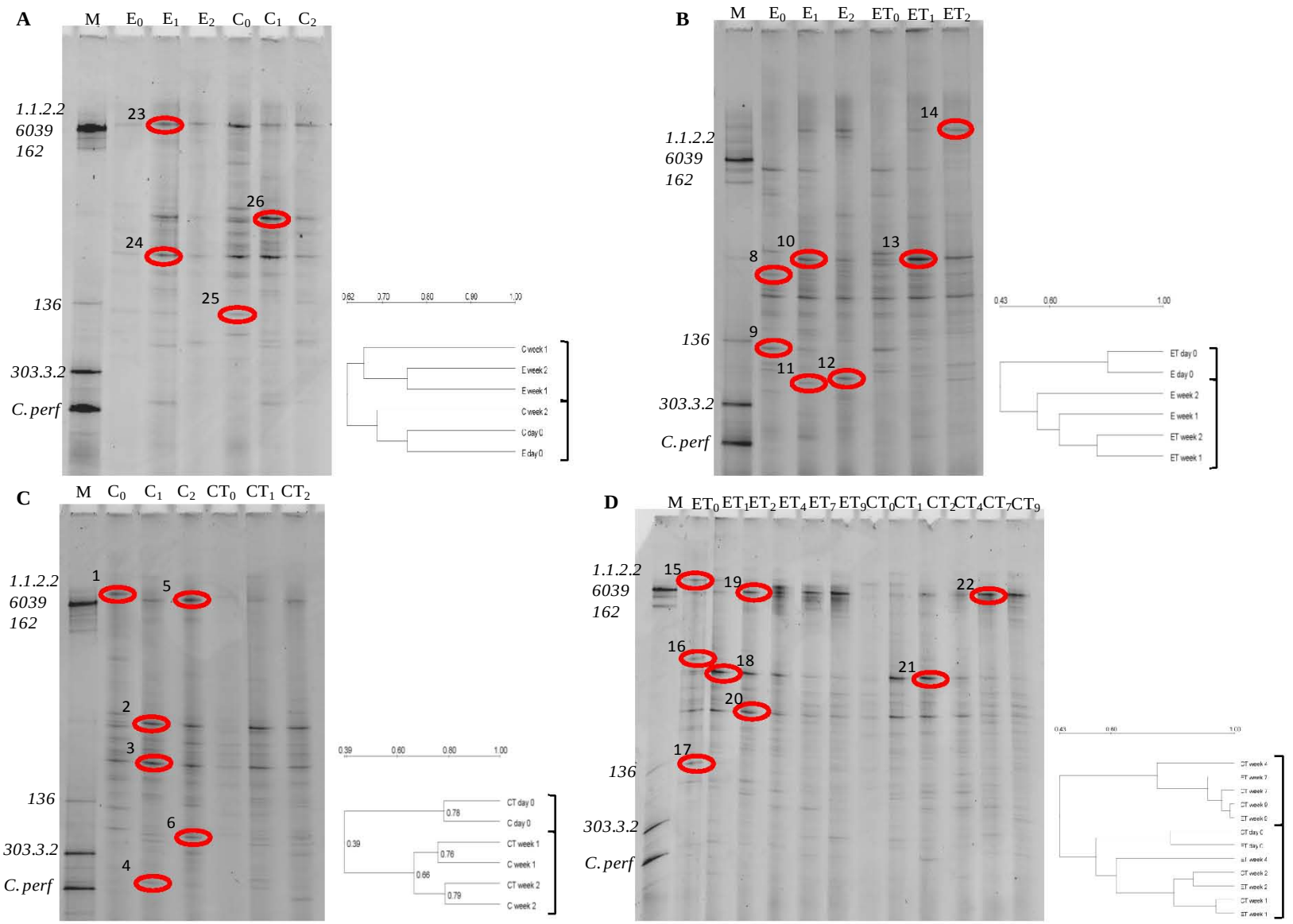

Figure 4.2 (A, B, C and D). DGGE and cluster analysis of total bacteria using universal primers Univ-341-f and Univ-515-r. A. The effect of probiotics on the normal microbiota. B. The effect of tylosin on the micrbiota of chicks receiving probiotics. C. The effect of tylosin on the normal microbiota of chicks. D. The effect of probiotics on chicks receiving tylosin. Universal 16S r RNA gene primers were used to amplify total bacteria isolated from faecal samples provided by groups given probiotics and tylosin (ET), only tylosin (CT), only probiotics (E) and the control group (C). PCR products were separated on 45 – 65% DGGE. Lane M is the mixed strain reference ladder. Excised bands are circled and numbered.

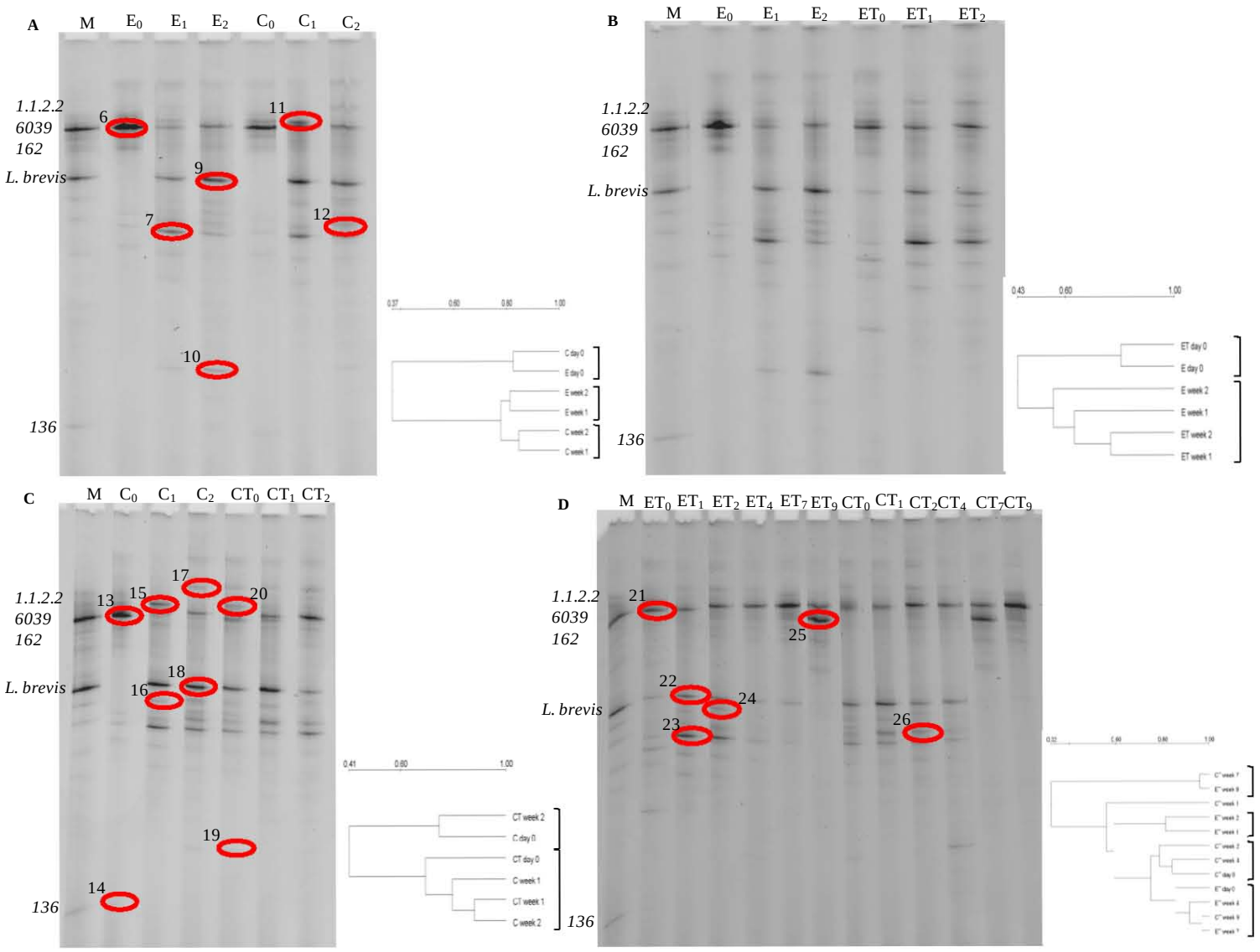

Figure 4.3 (A, B, C and D). **DGGE and cluster analysis of *Lactobacillus* using lactobacilli-specific primers Univ-341-f and Lab-0667-r.** A. The effect of probiotics on the normal microbiota. B. The effect of tylosin on the microbiota of chicks receiving probiotics (no bands were excised from this gel). C. The effect of tylosin on the normal microbiota of chicks. D. The effect of probiotics on chicks receiving tylosin. 16S rRNA gene primers were used to amplify *Lactobacillus* isolated from faecal samples provided by groups given probiotics and tylosin (ET), only tylosin (CT), only probiotics (E) and the control group (C). PCR products were separated on 45 – 55% DGGE. Lane M is the mixed strain reference ladder. Excised bands are circled and numbered.

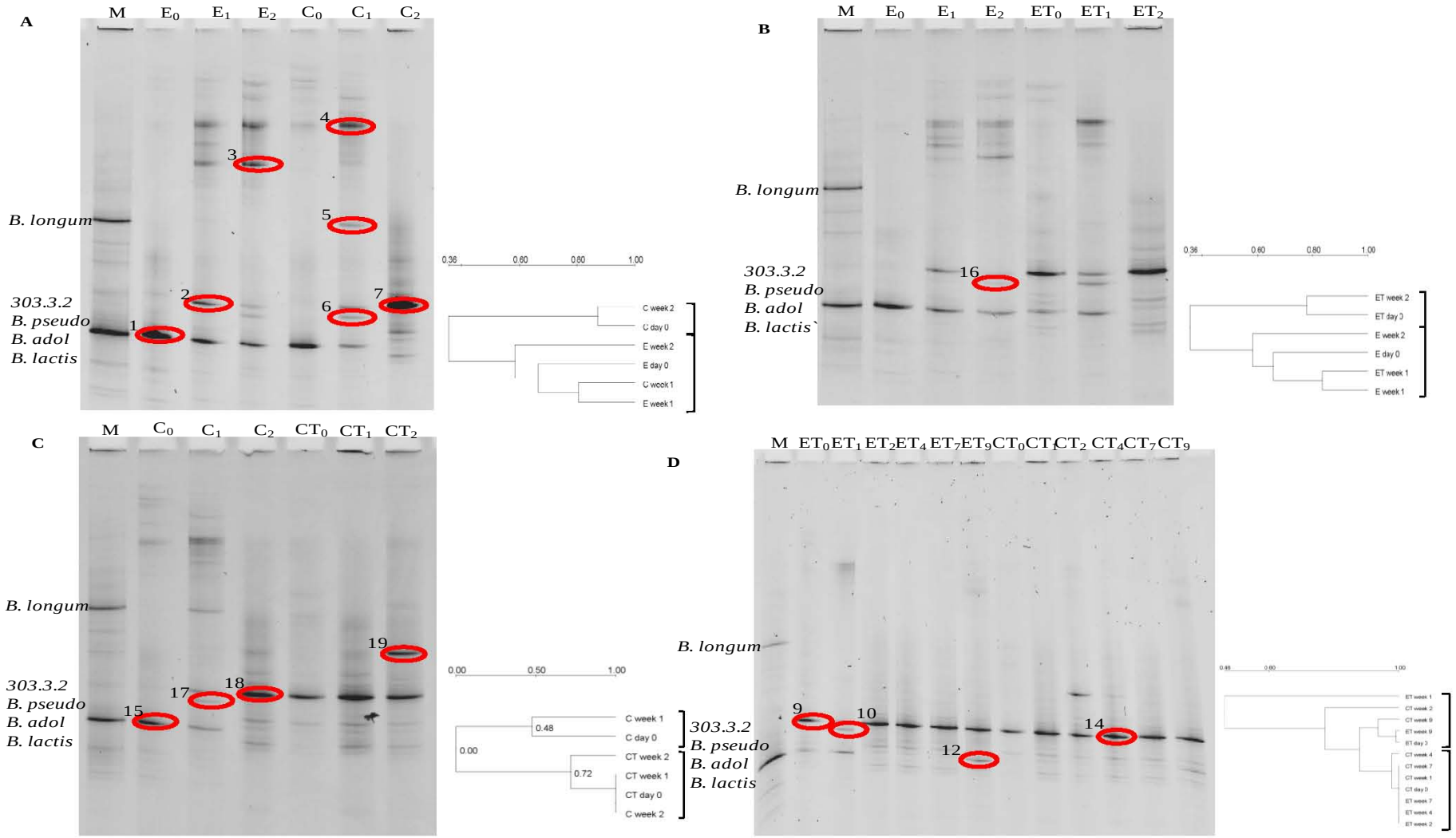

Figure 4.4 (A, B, C and D). DGGE and cluster analysis of *Bifidobacterium* using bifidobacteria-specific primers Bif-164-f and Bif-662-r. A. The effect of probiotics on the normal microbiota B. The effect of tylosin on the microbiota of chicks receiving probiotics. C. The effect of tylosin on the normal microbiota of chicks. D. The effect of probiotics on chicks receiving tylosin. 16S r RNA gene primers were used to amplify *Bifidobacterium* isolated from faecal samples provided by groups given probiotics and tylosin (ET), only tylosin (CT), only probiotics (E) and the control group (C). PCR products were separated on 55 – 65% DGGE. Lane M is the mixed strain reference ladder. Excised bands are circled and numbered.

4.4.5.2. *Lactobacillus*-specific primers

The DGGE comparing the probiotic treatment E and control group C with respect to the lactobacilli present shows clustering of the treatments (Fig. 4.3A), showing that the probiotic mix alters the normal microbiota of the ostrich chicks in the first two weeks after hatching, as seen with the universal primers. The specific effect may be due to band ten (*Lactobacillus bifermentans*), present in treatment E at both week one and two, but absent in treatment C. Aside from this, the DGGE fingerprints of treatment E and C show similar trends, with *Aerococcus urinaeequi* (band 11) disappearing in both groups after week two and *L. curvatus* (band seven) appearing in both groups at week one and two. The probiotic mix administered to the chicks in this study did alter the microbiota, agreeing with the numerous studies showing that the inclusion of *Lactobacillus* in a probiotic feed additive affects lactobacilli numbers, which significantly increase in treated animals compared to controls (Pollmann *et al.,* 1980a; Pollmann *et al.,* 1980b; Jonsson and Olsson, 1985; Jenny *et al.,* 1991; Mountzouris *et al.,* 2010).

The chicks fed probiotics (E) and those fed probiotics and tylosin (ET) did not show clustering according to treatment (Fig. 4.3B). E day zero and ET day zero grouped together, as expected. These two treatments showed similar DGGE fingerprints, with treatment E differing only with the presence of the band previously identified as *Lactobacillus bifermentans* (Fig. 4.3A). This band was present in the control group, and not in treatment ET, and may be a tylosin sensitive, commensal *Lactobacillus* of the ostrich GIT. The lactobacilli included in the probiotic mix were shown to tolerate tylosin at the dose administered in this feeding trial, and this is seen in the similarity between fingerprints of these two treatments.

Group C showed no grouping of control groups, according to treatment or time (Fig. 4.3C). There is no clustering of initial time points which is unexpected. This could be due to band 14 (*Lactobacillus plantarum*), present in C day zero, but not in CT day zero, band 19 (*Weissella paramesenteroides*) present in CT day zero and absent in C day zero, or to band 18 (*Weissella paramesenteroides*) which only appears in C at week one, and is in CT from day zero onwards. This initial difference in gut communities may reflect the random inter-individual differences between birds. It may also be due to the origin of the chicks as they were obtained from three different ostrich hatcheries (see Chapter 3).

No clustering is seen when comparing the effect of tylosin on probiotic-fed (ET) and control (CT) groups over a longer time frame, with regards to *Lactobacillus* spp., and day zero samples did not group together, although they are closely related (Fig. 4.3D). Again, this is unexpected. Overall, the clustering is not clear and it can be concluded that feeding probiotics to chicks receiving tylosin did not appear to alter the *Lactobacillus* diversity of the gut compared to birds only receiving the antibiotic. Band 21 (*Leuconostoc pseudomesenteroides*/ *Weissella confuse*) is present, constantly, for both of these treatments and is therefore considered insensitive to tylosin. Treatment CT at week seven and ET at week nine cluster together, probably due to the appearance of band 25 (*L. johnsonii*/*Aerococcus urinaeequi*) at these respective time points, indicating this strains tolerance to tylosin. Band 22 (*Weissella paramesenteroides*) and 23 (*Pediococcus pentosaceus*) are both present initially in treatments ET and CT, but decrease gradually, not detected by week seven.

4.4.5.3. *Bifidobacterium*-specific primers

Regarding the *Bifidobacterium* population, the probiotic mix did not appear to have an effect when compared to the control, as no specific grouping of treatments were seen (Fig. 4.4A; Table 4.5). Day zero of these treatments did not cluster together, possibly due to the appearance of band four (*Bifidobacterium animalis* subsp. *lactis*) in C day zero, but not in E day zero. Interestingly, this band appears in treatment E by week one. Band one (*Bifidobacterium ruminantium*) was present throughout for both treatments except in the control by week two. Although *B. pseudolongum* subsp. *pseudolongum* was detected in both these treatments (bands two, six and seven), treatment C week 2 showed a much more intense band (band seven) compared to treatment E and the earlier time points of treatment C. This suggests that this strain develops naturally in the maturing GIT, and is its colonisation is unaffected by the ingestion of probiotics which are thought to increase the presence of bifidobacteria in the gut. This lack of grouping of treatments shows that the probiotic treatment does not have an effect on the *Bifidobacterium* spp. diversity when compared to the control, in ostrich chicks. Harmsen *et al.* (2002) fed *B. longum* to healthy human volunteers and found, using DGGE and fluorescent *in-situ* hybridisation, that this probiotic had no effect on the gut *Bifidobacterium* populations. In contrast, Mountzouris *et al.* (2010) found that feeding broilers a probiotic mix containing *B. animalis* increased the *Bifidobacterium* spp. counts compared to the control not receiving the probiotics.

Again, the initial time points do not cluster together when testing the effect of tylosin on the bifidobacterial community for the groups receiving probiotics (E vs ET; Fig. 4.4B; Table 4.5). Week one of treatments E and ET cluster together and then each treatment at week two clusters with their respective day zeros. This could be another example of the tylosin-sensitive strains being replaced by the tylosin-resistant strains by week two, as previously reported by Collier *et al.* (2003b). However, the fingerprints obtained for these samples show an increase in diversity with time, the banding becoming more diverse, indicating that the probiotics increase the diversity of *Bifidobacterium* in the faecal samples.

The use of tylosin on the control groups (C and CT) appeared to have an effect on the autochthonous *Bifidobacterium* spp. population, with CT clustering together but group C only showed grouping of day zero and week one, while week two clustered close to CT day zero (Fig. 4.4C; Table 4.5). The initial time points are clearly different too, with band 15 (*Bifidobacterium animalis* subsp. *lactis*) present in C day zero and not in CT day zero, and band 18 (*B. pseudolongum* subsp. *pseudolongum*) present in CT day zero and not in the control, initially. Day zero and week one from both treatments clustered together, indicating that initially tylosin does make a difference to the *Bifidobacterium* spp.

In the comparison of treatments ET and CT over nine weeks, group ET and CT did not show clear clustering (Fig. 4.4D; Table 4.5). The initial time points did not cluster together either due to the presence of the band in ET day zero, aligning with band 12 (*B. pseudolongum* subsp. *pseudolongum*) and its absence in CT day zero. The band corresponding to *B. pseudolongum* subsp. *pseudolongum* is present in all samples except, ET week one, which also contains *B. pseudolongum* subsp. *pseudolongum* but which migrated to a unique position on the gel. The lack of clustering seen here shows that administration of probiotics to groups receiving tylosin had no effect on the diversity of the *Bifidobacterium* spp. population or that tylosin tended to reduce this groups diversity to similar states over nine weeks.

4.4.6. Band richness

Since each band in DGGE is likely to represent a phylogenetically distinct population, an estimation on the species number can be based on the total number of bands in the profile. This measure of diversity is very similar to the Shannon-Wiener index, however the Shannon-Wiener has the added level of measurement, including not only the number of bands per lane, but also their proportions. The bacterial richness was calculated for each treatment time point for total bacteria, *Lactobacillus* and *Bifidobacterium* populations (Fig. 4.5A-C).

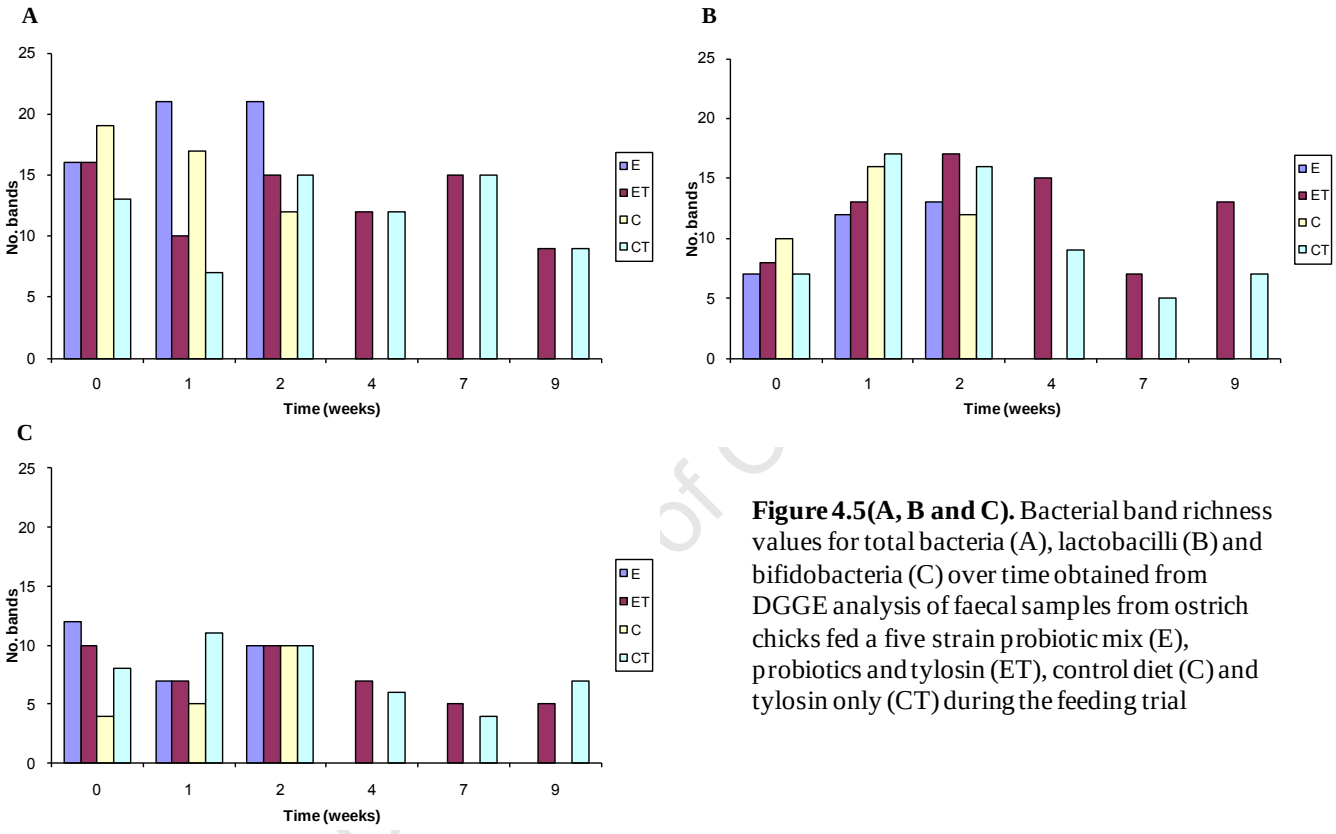

Figure 4.5(A, B and C). Bacterial band richness values for total bacteria (A), lactobacilli (B) and bifidobacteria (C) over time obtained from DGGE analysis of faecal samples from ostrich chicks fed a five strain probiotic mix (E), probiotics and tylosin (ET), control diet (C) and tylosin only (CT) during the feeding trial

4.4.6.1. Universal primers for total bacteria

Treatment E showed an increase in band richness in comparison to the control group, which showed a constant decrease in bacterial richness (Fig. 4.5A). This is unexpected as the diversity of the gut microbiome is expected to increase with age, thus providing a more solid competitive exclusion barrier to invading pathogens. It can be seen that feeding the five strain probiotic mix alone does increase microbial diversity when studying the total bacteria, but this increase seen here did not protect the chicks from an early death either, suggesting that microbial richness is not a dependable measure when studied alone, as it may include

potential pathogens. Tylosin seems to have a varying effect on the band richness for both ET and CT, and this may be due to tylosin-sensitive strains being replaced by tylosin-resistant strains repetitively.

4.4.6.2. *Lactobacillus*-specific primers

With regard to the *Lactobacillus* population (Fig. 4.5B), all treatments increased in richness in the first two weeks, except treatment C which decreased sharply at week two. This is interesting and suggests that feeding tylosin alone (group CT) increases the lactobacilli population in the gut. However, from week two onwards, treatment ET shows a greater *Lactobacillus* richness than treatment CT suggesting that the feeding of probiotics did increase the *Lactobacillus* richness. This agrees with previous work (Pollmann *et al.,* 1980a; Pollmann *et al.,* 1980b; Jonsson and Olsson, 1985; Jenny *et al.,* 1991; Mountzouris *et al.,* 2010) showing that in-feed *Lactobacillus* probiotics increases the diversity of this bacterial group in the gut.

4.4.6.3. *Bifidobacterium*-specific primers

Treatments E and ET follow the same trend, decreasing by week one and then increasing again by week two (Fig. 4.5C). Treatment ET decreases steadily hereafter, suggesting tylosin has a detrimental effect on the richness of the *Bifidobacterium* population. Groups C and CT gradually increase until week two, thereafter CT decreases, finally increasing slightly by week nine.

4.4.7. Statistical analysis of microbial diversity using the Shannon-Wiener index (H')

For the statistical analysis, the five faecal samples collected from each pen were pooled but the three replicates of each treatment pen were run separately on the DGGE to obtain three H' values on which to perform statistics (gels not shown). The H' is a more sensitive method of measuring bacterial diversity as it considers the intensity of each band which it includes. The Kruskal-Wallis test was used when comparing all four treatments over the feeding trial period**.** This was only possible in the first two weeks, as thereafter birds undergoing treatments E and C died. The Kruskal-Wallis test is a non-parametric test used to assess the equality of a population among groups, and is an extension of the Mann-Whitney U test to

three or more groups. To compare the remaining two treatments, ET and CT, the Mann-Whitney U test was used. This is a non-parametric statistical hypothesis test used to assess two independent samples.

4.4.7.1. Diversity of the total bacteria found in the faecal samples over time

Group E had all died after week two and it was therefore only possible to analyse the H' values for all four groups up to this time point. The Kruskal-Wallis test showed only treatment ET having a significant difference in diversity ($p = 0.05$), increasing from day zero to week two suggesting that feeding tylosin together with probiotics increases the diversity of the total bacteria over two weeks (Fig. 4.6A(i)). If the confidence interval is dropped to 90 % (as n is small), treatment CT shows a significant decrease in total bacterial diversity from day zero to week one ($p = 0.097$).

The Mann-Whitney U test between treatments ET and CT over the full duration of the feeding trial (nine weeks) showed no significant differences for either group (Fig. 4.6A(ii)). This differs from the results above using the Kruskal-Wallis test where ET showed a significant increase in H' and is most likely due to the fact that the Mann-Whitney U test is biologically more stringent than the Kruskal-Wallis test. Shimada *et al.* (2010) found that addition of tylosin at 1.67mg/L to an anaerobic batch reactor had no effect on total bacteria in the first 50 days.

4.4.7.2. Diversity of the total bacteria found in the faecal samples between treatments

The Kruskal-Wallis test showed no significant difference between the four treatments at day zero or at week two, but a significant difference ($p = 0.044$) was seen at week one, group ET having the highest H' value (Fig. 4.6A(i)).

A(i)

Pen	Time point: Day 0	Week 1	Week 2	p value **
E1	1.67	1.56	1.82	
E2	2.06	1.57	2.41	0.37
E3	1.79	1.71	1.48	
C1	1.71	1.72	2.09	
C2	1.75	1.31	1.52	0.26
C3	1.89	1.52	2.05	
ET1	1.75	1.90	2.33	
ET2	1.75	1.91	1.95	**0.05**
ET3	1.77	1.91	2.03	
CT1	2.02	1.35	2.38	
CT2	2.06	1.34	2.07	0.097
CT3	2.13	1.37	1.79	
p value *	0.13	**0.04**	0.93	

B(i)

Pen	Time point: Day 0	Week 1	Week 2	p value **
E1	1.84	1.93	1.59	
E2	1.98	1.09	1.82	1.00
E3	1.57	1.76	1.94	
C1	1.27	1.33	2.31	
C2	0.68	1.64	2.36	**0.05**
C3	0.69	1.25	2.14	
ET1	1.91	1.91	1.81	
ET2	0.65	1.86	2.20	0.37
ET3	1.49	1.56	2.15	
CT1	1.44	1.35	2.17	
CT2	1.68	1.92	2.12	0.10
CT3	1.45	0.49	2.14	
p value *	0.13	0.60	0.10	

C(i)

Pen	Time point: Day 0	Week 1	Week 2	p value **
E1	0.68	0.84	1.12	
E2	1.40	0.78	1.51	0.10
E3	0.55	0.43	0.89	
C1	0.00	0.48	0.85	
C2	0.22	0.82	0.78	0.76
C3	0.68	0.00	0.00	
ET1	0.00	0.79	0.69	
ET2	0.29	0.19	0.66	0.37
ET3	0.43	0.64	0.63	
CT1	1.51	0.19	0.42	
CT2	0.00	0.30	0.63	0.72
CT3	1.06	0.90	0.70	
p value *	0.28	0.86	0.08	

A(ii)

Pen	Time point: Day 0	Week 1	Week 2	Week 4	Week 7	Week 9	p value **
ET1	1.75	1.90	2.33	1.82	1.66	2.02	
ET2	1.75	1.91	1.95	2.11	2.11	1.53	0.21
ET3	1.77	1.91	2.03	2.02	1.96	1.47	
CT1	2.02	1.35	2.38	1.95	2.05	1.59	
CT2	2.06	1.34	2.07	2.03	2.16	1.75	0.15
CT3	2.13	1.37	1.79	2.13	1.36	1.58	
p value *	**0.05**	**0.05**	0.83	0.51	0.83	0.51	

B(ii)

Pen	Time point: Day 0	Week 1	Week 2	Week 4	Week 7	Week 9	p value **
ET1	1.91	1.91	1.81	0.83	1.09	1.50	
ET2	0.65	1.86	2.20	1.21	0.34	1.11	**0.05**
ET3	1.49	1.56	2.15	1.35	0.14	1.18	
CT1	1.44	1.35	2.17	1.59	0.39	1.12	
CT2	1.68	1.92	2.12	1.55	0.57	1.61	0.07
CT3	1.45	0.49	2.14	0.17	0.32	1.55	
p value *	0.83	0.51	0.83	0.51	0.83	0.28	

C(ii)

Pen	Time point: Day 0	Week 1	Week 2	Week 4	Week 7	Week 9	p value **
ET1	0.00	0.79	0.69	0.73	0.73	0.91	
ET2	0.29	0.19	0.66	0.69	0.74	0.89	0.06
ET3	0.43	0.64	0.63	0.64	0.60	0.94	
CT1	1.51	0.19	0.42	0.85	0.58	0.75	
CT2	0.00	0.30	0.63	0.56	0.74	0.80	0.89
CT3	1.06	0.90	0.70	0.77	0.63	0.16	
p value *	0.38	0.83	0.51	0.51	0.51	**0.05**	

Figure 4.6. The Shannon-Wiener diversity indices calculated from DGGE banding patterns from the three different primer sets: A, Eubacteria primers Univ-341-f and Univ 515-r; B, Lactobacillus-specific Univ-341-f and Lab-0667-r; C, Bifidobacterium-specific Bif 164-f and Bif-662-r. The Kruskal Wallis test (i) was used to statistically differentiate between all four treatments, and the Mann-Whitney U test (ii) was used to test between two treatments. P value * value comparing treatments; P value ** value over time.

This shows that the antibiotic-probiotic mix has an effect on the microbial diversity as soon as one week. However, when the Mann-Whitney U test was done on H' values from treatments CT and ET at all time points (day 0 to week 9), no significant differences were seen between these groups at any time point (Fig. 4.6A(ii)). Again this is most likely due to the stringency of the Mann-Whitney U test.

4.4.7.3. Diversity of the *Lactobacillus* found in the faecal samples over time

The Kruskal-Wallis statistical analysis only showed a significant increase in lactobacilli diversity for treatment C over the two week time frame (p = 0.05; Fig. 4.6B(i)). The other treatments have no effect on the diversity of this taxon. At 90 % confidence interval, group CT showed a significant increase in lactobacilli diversity from day zero to week two (p = 0.1), suggesting tylosin selects for *Lactobacillus* spp. This agrees with previous work by

Collier *et al.* (2003a, b) where tylosin was shown to enrich *Lactobacillus* spp. in both chickens and pigs.

Again the Mann-Whitney U test showed different results, showing group ET to have a significant increase in lactobacilli diversity (p = 0.05) from day zero to week two (Fig. 4.6B(ii)). Hereafter, the H' decreased again. The confidence interval of 90 % also shows treatment CT to have a significant increase in this phylum from day zero to week two, confirming the above results.

4.4.7.4. Diversity of the *Lactobacillus* found in the faecal samples between treatments

No significant differences were observed between the treatments at any of the time points using either the Kruskal-Wallis or Mann-Whitney U test (Fig. 4.6B(i) and (ii)). This implies that none of the treatments altered the lactobacilli diversity profiles in the ostrich gut even though *Lactobacillus* were included in the probiotic mix. However, at 90 % confidence interval, group E has a significantly lower H' value (p = 0.1) than the other three treatments using the Kruskal-Wallis test.

4.4.7.5. Diversity of the *Bifidobacterium* found in the faecal samples over time

Neither of the statistical tests used here showed any significant differences in *Bifidobacterium* diversity over time for any of the treatments (Fig. 4.6C (i) and (ii)). This may suggest that the *Bifidobacterium* population is acquired among farmed ostrich chicks as soon as they hatch and becomes well established so that exogenous *Bifidobacterium* do not survive the conditions and competition of the endogenous microbiota. When the confidence level is altered to 90 % however, group E showed a significant increase from day zero to week two (p = 0.1) using the Kruskal-Wallis test, and the Mann-Whitney U test showed the *Bifidobacterium* diversity in group ET to increase significantly (p = 0.06) over the course of the feeding trial.

4.4.7.6. Diversity of the *Bifidobacterium* found in the faecal samples between treatments

Using the Kruskal-Wallis test of significance, no differences were seen between any of the treatments (Fig. 4.6C(i)). However, at the 90 % confidence interval, week two showed treatment E to have a significantly higher *Bifidobacterium* H' than the other three treatments (p = 0.08). This suggests a positive role for this probiotic mix in the diversity of the *Bifidobacterium* population.

The Mann-Whitney U test however did show a significant increase in *Bifidobacterium* diversity at week nine for group ET compared to group CT (p = 0.05; Fig. 4.6C(ii)). This infers that feeding the combination of tylosin and probiotics increases the diversity of the *Bifidobacterium* population, but only after nine weeks.

4.4.8. Sequence analysis

Chosen dominant bands from all treatment groups were excised from the DGGE gel and re-amplified using Univ-341-f and Univ-515-r, and the PCR purified products were sequenced. This approach showed mixed DGGE bands were present due to co-migration. Therefore, the PCR purified products were cloned into the pTZ57R/T vector and three random clones were selected for sequencing.

4.4.8.1. Universal primers

The primer combination used in this section is specific to all eubacteria. Figure 4.2 shows the bands which were excised and sequenced. The majority of cloned bands were identified as uncultured bacteria as the primary hit using BLAST searches, suggesting that most of the amplified bacterial DNA isolated from the ostrich gut have not previously been identified to species level in the database. To get an indication of actual bacteria present, the sequences from this study returned as uncultured were further used to search for the closest culturable relatives to the excised bands using BLAST and are shown in Table 4.3.

Table 4.3. The closest relatives of the amplicons excised from DGGE gels using the universal primers. The results are based on BLAST searches.

Treatment	Band Number	16S rRNA gene sequence identification	% similarity	Accession Number
E day 0	8a,b	*Clostridium hathewayi*	100	NR_036928.1
	8c	*Clostridium ramosum*	96	AB595128.1
E day 0	9a, c	*E. coli* KO11	99	CP002516.1
	9b	*Klebsiella oxytoca*	100	HQ694732.1
E week 1	10a, b	*Akkermansia muciniphila* ATCC BAA-835	98	CP001071.1
E week 1	11a, b	*Klebsiella pneumoniae* 342	99	CP000964.1
E week 1	23a	*Anaerostipes butyraticus*	97	AB616134.1
	23b	*Ruminococcus albus* 7	95	CP002403.1
	23c	*Odoribacter splanchnicus*	94	AB547649.1
E week 1	24a	*Turicibacter sanguinis* strain PC909	97	HQ428099.1
	24b	*Clostridium* sp. MK12	98	AB275141.1
	24c	*Clostridiales* bacterium	94	HQ452851.1
E week 2	12a	*Eubacterium xylanophilum*	97	L34628.1
	12b	*Clostridiales* bacterium	95	HQ452852.1
ET day 0	15a, b, c	*Clostridium ramosum*	98	AB595128.1
ET day 0	16a, b, c	Uncultured *Lachnospiraceae* bacterium	99	EF708581.1
ET day 0	17a	*Anaerostipes butyraticus*	100	AB616134.1
ET week 1	18a, b	*Akkermansia muciniphila* ATCC BAA-835	89	CP001071.1
	18c	*Clostridium ramosum*	96	AB595128.1
ET week 1	13a	*Clostridium ramosum*	98	AB595128.1
	13b	*Oscillibacter valericigenes*	99	AB238598.1
	13c	*Coprobacillus cateniformis*	100	GU811876.1
ET week 2	14a, b, c	*Dethiosulfovibrio acidaminovorans*	92	NR_029034.1
ET week 2	19a, b, c	*Roseburia intestinalis* strain DSM	94	HM007565.1
ET week 2	20a	*Clostridium* sp. MK12	98	AB275141.1
	20b	*Lactobacillus* sp. DI71	87	AB290831.1
	20c	*Akkermansia muciniphila* ATCC BAA-835	96	CP001071.1
C day 0	1a	*B. subtilis* BSn5	100	CP002468.1
	1b	Uncultured bacterium clone from okapi faeces	93	EU468485.1
C day 0	25a	*Escherichia coli* O157:H7 EDL933	99	AE005174.2
	25b	*Enterobacter* sp. KN/03/24	100	HQ235641.1
	25c	*Acinetobacter* sp. CNE 4	100	FR749854.1
C week 1	26a	*Akkermansia muciniphila* ATCC BAA-835	97	CP001071.1
	26b	Rumen bacterium NK4B114 from rumen of sheep	95	GU324414.1
C week 1	2a	*Clostridium clostridioforme*	99	HM008264.1
	2b	*Anaeroplasma bactoclasticum*	92	M25049.1
C week 1	3a	*Lachnospiraceae bacterium*	96	AF550610.1
C week 1	4a	*Anaerostipes butyraticus*	95	AB616134.1
	4b	*Pediococcus pentosaceus*	100	HQ834496.1
	4c	*Lachnospiraceae* bacterium	95	EU728787.1
C week 2	5a	*Odoribacter splanchnicus*	94	AB547649.1
	5b	Uncultured bacterium clone from Trancaspian Urial feces	97	EU779235.1
C week 2	6a, b	*Clostridiales* bacterium from chicken caecum	95	HQ452851.1
CT week 2	21a, b, c	*Akkermansia muciniphila* ATCC BAA-835	98	CP001071.1
CT week 7	22a, b	*Bacteroides dorei* strain F4071	100	FJ460592.1

If no species names were found, the result was recorded as "uncultured". Using the 97% or higher threshold for identification, only just over half of the sequenced clones could be

identified to the species level. This data was obtained from limited sequencing of major bands from DGGE and is therefore not a comprehensive indication of the microbiota found in ostrich chicks. Six phyla were identified as representatives of the bacterial groups present in this study from the clones identified by nucleotide sequence analysis, the majority belonging to the *Firmicutes*.

When looking at the effect of the treatment on the distribution of total bacteria, all groups show a great diversity, including species from various phyla. The *Firmicutes* were the major phylum found in all treatments, with 15 out of the 25 excised bands belonging to this phylum (Table 4.3). In addition, *Akkermansia muciniphila* was detected in all four treatment groups, from as early as week one, showing it is an early coloniser and possibly a common commensal. It also appears to be resistant to tylosin. Birds from treatment E harboured the pathogenic *Klebsiella pneumoniae* which causes ostrich chick fading syndrome and *Klebsiella oxytoca,* also a potential pathogen. On the other hand, birds from treatment C harboured pathogenic *Escherichia coli* O157:H7 and *Acinetobacter* sp. Although the *Acinetobacter* band appears to be present in treatment E too, it was not positively identified using sequencing and so cannot be confirmed. *Lachnospiraceae* spp. is frequently detected in C but not in E, highlighting another effect of the probiotic mix. Sequencing results do not differ significantly for groups receiving probiotics compared to those receiving both probiotics and tylosin. One difference is that treatment ET appeared to have fewer pathogens such as *Klebsiella* and tylosin seems to favour *Clostridium*, especially cluster XVIII and XIVa strains.

4.4.8.1.1. Phylum Firmicutes

The *Firmicutes* represented the majority of the sequences identified in this study using the universal primers (Table 4.3). Of these most belonged to the Class *Clostridium.* Of the 25 bands sequenced, 14 were identified as members of the *Clostridium* group. This finding agrees with Matsui *et al.* (2010b), who in a metagenomics study screened a 16S rRNA gene clone library obtained from caecal digesta of adult ostriches and found the *Firmicutes* to dominate the sequences obtained from the ostrich caeca (51% of the total number of sequences) and of these most were from the class *Clostridium* (*Clostridium leptum* and *Clostridium coccoides*), and none from *Lactobacilliales.* Previous work has shown the

Firmicutes to be the main component of the microbiota of broiler chickens, followed by *Bacteroidetes* (Apajalahti and Kettunen, 2006).

This is in contrast to previous work by Lu *et al.* (2008) where they showed this genus to be present in avian faeces at only 11%. However, Apajalahti *et al.* (2004) and Dumonceaux *et al.* (2006) showed the chicken caeca to be dominated by the *Clostridiales* order, followed by *Lactobacillales* and *Bacteroidales.* Matsui *et al.,* (2010a) confirmed this, showing *Clostridium* clusters XIVa and XIV to be dominant in the ostrich caeca. Our current study found the *Clostridiales* being most frequently detected, the majority being members of *Clostridium* cluster XIVa (*C. coccoides*) and XIV (*C. leptum*), both lineages containing many butyrate producers. *Clostridium hathewayi* (cluster XIVa) and *C. ramosum* (cluster XVIII) were the most common, being found in treatments E, C and ET. Treatment C at week one also contained *Clostridium clostridioforme* (cluster XIVa). Tylosin has been shown to be effective in decreasing the genus *Clostridium* (Shimada *et al.,* 2010) and it is therefore interesting to find this genus represented at such an extent in treatment ET.

4.4.8.1.2. Butyrate producing bacteria

Butyrate-producing bacterial strains are considered important in the maintenance of a healthy gut via butyrate production, maintenance of luminal pH by consumption of lactate or the anti-inflammatory effect of some of these strains (Sokol *et al.,* 2008; Guilloteau *et al.,* 2010). Ostriches have been shown to be particularly dependent on these bacteria. Swart *et al.* (1993b) showed that volatile fatty acids such as butyrate, propionate and acetate, from hemi-cellulose and cellulose fermentation, are responsible for about 76% of the metabolizable energy in a growing ostrich chick, especially acetate, and that propionate and butyrate were only present in small amounts in the hindgut. Swart *et al.* (1993b) also suggested that in ostriches, similar to other animals, butyrate is absorbed preferentially over propionate which in turn is absorbed faster than acetate. So although acetate is the predominant VFA in the ostrich gut, butyrate is the most readily absorbed.

Butyrate-producing strains are well represented in the ostrich gut and most of the *Clostridium* identified in this study are butyrate producers and belong to clusters XIVa and IV. The major species identified from the DGGE analysis was *Anaerostipes butyraticus* (band six), a novel butyrate producer and part of the *Clostridium* cluster XIVa, previously isolated from chicken

caeca (Eeckhaut *et al,* 2010). This species was detected in treatments E, ET and C and is therefore considered resistant to tylosin at the concentration used here. *Roseburia intestinalis* (band 19), which is a dominant band in the DGGE in weeks four to seven, is an important butyrate producer isolated from many animals including the caecal contents of broiler chickens (Eeckhaut *et al.,* 2011). It is very similar genomically to *Eubacterium rectale,* both part of the *Clostridium* cluster XIVa, and in addition to butyrate, it also produces lactate and formate and utilizes acetate. *R. intestinalis* was also shown to degrade cellobiose as well as arabinose, fructose, maltose, raffinose, sucrose, xylose and starch (Duncan *et al.,* 2002). *Oscillibacter valericigenes* (band 13) is found in *Clostridium* cluster IV and has been shown to be a valerate-producing anaerobic bacteria, producing butyrate and valeric acid from D-xylose (Iino *et al.,* 2007).

4.4.8.1.3. Fibre digesting bacteria

Fibre digesting bacteria are also important in terms of their function in ostrich digestion. An interesting difference between the well-characterised poultry model we use and the ostrich is that chickens do not digest plant fibre, especially not cellulose, to any significant extent (Swart *et al.,* 2003a). This is most likely due to the short retention time in the chicken gut, compared to the long (40 hour) transit time in the ostrich. Ostriches are capable of digesting hemicellulose (68% of that ingested) and cellulose (38% of that ingested) very efficiently and this is presumably due to microbial digestion as well as the highly developed hindgut. Van Gylswyk *et al.* (1998) isolated five fibrolytic bacteria from ostrich faeces, two of which were identified as *Propionibacterium acnes* (97%) and *Ruminococcus flavefaciens* (97%). Matsui *et al.* (2010) also detected an uncultivated *Fibrobacter* in fresh ostrich faeces.

In Table 4.3, a number of fibre-digesting bacterial strains have been identified. *Eubacterium xylanophilum* (97%), part of *Clostridium* cluster IV, is a novel fibre digesting bacteria, utilising cellobiose and xylan and producing formic, acetic and butyric acids. *Ruminococcus albus*, a member of the Family *Lachnospiraceae,* a member of *Clostridium* cluster IV, was also detected in treatment E (band 23) but the similarity was only 95%. Although *R. albus* was only detected in one clone, the corresponding band appears to be present in all lanes across the gel in the DGGE with treatments E and C. This phylotype is a major fibrolytic bacteria in the rumen of ruminants (Koike and Kobayashi, 2001). However, in a study by Matsui *et al.* (2010), where a specific primer was used for this phylotype, no *R. albus* was detected in ostrich caecal digesta. The amplicon corresponding to band three was identified as

a member of *Lachnospiraceae* (96%; Accession number AF550610.1), which is phylongenetically close to clones RA-01 and RA-02 previously isolated from ostrich faeces, belonging to *Clostridium* cluster XIVa (Matsui *et al.,* 2010). *Lachnospiraceae* have also been isolated from the pig intestines (NCBI, Accession number AY587806). More importantly, Matsui *et al.* (2010) found several operational taxonomic units (OTU's) isolated from ostrich faeces, which when sequenced, clustered closely with *Ruminococcus flavefaciens* and *Lachnospiraceae bacterium.* These two bacteria are closely related and *R. flavefaciens* has been isolated from ostrich faeces previously (van Gylswyk *et al.,* 1998) and is thought to be a major fibrolytic bacterium in the large intestine of the ostrich. Tanikawa *et al.* (2011) found uncultured *Lachnospiraceae* to be the main *Firmicute* present in the caeca of chicks. Leng *et al.* (2010) detected this bacterial genus using DGGE in the rumen of Indian Gayals and reported it is also commonly found in the rumen of cattle and is able to degrade pectin, phenolyic compounds and quercetin.

4.4.8.1.4. Potential pathogenic bacteria

Proteobacteria were found to account for 20.8% of the total bacteria in the chicken caeca (Zhu *et al.,* 2002). Tanikawa *et al.* (2011) also found *Proteobacteria* to be one of the main phyla present in chick caeca in the first two weeks of life, especially *E. coli,* and *Klebsiella pneumonia* was also detected. However, Apajalahti and Kettunen (2006) found this phylum only as a minorityin chicken caeca.

E. coli was detected in treatments E and C. Aside from commensal *E. coli*, there are also pathogenic strains of *E. coli* which cause colibacillosis in chickens (Rodriguez-Siek *et al.,* 2005) and pigs (Chin and Chapman, 2009) and leads to major economic loss in the farming industry. The *E. coli* detected in treatment C (band 25) shows 99% similarity to *E. coli* O157:H7, a strain posing a major threat to public health, causing haemorrhagic colitis which can be fatal (Perna *et al.,* 2001). It is known that stress can lead to intestinal dysbiosis, and has lead to the presumption in avian pathology that this aids the penetration of *E. coli* into the blood stream from the gut mucosa, causing septicaemia (Herráez *et al.,* 2005). Ostriches are extremely sensitive to stress. On the other hand, *E. coli* K011 detected in treatment E (band nine) is non-pathogenic and is most likely a commensal of the ostrich gut, capable of utilising cellobiose, xylose and arabinose (Dien *et al.,* 2000). These two strains appear to migrate to the same position on the DGGE gel, just below the reference ladder strain Lr136.2.2 and the band seems to be present in treatment ET also. This is not surprising as *E. coli* is intrinsically resistant to tylosin and has been shown to increase in numbers in dogs (Suchodolski *et al.,*

2009) and pigs (Smith, 2009) when fed tylosin. In an anaerobic continuous fermentation culture of chicken microbiota, Poole *et al.* (2003) showed tylosin to facilitate the persistence of pathogenic *E. coli* O157:H7.

Klebsiella pneumonia (band 11) was detected in treatment E. This species has previously been isolated from ostrich chicks and was shown to cause ostrich chick fading syndrome (OCFS) (Terzich and Vanhooser, 1993). OCFS is characterised by depression, anorexia and death three to five days after onset of clinical signs in ostrich chicks less than three weeks old. *Klebsiella oxytoca* (band nine), a cellulose degrader (Wood, 1997) and cause of antibiotic-associated hemorrhagic colitis in humans (Högenauer *et al.,* 2006) was also present in treatment E at day zero. Both of the above species have been isolated from ostrich cloaca and oropharynx previously (Melville *et al.,* 2004), *K. pneumonia* reaching as high as 32% in the oropharynx, and *K. oxytoca* present at 2% in both locations.

Band 25 shows 100% similarity to *Enterobacter* sp. isolated from human urine and *Acinetobacter* sp. (100%), an opportunistic pathogen. Both of these Classes have been isolated previously from ostrich eggs, in a study related to ostrich embryo death (Cabassi *et al.,* 2004).

Anaeroplasma bactoclasticum (band two), a species belonging to *Anaeroplasma*, a genus of the *Mollicutes,* was detected in group C. Although mycoplasma are rife in South African ostrich farming and most ostrich mycoplasma related illnesses are due to species found in the poultry industry (Verwoed, 2000), only three ostrich specific mycoplasmas have been isolated, to date which cause respiratory disease (Botes *et al.,* 2005). These isolates, Ms01, Ms02 and Ms03 (accession numbers DQ223545, DQ223546 and DQ223547, respectively) all fall into the Hominis group of mycoplasma, whereas the *Anaeroplasma bactoclasticum* isolated in this study falls under the Anaeroplasma group, separating it too from the most common poultry mycoplasma.

4.4.8.1.5. Phylum Verrucomicrobia

This phylum, represented by the species *Akkermansia muciniphila* only, makes up 13% of the sequences from ostrich faeces identified from the gels using total bacteria primers, and was found in samples from all treatments. It was therefore assumed to be resistant to tylosin. In comparison, Matsui *et al.* (2010b) found this phylum to be present in ostrich caecal contents at only 0.3% and Frey *et al.* (1999) found it to be present at 17.2% in the faeces of wild

gorillas. *A. muciniphila* is capable of using mucin as its sole carbon and nitrogen source (Derrien *et al.*, 2004). The exact relationship between mucin-degraders and the host remains unknown. Mucins are the main constituents of mucus which provides the protective barrier against pathogens. Degradation of this mucus layer can therefore lead to reduced protection which can in turn become pathological (Sonoyama *et al.*, 2009). However, van Passel *et al.* (2011) showed that *A. muciniphila* colonises the gut of humans early and is found less abundantly in humans with gut disease, suggesting it may be a good biomarker for a healthy gut. Since mucins are highly sulphated acidic monopolysaccharides, their degradation by gut microbes release sulphate, encouraging sulphate-reducing microbes such as *Dethiosulfovibrio acidaminovorans* (Gibson *et al.*, 1988), also detected in this study.

4.4.8.1.6. Phylum Bacteroidetes

Matsui *et al.* (2010b) found *Bacteroidetes* to be the second most dominant phylum present in ostrich faeces at 39%. However, Zhu *et al.* (2002) found that in broiler chickens, the *Bacteroides* group only represented 1.9% of total bacteria detected using TGGE. Lu *et al.* (2009) reported that this phylum is generally very low in avian faecal samples such as the waterfowl, having only 7.1%. In comparison, the turkey harbours *Bacteroidetes* in abundance (Scupham *et al.*, 2007), as do humans. Only one DGGE band was identified as *Bacteroides* species, which is surprising given that *Bacteroides* are thought to have essential roles in the degradation and fermentation of organic material in the colon (Salyers, 1995). Sonnenburg *et al.* (2005) showed that *Bacteroidetes* are able to detect, bind, degrade and import carbohydrates, providing substrates to a myriad of bacteria, including the butyrate producers. *Bacteroides dorei* (band 22), a species isolated from human faeces and found to ferment xylose (Bakir *et al.*, 2006), has not been isolated in avian faeces before. This is interesting as *Bacteroidales* populations studied in animal and human faeces was shown to be host-specific and show host-specific distributions to such an extent that it was possible to design a host-specific marker, a useful addition to the microbial source tracking toolbox (Mieszkin *et al.*, 2009).

4.4.8.1.7. *Lactobacillus*-specific primers

The bands which were excised and sequenced are shown in Fig. 4.3. The specificity of the *Lactobacillus* primers for the lactic acid group is confirmed by the sequencing results as the identities of the different bands were *Lactobacillus, Leuconostoc*, *Weissella*, *Pediococcus* and *Aerococcus* as expected (Heilig *et al.*, 2002; Table 4.4). Low similarities were also obtained to *Anaeroplasma bactoclasticum* (91 and 93%), a Mollicute, closely related to *Lactobacillus*

(Woese *et al.*, 1980). *A. bactoclasticum* (Accession number M25049.1) was detected at week one with both the *Lactobacillus*-specific and the universal primers in treatment C (table 4.3). Of the 20 bands excised, the majority of sequences were identified at *Weisella* (52% of the total number of sequences) and only six bands corresponding to *Lactobacillus* species and the rest were made up of *Pediococcus*, *Leuconostoc* and *Aerococcus.* Again, some excised bands showed identity to more than one species, indicating co-migration. These primers resulted in good sequences with high similarities, above the 97% threshold.

On closer examination of the sequence identities of major bands present in DGGE analysis of the faecal samples, it can be seen that neither the administered probiotic strain nor tylosin had a profound effect on the LAB diversity of the ostrich chicks. None of the *Lactobacillus* strains included in the probiotic mix were detected in the faecal samples of the groups receiving probiotics, and no distinct species grouping was noted according to treatment. Although bands six and 13 both aligned with the *L. plantarum* found in the reference ladder on the DGGE, they were identified as *Weissella confusa.* The *L. plantarum* strain which was detected in treatment C on day zero, where no probiotics were fed, shows an exact sequence match to Lp1.1.2.2 (the *L. plantarum* included in the probiotic mix). This may suggest that this strain is a normal member of the ostrich flora, acquired immediately after hatching. It is interesting to see that the band corresponding to this bacterium migrates to the same position as Lr136.2.2 (*L. reuteri*) in the reference ladder.

Table 4.4. The closest relatives of the amplicons excised from DGGE gels using *Lactobacillus*-specific primers. The results are based on BLAST searches.

Treatment	Band Number	16S rRNA gene sequence identification	% similarity	Accession Number
E day 0	6a, b, c	*Weissella confusa*	100	AM117179.1
E week 1	7a, b	*Lactobacillus bifermentans*	98	AB289045.1
	7c	*Lactobacillus curvatus* strain NM33-2	100	HM218173.1
E week 2	9a, b	*Weissella paramesenteroides* strain FMA246	100	HQ721270.1
E week 2	10a, b, c	*Lactobacillus bifermentans*	98	AB289045.1
C day 0	13a	*Weissella confusa*	100	AM117179.1
C day 0	14a, b, c	*Lactobacillus plantarum* strain P1N	99	GU372710.1
C week 1	15a, b, c	*Weissella paramesenteroides* strain FMA246	100	HQ721270.1
C week 1	16a, b, c	*Pediococcus pentosaceus* strain SC1	99	HQ834496.1
C week 1	11a, c	*Anaeroplasma bactoclasticum*	93	M25049.1
	11b	*Aerococcus urinaeequi* strain 11LS3	98	HQ284933.1
C week 2	12a	*Weissella paramesenteroides* strain FMA246	100	HQ721270.1
C week 2	17a	*Weissella paramesenteroides* strain FMA246	98	HQ721270.1
	17b	*Lactobacillus bobalius*	99	AB626063.1
C week 2	18a	*Pediococcus dextrinicus*	99	FM877685.1
	18b, c	*Weissella paramesenteroides* strain FMA246	100	HQ721270.1
ET day 0	21a, c	*Leuconostoc pseudomesenteroides*	100	AB572039.1
	21b	*Weissella confusa*	100	AM117179.1
ET week 1	22a, b, c	*Weissella paramesenteroides* strain FMA246	98	HQ721270.1
ET week 1	23a	*Weissella confusa*	99	AM117179.1
	23b, c	*Pediococcus pentosaceus* strain SC1	99	HQ834496.1
ET week 2	24a	*Weissella paramesenteroides* strain YSY-M4	100	GU223367.1
	24b, c	*Pediococcus pentosaceus* strain SC1	99	HQ834496.1
ET week 9	25a	*Lactobacillus johnsonii* strain Sun	100	HQ641209.1
	25b	*Aerococcus urinaeequi* strain 11LS3	100	HQ284933.1
CT day 0	19a, b	*Weissella paramesenteroides* strain FMA246	100	HQ721270.1
CT day 0	20a	*Lactobacillus* sp. SXVIII11(2011)	97	HQ728335.1
	20b	*Pediococcus dextrinicus*	99	FM877685.1
CT week 1	26a	*Pediococcus argentinicus*	98	AM709786.1
	26b	*Pediococcus pentosaceus* strain SC1	100	HQ834496.1
	26c	*Lactobacillus curvatus* strain NM33-2	100	HM218173.1

The LAB distribution in the ostrich gut between treatments appears to be homogenous. *Weissella paramesenteroides* is detected in all treatments and is therefore appears to be a common commensal member of the ostrich gut, which is resistant to tylosin used in this study. The *Lactobacillus* strains which were detected include *L. bifermentans* and *L. curvatus* in treatment E, while treatment C contained *L. plantarum* and *L. bobalius*, and group ET had a single report of *L. johnsonii*. Treatments ET, C and CT all show a high presence of

Pediococcus pentosaceus, and ET is also the only treatment harbouring *Leuconostoc pseudomesenteroides* although the corresponding band seems to be present throughout in ET and CT.

4.4.8.2. *Bifidobacterium*-specific primers

The specificity of the *Bifidobacterium*-specific primer set was seen from the sequencing results in Table 4.5, with only *Bifidobacterium* being detected, showing high sequence similarities to the species in the database, again all above the 97% threshold. The range of *Bifidobacterium* present was very limited, only four species being reported, *B. pseudolongum* subsp. *pseudolongum* being the most common (55% of clones).

The next most common strain was *B. animalis*, with subspecies *animalis* and *lactis* present. *B. ruminatium* and *B. adolescentis* were found to occur only in E day zero. *B. pseudolongum* subsp. *pseudolongum* was the strain incorporated into the probiotic mix and was not found at day zero except in treatment ET. It did, however, occur, in the faecal samples from all treatments, indicating it may be a commensal member of the ostrich GIT. Faecal samples from day zero showed a high *Bifidobacterium* diversity with *B. pseudolongum* subsp. *pseudolongum, B. ruminatium, B. adolescentis* and *B. animalis* present. This variety decreased by week two to mainly *B. pseudolongum* subsp. *pseudolongum* with few *B. animalis.* Treatment C and ET showed three species present, *B. pseudolongum* subsp. *pseudolongum, B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis.* Treatment CT only showed the presence of *B. pseudolongum* subsp. *pseudolongum.* Petersson *et al.* (2009) showed that feeding pigs *Bifidobacterium* spp. resulted in more *Bifidobacterium*-related DGGE bands. This is not so in this study where feeding the ostrich chicks a probiotic mix including *B. pseudolongum* subsp. *pseudolongum* did not result in any significant differences when compared to the control treatments. Vasquez *et al.* (2009) found a similar low *Bifidobacterium* diversity in the caecal contents of 36 rats, all harbouring one dominant *Bifidobacterium* DGGE band which co-migrated with *B. pseudolongum* strain *patronus*.

Table 4.5. The closest relatives of the amplicons excised from DGGE gels using *Bifidobacterium*-specific primers. The results are based on BLAST searches**.**

Treatment	Band Number	16S rRNA gene sequence identification	% similarity	Accession Number
E day 0	1a	*Bifidobacterium ruminantium* strain KCTC 3425	100	GU361831.1
	1b	*Bifidobacterium adolescentis* strain LCR4	99	HQ259739.1
E week 1	2a	*Bifidobacterium pseudolongum* subsp. p*seudolongum*	98	AB507147.1
E week 2	3a, b	*Bifidobacterium animalis* subsp. *lactis*	99	HQ293104.1
E week 2	16a, c	*Bifidobacterium animalis* subsp. *lactis* strain NWL83	99	HQ293104.1
	16b	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	99	AB507147.1
C day 0	15a, b, c	*Bifidobacterium animalis* subsp. *lactis* strain NWL81	99	HQ293104.1
C week 1	4a	*Bifidobacterium animalis* subsp. *lactis*	100	HQ293104.1
	4b	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1
C week 1	5a	*Bifidobacterium animalis* subsp. *animalis*	98	GQ913435.1
C week 1	6a	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1
	6b	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	GU361829.1
C week 1	17a, b	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1
C week 2	18a, b, c	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	99	AB507147.1
C week 2	7a, b	*Bifidobacterium pseudolongum* subsp. p*seudolongum*	100	GU361829.1
ET day 0	9a	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1
ET week 1	10a	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1
	10b, c	*Bifidobacterium animalis* subsp. *animalis* strain TEEV	98	GQ913435.1
ET week 9	12a	*Bifidobacterium animalis* subsp. *lactis* strain NWL81	99	HQ293104.1
	12b, c	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	99	AB507147.1
CT week 2	19a	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1
CT week 4	14a, b	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	100	AB507147.1

The lack of *Bifidobacterium* diversity in the ostrich gut could be due to this set of primers not picking up ostrich specific species, or due to the ostrich GIT not having a diverse population of this genus, or the strains being below the detection level of PCR-DGGE. Janczyk *et al.* (2007) showed that *Bifidobacterium* 10^5 per gram digesta) compared to the high numbers of lactobacilli present (10^9 per gram chyme). Zhu *et al.*, (2002) investigated the chicken caecal microbiota and found *Bifidobacterium* to be present at less than 2% of the total bacteria. Zhu and Joerger (2003) confirmed this. In a study by Endo *et al.* (2010), *Bifidobacterium* were isolated from the faeces of only four out of nine herbivores (two springbok and two wallaby). Ostrich faecal material was tested for the presence of *Bifidobacterium*, but none were detected. The *Bifidobacterium* identified from these herbivores belonged to *B. pseudolongum*. This species is reported to be the most

common *Bifidobacterium* in animal faeces (Lamendella *et al.*, 2008) and possesses probiotic properties (Vasquez *et al.*, 2009).

When the sequences from probiotic strain Bp303.3.2 and *B. pseudolongum* subsp. *pseudolongum* (AB507147.1), detected here were aligned, only five mismatches were detected, whereas 19 mismatches were found between Bp303.3.2 and *B. pseudolongum* subsp. *pseudolongum* (GU361829.1; data not shown). This may indicate that the *Bifidobacterium* included in the probiotic mix was not detected in the faeces, but that this species is a commensal of the ostrich chick gut from as early as day zero, as it is frequently detected in all treatments. These results also suggest that ingesting a probiotic *Bifidobacterium* does increase the diversity of this species found in the gut, as seen in treatment E. This agrees with work by Petersson *et al.* (2009) who found more *Bifidobacterium*-related DGGE bands in pigs ingesting these bacteria. The only specific treatment-related pattern observed here is treatment CT which shows the presence of only one species of *Bifidobacterium*, suggesting tylosin does not favour the diversification of the genus.

4.5. Conclusion

The development of molecular techniques such as PCR-DGGE has allowed for more accurate and rapid microbial community profiling. Probiotic LAB and *Bifidobacterium* are common inhabitants of the gut and are generally regarded as markers of a healthy gut..

The effects of the probiotic and tylosin treatments analysed by the clustering of the DGGE banding patterns showed interesting results. Optimally, initial time points would have been shown to cluster together given the chicks had not started their respective treatments, and that 15 faecal samples had been pooled together to reduce intra chick variability. This grouping of day zero samples from different treatments was not the case in some instances, presumably because the chicks came from three different farms. Although the eggs are incubated in sterile incubators, once hatched the chicks would have been exposed to their environments and this, on top of their genetic influence, would affect their microbiota differently, as previously described (Zoetendal *et al.*, 2001). In addition, an individual's gut microbiota has been reported to show high intra-individual variation (Apajalahti *et al.*, 2004). In future, it

would be advantageous to use chicks from the same origin and batch to eliminate this variable as much as possible.

The effect of the various treatments on the diversity of bacterial populations found in faecal samples showed that feeding probiotics altered the diversity of total bacteria and the lactobacilli population when compared to the control chicks, as seen by the clustering of these two treatments observed. When compared to the control birds, ingesting probiotics did not affect the diversity of the bifidobacteria population. Tylosin causes a shift in total bacteria in chicks receiving probiotics but does not appear to affect the autochthonous total bacterial microbiota, as seen by the lack of clustering of groups C and CT. It is therefore assumed that tylosin affects the ingested probiotics. As the probiotics were shown to tolerate the level of tylosin used in this trial, the clustering may be due to tylosin enriching the probiotic strains, altering their proportions. Tylosin did not alter the lactobacilli population diversity when compared to the group only receiving probiotics, or the control group. When looking at the bifidobacteria population, tylosin did not alter the diversity of this bacterial group when tested on chicks receiving probiotics, but there was clustering observed for the control chicks receiving tylosin. Probiotics do not appear to change the diversity of the total bacteria, lactobacilli or bifidobacteria components of the microbiota of chicks receiving tylosin as no clustering according to treatment is observed for ET and CT. However, the effect of time on the development of the microbiota is evident from the way that weeks four, seven and nine form a cluster, as do day zero, week one and week two.

The bacterial richness measured by the number of bands per lane did not always agree with the Shannon-Wiener index of diversity. Given that the Shannon-Wiener is a more sensitive measure of diversity, our conclusions shall be drawn from it, rather than the band richness. Feeding the probiotic mix alone had no effect on the diversity of the total bacteria or the *Lactobacillus* spp. populations overtime. However, it did result in an increase in *Bifidobacterium* spp. from week one to two. This is in contrast to the control group which showed an increase in *Lactobacillus* spp. The combination of the probiotics and tylosin increased the total bacterial diversity and the *Bifidobacterium* spp., but decreased the *Lactobacillus* diversity. In comparison, the group tylosin alone increased the *Lactobacillus* spp. and had no effect on the *Bifidobacterium* spp. In conclusion, the probiotics, alone or together with tylosin increase the diversity of bifidobacteria, but reduce that of the lactobacilli.

When comparing treatments, the total bacteria show their highest prevalence in treatment ET at week one, compared to the other three treatments indicating this combination enhanced bacterial numbers in the gut microbiota. At week two, the control birds exhibited the highest lactobacilli prevalence and treatment E the highest bifidobacteria prevalence, showing that the probiotics alone enhance diversity of this genus, but not that of the lactobacilli. Hereafter, no differences between treatments were observed except at week nine where bifidobacteria were more prevalent in ET than CT.

The sequencing of excised DGGE bands returned results similar to those obtained by Matsui *et al.* (2010b) for ostrich caecal contents, showing similar bacterial group patterns, the dominant bacterial group being members of the *C. coccoides* and *C. leptum* group. However, *Bacteroidetes* were frequently isolated by Matsui *et al.*, (2010b) whereas in this current study, *Proteobacteria* and *Verrucomicrobia* were more common than *Bacteroidetes*. However, this study was a very limited study with respect to DGGE bands excised and identified by sequencing. A very much larger clone library would need to be included in the future to draw any conclusions on the bacteria present in the ostrich gut. No bands aligning with the *C. perfringens* strain included in the reference ladder were observed, as were no excised bands identified as this pathogen. This is interesting, given its role in ostrich chick mortality, however, this pathogen may not be a transient species and would therefore not be expected to be found in the faeces. The *L. plantarum* strains included in the probiotic mix were not detected in the faeces of the chicks receiving the mix, despite having been recovered from the day zero samples of treatment C. *B. pseudolongum* subsp. *pseudolongum* was the most frequently isolated *Bifidobacterium*, being detected in all treatments, indicating it may be a commensal member of the ostrich gut from the day of birth. The probiotic mix did appear to result in higher number of *Bifidobacterium* species and tylosin seemed to decrease the number of species found.

The limitations of the DGGE technique notwithstanding, one can tentatively claim that feeding the probiotics did not significantly alter the microbiota of the chicks, and the probiotics used here were not detected in the DGGE and could be due to their lack of ability to survive the GIT or, that they successfully adhere to the mucosa, and are not passed out in the faeces. Tylosin did not appear to have a detrimental effect on the bacteria in the ostrich gut when used in combination with the probiotic mix, but when used alone it appeared to limit the diversity of the *Bifidobacterium* population.

This was a limited sequence analysis of the ostrich faecal content, from the time of hatching to nine weeks. It gives a brief overview, but a much more comprehensive study of the bacteria present is necessary. The PCR amplicons generated using the primers described here are short. Longer amplicons would result in better identification of excised bands to allow for better differentiation between strains. The future of microbiota analysis lies with metagenomics, a powerful tool for expanding our knowledge of the communities harboured in the gut. Although identifying the bacterial groups present, it would be extremely useful to get a functional description of the microbiota, by detecting functional genes, for example those encoding proteins involved in cellulose degradation and butyrate production. Functional gene primers have been shown to be appropriate targets for DGGE to characterise subpopulations that are responsible for particular metabolic functions. Some examples already characterised with DGGE include methyl coenzyme-M reductase (Wilms *et al.*, 2007), dissimilatory sulfite reductase (Karr *et al.*, 2005; Geets *et al.*, 2006; Wilms *et al.*, 2007), monooxygenase genes including ammonia and methane monooxygenases (Oved *et al.*, 2001; Hoffmann *et al.*, 2002; Hendrickx *et al.*, 2006), dioxygenase genes (Morimoto *et al.*, 2005), and nitrite and nitrous oxide reductases (Throback *et al.*, 2004).

This chapter utilized DNA obtained from faecal samples. Discrepancies are thought to occur between faecal samples and mucosal samples (Zoetendal *et al.*, 2002), although Palmer *et al.* (2007) argue that the differences are minimal. It is therefore recommended to do a comparison of the two sample sites. Faecal matter poses a potential bias when it comes to genomic DNA extraction, the presence of certain insoluble molecules binding to bacteria and making them difficult to lyse (Walker *et al.*, 2008). In addition, faecal matter is thought to contain several PCR inhibitors which may lead to inaccurate representations of the bacterial communities present in the sample (de la Cochetière *et al.*, 2004).

Although DGGE has given a good representation of the diversity found in ostrich faeces, Green *et al.* (2009) report that DGGE is not ideal for monitoring highly diverse and complex bacterial communities such as those found in ostrich faeces. They are thought to give indistinct bands which may not be discernable, leading to smearing or poorly resolved patterns, as seen in the total bacterial DGGE gels. In addition, although the use of the diversity index, H', is useful in following the changes procured by different treatments, it is difficult to say whether the change in diversity is beneficial or detrimental to the ostrich gut. It is much more useful to follow the changes in population density of certain bacterial groups.

Therefore, a quantitative approach, fluorescent *in-situ* hybridisation (FISH), is used in the following chapter to obtain essential information on the fluctuations of important bacterial species found in the samples over time and according to treatment. Chapter five explores enumeration of important bacterial groups in both faecal and mucosal samples, with the intention of obtaining a more precise understanding of the ostrich microbiota and the abundance of certain bacterial groups.

CHAPTER 5

Investigation of the ostrich chick intestinal bacterial communities by 16S rRNA targeted fluorescent *in-situ* hybridisation and the observed differences when fed tylosin or a probiotic mix

Contents

5.1.**Summary** 154

5.2.**Introduction** 154

5.2.1 Aims and objectives of this study 157

5.3.**Methods and Materials** 158

5.3.1. Faecal collection 158

5.3.2. Housing of ostrich chicks 158

5.3.3. Gut mucosa sample collection 158

5.3.4. Fixation of faecal samples for FISH analysis 158

5.3.5. FISH 158

5.3.6. Statistical analysis 159

5.4.**Results and Discussion** 160

5.4.1. Total bacterial numbers in faecal samples from ostrich chicks 160

5.4.2. FISH enumeration of faecal samples over time 162

5.4.3. FISH enumeration of faecal samples between treatments 166

5.4.4. FISH enumeration of gut mucosal samples between two slaughter times 169

5.4.5. FISH enumeration of gut mucosal samples between treatments at each slaughter time 172

5.4.6. FISH enumeration of gut mucosal samples between different areas of the ostrich gastrointestinal tract 173

5.4.7. Comparison of microbiota from the GIT of various animals 175

5.5. **Conclusion** 177

5.1. Summary

Microorganisms in the gut of the ostrich are considered vital for their nutritional contribution as well as their health benefits to the host. The ostrich is a hindgut fermenter, possessing a well-developed, paired caecum and colon which allows for extensive microbial fibre digestion. Fluorescent *in situ* hybridization combined with flow cytometry (FISH-FC) was used in this study to quantify specific bacterial groups in ostrich faecal samples as well as gut mucosal scrapings from five days post-hatching up to nine weeks of age. The ostrich chicks were fed a control diet (C), a diet supplemented with a five strain probiotic mix (E), tylosin alone (CT) or probiotics and tylosin (ET). Faecal samples were collected from farmed ostrich chicks on the first day of the feeding trial, and one, two, four, seven and nine weeks thereafter. At two and five weeks old, one chick per pen was slaughtered and mucosal scrapings obtained from the duodenum, caecum and colon. These, along with the faecal samples, were analysed using seven bacterial group-specific probes, specifically chosen based on previous studies on the avian gut as well as knowledge on ostrich pathogens. The effect of feeding probiotics and the antibiotic, tylosin, as well as the change in the major bacterial groups, was observed with respect to age. Ostrich chicks were found to be rapidly colonised by high numbers of bacteria, having a total bacteria count in faecal matter of 10^8 cfu/g of faeces one week post-hatching. The faecal samples showed a shift of predominant bacteria, from the *C. coccoides*/*E. rectale* and *Bacteroides* groups, to *Bacteroides* by week seven. Studies on the gut samples showed the caecum and colon to be predominated by butyrate-producing *Clostridium* cluster XIVa and the duodenum mostly by *Bacteroides*. Both of these groups contain important fibre-digesting bacteria, producing short-chain fatty acids as their main by-products. These provide growing ostrich chicks with up to 76% of their metabolic energy. This is the first study using FISH-FC for enumeration of the ostrich gut microbiota.

5.2. Introduction

The commensal microbiota of the animal gastrointestinal tract (GIT) plays an important role in the health status of the host due to its nutritional, immunological and physiological functions (Rigottier-Gois *et al.*, 2003). Gut microbiota is influenced by different factors including age, environmental conditions, diet and dietary supplements such as probiotics, prebiotics or antibiotics. An altered bacterial community in the gut can lead to digestive disorders and possible microbial infections, the leading cause of economic loss and mortality

in farmed animals (Anadón *et al.*, 2006). *Clostridium perfringens* is a particularly important pathogen in many animals, causing necrotic enteritis and leading to severe and often fatal lesions in the gut (Songer, 1996). This cause of death is especially rampant in chicken and ostrich farming (Huchzermeyer, 2002; Hofacre *et al.*, 2003). Antibiotics are commonly used in animal farming to control these pathogenic infections, as well as for their growth promoting effects. In this study, the macrolide antibiotic, tylosin, was used (Chapter 3). Tylosin has a broad spectrum of activity against gram positive bacteria, and a narrower range against gram negatives (Shimada *et al.*, 2010). The enteric bacteria *E. coli* and *Salmonella* spp. are intrinsically resistant to tylosin (Shryock *et al.*, 1998). When used as a growth promoting agent, it is used at subtherapeutic levels (10-40mg/kg feed). These concentrations have an effect on the commensal microbiota which is not fully understood. However, Poole *et al.* (2003) concluded that the low levels of tylosin adversely affected the microbial ecology of the GIT in chickens, with respect to its ability to exclude exogenous and transient bacteria such as probiotics or other food-related bacteria. In this study, tylosin was administered to ostrich chicks at 20mg/kg feed for nine weeks for its growth promoting properties and the probiotics used in the trial were found to tolerate this concentration well (Chapter 3).

Fluorescent *in situ* hybridization (FISH) is a commonly used technique in microbial ecology and it has been applied for the detection of bacteria that cannot yet be cultivated. It utilizes specific regions of the 16S rRNA gene of the bacteria as a target for fluorescently labelled oligonucleotide probes, termed "phylogenetic stains" because of their unique character (Amann *et al.*, 1990b). By designing probes based on the 16S rRNA genes and some 23S rRNA genes, probe specificity can generally be freely adjusted, distinguishing species and subspecies by probe hybridisation to the most variable regions of the molecule (Woese *et al.*, 1985; Stahl *et al.*, 1988). Achieving this specificity in a complex community such as in the GIT, however, is difficult and therefore, probes are more frequently targeted at the different bacterial groups. By targeting areas of more highly conserved regions, probes can be used to include genera or taxons (Woese *et al.*, 1985) and areas which have remained basically unchanged between species, allow for the binding of a universal probe to measure total rRNA abundance (Stahl *et al.*, 1988). It allows for identification of subpopulations as well as their habitat and accurately enumerates defined cell populations (Vaughan *et al.*, 2000). Some disadvantages of this technique include: its dependence on SSU rDNA sequences which are available on the databases, the fact that only a few probes can be used together per analysis, and it is dependent on the permeability of the bacterial cell, accessibility of the target and the

number of ribosomes per cell (Zoetendal *et al.,* 2004). In addition, optimal hybridisation conditions need to be determined, taxa occurring in low abundance are not detected, bad signal/noise ratio occurs, biofilms and cell aggregates hamper manual counting, and only a few samples can be visually counted (Wagner *et al.*, 2003).

Flow cytometry is a method used for measuring selected physical and chemical characteristics of individual cells. Multiple parameters, such as forward and side scatter as well as measurement of fluorescence at different wavelengths, can be adjusted and used to measure a large number of cells in a very short time, allowing more samples to be analysed (Amann *et al.,* 1990). Combined with flow cytometry (FC), FISH provides a high throughput screening method, for accurate bacterial counting (Riggotier-Gois *et al.,* 2003). Further, FISH-FC offers the possibility of using multiple probes to simultaneously detect several bacterial groups (no overlapping of fluorochromes) while enabling reliable quantitation of bacteria present and has the added benefit of a low detection limit and cell sorting ability. Large scale studies have shown FISH-FC to be a reproducible method for microbiota assessment which provides additional information to that obtained by traditional culture techniques (Collado and Sanz, 2007). However, flow cytometry also has its downfalls, including relying on a machine to do the counting and the need for the microorganism to occur as single cells (or be able to be separated into single cells by pre-treatment). There are many studies on the use of FISH-FC on the human microbiome and probes are available for bacterial species in intestinal samples. About 90% of faecal bacteria have been estimated to be detectable with a set of just a few probes (Sghir *et al.,* 2000). There are limited reports on the microbiota of animal GITs, and very few on the ostrich microbiota (Melville *et al.,* 2004; Matsui *et al.,* 2009; Matsui *et al.,* 2010). However, the chicken model has been well characterised using several techniques including FISH-FC (Zhu and Joerger, 2003; Collado and Sanz, 2007; Lebeer *et al.,* 2010).

Characterisation of the microbiota is frequently done on faecal samples using techniques based on 16S rRNA amplicon analysis (Zoetendal, *et al.,* 2002). These studies have shown that the predominant bacterial communities in mammalian faeces are stable in time, host specific, affected by ageing and not changed by consumption of certain probiotic strains (Vaughan *et al.,* 1999; Simpson *et al.,* 2000; Zoetendal *et al.,* 2001). This technique does not, however, provide information on the spatial orientation of the specific bacteria in their niches in the GIT. In chickens, the GIT begins to be colonised by bacteria immediately after

hatching (Brisbin *et al.,* 2008) and the majority of the bacteria residing in the GIT are found in the caeca (Rehman *et al.,* 2007). There are numerous studies on the bacteria found in avian crops (Collado and Sanz, 2007; Gong, *et al.*, 2007; Lebeer *et al.,* 2010), but since ostriches do not have a crop, and also possess an enlarged caeca and an unusually long intestine of 14 meters (Swart *et al.,* 1993a), this study chose to examine the microbiota of three sections of the ostrich large intestine: the duodenum, the caecum and the colon.

5.2.1 Aims and objectives of this study

The aim of this chapter was to use the FISH-FC technique to quantify specific bacterial groups in faecal samples and gut mucosal scrapings, and to confirm the diversity profiles obtained previously by DGGE patterns (Chapter 4) and selective culture counts (Chapter 3). The total numbers of bacteria in faecal matter as well as in different areas of the gut were studied, and the effect of tylosin on the total bacteria as well as on specific bacterial taxa was observed. The probiotic strains used in this mix were shown to tolerate tylosin well, and grew similarly in the presence or absence of tylosin (Chapter 3). The probes were specifically chosen based on previous studies of the microbiota of the avian gut as well as knowledge of ostrich pathogens. Probe Eub338 was used for total bacterial counts as, to our knowledge; this is not known for ostriches. Probe Bif 164 and Lab 158 (Table 5.1) were used to enumerate the *Lactobacillus* and *Bifidobacterium* populations, as these two taxonomic groups are known to represent the health of a gut (Isolauri *et al.,* 2002) and they have beneficial properties which favour their use as probiotics. Probes Clit 135, Chis 150 and Erec 482 (Table 5.1) hybridise to bacteria from various *Clostridium* clusters. This phylum has been shown to be dominant in the avian gut and appears to play an important role. Chis 150 detects *Clostridium histolyticum* (clusters I and II), which includes *C. perfringens*. As this probe would not give data specific to this pathogen, Cperf 191 (Table 5.1) was included, to get a more accurate enumeration of this species as it was the main pathogen anticipated here. Clit 135 targets the *Clostridium lituseburense* as part of the *Clostridium* cluster XI, and includes detection of *Clostridium difficile*. The *Clostridium coccoides-Eubacterium rectale* group (cluster XIVa) was detected using probe Erec 482. Bac 303 (Table 5.1) covers the *Bacteroides* group, thought to be dominant in the GIT of most mammals. We also wanted to monitor the effect of probiotics and tylosin administration on the major bacterial groups in the gut and to follow the development of the gut microbiota with age, and to identify the

microbiota in different areas of the ostrich gut. For this, statistical analysis was done over time and between treatments.

5.3. Methods and Materials

5.3.1. Faecal collection

As described in chapter 3.

5.3.2. Housing of ostrich chicks

As described in chapter 3.

5.3.3. Gut mucosa sample collection

As described in chapter 3.

5.3.4. Fixation of faecal samples for FISH analysis

Fresh faecal material (0.1 g) was added to 1ml 1 x PBS and homogenised. The samples were centrifuged at 11000 rpm for 2 min, washed twice in filter-sterilized 1 x PBS and resuspended in 1 ml filter-sterilized 1 x PBS. This was thoroughly vortexed for 3 min and centrifuged at 4500 rpm for 20 – 30 sec. This step was to remove debris, such as grass, and reduce the background fluorescence when counting. The supernatant (0.375 ml) was then added to 1.125 ml chilled 4% (w/v) paraformaldehyde in a capped eppendorf and left at 4 °C for 18 h. The cells were collected by centrifugation at 22000 rpm for 5 min and washed once in filtered 1 x PBS and then resuspended in 300µl of 50% v/v ethanol/1 x PBS. The fixed cells were stored at -20 °C. Gut mucosal scrapings were fixed in the same way.

5.3.1. FISH

Bacterial group-specific and genus-specific 16S rRNA gene targeted oligonucleotide probes were commercially synthesized (Thermo Biosciences, Ulm, Germany) and monolabelled, at the 5' end, with FITC (Eub 338 probe) or Cy3 (the rest of the probes). The probes used in this study are listed in Table 5.1.

The specificity of these probes has previously been tested (see individual references). Specific cell enumeration was performed by combining the fixed cells with the Eub 338-

FITC probe as well as the genus- or group-specific Cy3 probes. Hybridization was performed as previously described (Kalliomäki *et al.*, 2008). In brief, fixed cell suspensions were hybridized with each fluorescent probe (50ng/µl) and 2 x hybridisation buffer (40mM/L Tris-HCl, 1.8M NaCl/L, 10% sodium dodecyl sulphate) for 18h at the respective hybridisation temperatures. The cells were collected after washing and resuspended in PBS. All experiments were conducted in duplicate and analysis of the samples was performed with the use of a BD LSR II flow cytometer (Becton Dickinson & Co, Franklin Lakes, NJ) equipped with a 488nm laser at 15mW. Data was analyzed with the use of BD FACSDIVA software, version 4.1.1. (Becton Dickinson & Co). Absolute bacterial cell counts were determined by addition of Flow-Count fluorospheres (Beckman Coulter, Fullerton, CA). To avoid loss of signal intensity, hybridized cells were kept in the dark until analyzed.

Table 5.1. Sequence and hybridisation temperatures of group specific 16S rRNA probes used in this study together with their respective target organisms

Probe	Sequence (3' – 5')	Hybridisation temp (°C)	Target	Reference
Eub 338	GCTGCCTCCCGTAGGAGT	50	Total bacteria	Amman *et al.* (1990)
Bif 164	CATCCGGCATTACCACCC	50	Most *Bifidobacterium* spp	Langendijk *et al.* (1995)
Lab 158	GGTATTAGCAYCTGTTTCCA	50	Most *Lactobacillus* spp *Weisella*, *Enterococcus*	Harmsen *et al.* (1999)
Clit 135	GTTATCCGTGTGTACAGGG	50	*Clostridium* cluster XI (*C. difficile)*	Franks *et al.* (1998)
Chis 150	TTATGCGGTATTAATCTYCC TTT	50	Most *Clostridium* cluster I and II (*C. perfringens*)	Franks *et al.* (1998)
Erec 482	GCTTCTTAGTCARGTACCG	50	Most *Clostridium* cluster XIVa	Franks *et al.* (1998)
Bac 303	CCAATGTGGGGGACCTT	45	*Cytophaga-Flavobacter-Bacteroides*	Manz *et al.* (1996)
Cperf 191	GCTCCTTTGGTTGAATGATG	35	*C. perfringens*	Fallani *et al.* (2006)

5.3.2. Statistical analysis

For the faecal sample analysis, all the variables were $\log_{10}$ transformed before data analysis. Associations between outcome variables and treatment were studied using mixed models

separately at all time points. Subjects within a pen were nested to minimise natural individual variation. The effect of time on outcome variables was also studied using mixed models separately at each treatment. The selection of covariance structure for these analyses was based on the comparisons of Akaike Information Criteria (AIC). Statistical analyses were done using SAS for Windows version 9.2.

The gut mucosal samples had the added variable of gut location. Again, all the variables were $\log_{10}$ transformed before data analysis. The effect of slaughter on the outcome variables was studied using mixed models with slaughter, treatment and interaction between slaughter and treatment as independent variables. Treatment was used as a repeated measures factor and the pen was used for intra-individual variation minimisation. These two variables were similarly taken into account when the effect of location on the outcome variables was studied. The effect of treatment on the outcome variables was studied separately at each slaughter using the pens to minimise natural variation. The selection of covariance structure for these analyses was based on the comparisons of Akaike Information Criteria (AIC). Statistical analyses were done using SAS for Windows version 9.2.

5.4. Results and Discussion

5.4.1. Total bacterial numbers in faecal samples from ostrich chicks

Faecal samples of ostrich chicks were analysed using FISH-FC, investigating the numbers and relative proportions of each of seven bacterial groups present in faecal material and mucosal samples over the time course of the feeding trial. The purpose of this was to obtain data on probiotic efficiency in outcompeting pathogens, their ability to persist in the gut and also to observe the effect of feeding tylosin on the endogenous microbiota, as well as on the ingested probiotics *in vivo*.

The number of cells hybridised to probe Eub338 using FISH are shown in Figure 5.1 and allowed for the proportion of species-specific bacterial groups to be calculated (see section 5.4.2 – 5.4.5). It also gives essential information on the ostrich gut microbiome, about which very little is published. Ostrich chicks appear to be rapidly colonised by gut bacteria, having a total bacteria count of 10^8 cfu/g (treatments C and E) and10^7 cfu/g of faeces (treatment CT and ET), one week after hatching. Collado and Sanz (2007) found two month old broiler chickens and 12 month old pigs to harbour approximately 10^8 cfu/g faeces throughout their GITs, enumerated using FISH.

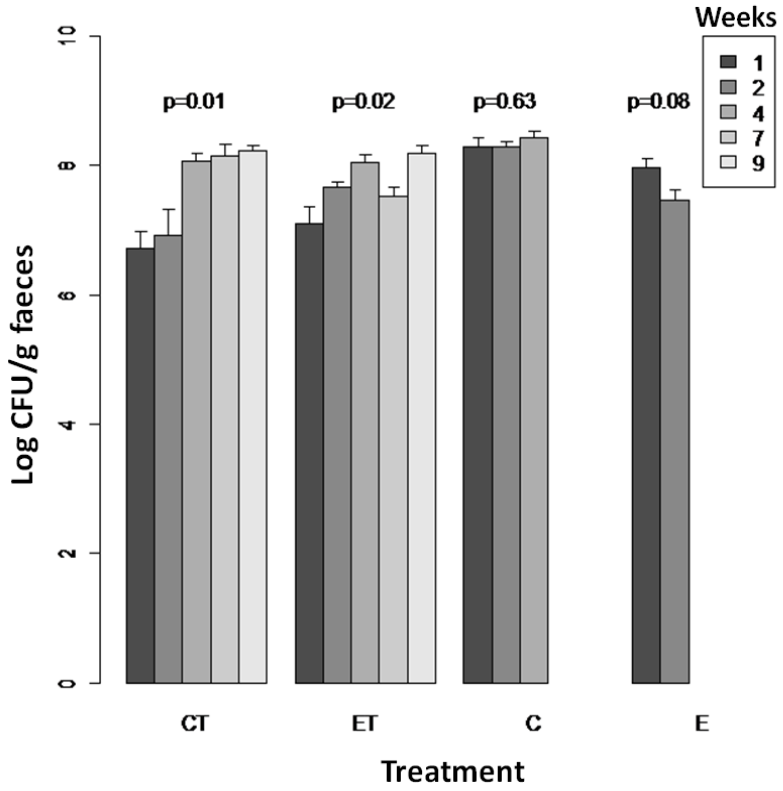

Figure 5.1 FISH assays showing total bacteria counts in faecal samples from ostrich chicks from week one to week nine of the feeding trial from newly-hatched chicks fed a control diet (C), tylosin (CT), probiotics (E) or probiotics and tylosin (ET) obtained using probe Eub 338. Values are the log of the average from two experiments done in duplicate and the error bars represent standard deviation from the mean. Statistical analysis showed any differences among the treatments over time ($p < 0.05$; n = 15).

The total bacterial count in the ostrich chicks appears to remain stable in chicks not treated with tylosin. However, due to mortality in these groups, the data for treatments E and C is only available up to week two and four, respectively, thus hindering the detection of any observable trends seen for the chicks not receiving tylosin, and making any comparisons to tylosin-receiving chicks impossible. Both treatments ET and CT showed an increase in total bacterial counts over time, having approximately 10^8 cfu/g of faeces by week nine, corresponding to data obtained for broiler chickens and pigs by Collado and Sanz (2007). Interestingly, after one week, treatments E and C have a ten-fold higher total bacterial count than the groups receiving the antibiotic suggesting that tylosin initially has an effect on bacterial numbers, lowering the total count. With time the microbiota levels or counts appear to be stable to the antibiotic effect. Suchodolski *et al.* (2009) suggested that antibiotics exhibit

their anti-diarrheal effects in dogs by either decreasing the total bacterial load in the gut or by altering the proportions of specific bacterial taxa present. They did not include enumerating total bacteria into their study and so could not make any conclusions from this. However, in this present study, it can be seen that tylosin does not lower the total bacteria load in the gut over time. Suchodolski *et al.* (2009) also showed an initial decrease in certain bacterial taxa initially, rebounding after 28 days of treatment with tylosin. The mucosal samples (data not shown) show similar total bacteria counts, but do not show any significant changes between the two slaughter times (week two and week five). The ostrich gut exhibits a lower total bacteria count than chickens, found to be between 10^9 and 10^{11} per gram of caecal contents three days post hatching, using nucleic acid staining and flow cytometry (Apajalahti *et al.*, 2002; Apajalahti *et al.*, 2004). This study demonstrates that tylosin does not have a detrimental effect on total bacteria populations.

5.4.2. FISH enumeration of faecal samples over time

Different bacterial groups in faecal samples were enumerated over time using FISH and the primers listed in Table 5.1. The primers chosen represent important bacterial taxa with regard to animal gut health, including commensal bacteria as well as pathogens, and they were monitored to evaluate the maturation of the ostrich microbiome and the effects of probiotics and tylosin on this.

The control group, C, which did not receive any treatment (Table 5.2A), showed a significant increase in *C. coccoides/E. rectale* group ($p = 0.0421$) and *C. perfringens* ($p = 0.008$). This is in contrast to the group fed probiotics (E) which showed no differences in these bacterial phyla suggesting the probiotics may prevent the growth or colonization of these clostridial bacteria. In addition, the *Bifidobacterium, Lactobacillus* and *Bacteroides* populations showed an increasing trend, although not statistically significant.

Group E, which received only the five strain probiotic mix (10^9 CFU/day), showed no significant differences (Table 5.2B) in the relative proportions of bacteria present for any of the bacterial groups tested over time. Again, the *Bifidobacterium, Bacteroides* and *C. perfringens* showed increasing trends from week one to week two. In contrast to the control group, E showed decreasing trends in *Lactobacillus* populations as well as bacteria hybridising to the Erec 482 probe. The animals in this group died after week two and post-mortems indicated pseudomembranous colitis with lesions resembling those caused by *C. perfringens* in the guts. It was thus assumed that this pathogen was the cause of death.

Table 5.2. Proportions of *Bifidobacterium* (Bif164), *Lactobacillus* (Lab158), *Clostridium* cluster XI including *C. difficile* (Clit135), *Clostridium* cluster XIVa including *C. coccoides* and *E. rectale* (Erec482), *Clostridium* cluster I and II including *C. perfringens* (Chis150), *Bacteroides* (Bac303) and specifically *C. perfringens* (C.perf 191)in faecal samples in the group fed the five strain probiotic mix (E), the group fed the five strain probiotic mix and the antibiotic Tylson (ET), the control group (C) and the control group fed Tylosin (CT). The relative proportions were calculated using the universal probe Eub 338 and the respective group specific probe. Results are reported as medians of cell numbers per gram of faecal sample; interquartile ranges in parentheses. Significant differences over time were calculated using mixed models, $P < 0.05$.

A

E			
Time point	**Wk 1**	**Wk 2**	**p value**
Probe			
Bif 164	8.4 (5.9,11.9)	10.6 (6.5,17.1)	**0.3311**
Lab 158	9.4 (6.3,14.0)	8.6 (5.0,14,6)	**0.7674**
Clit 135	6.5 (4.2,10.1)	4.5 (2.4,8.6)	**0.1216**
Erec 482	13.1 (8.4,20.5)	12.2 (8.7,17.2)	**0.5558**
Chis 150	7.1 (4.4,11.4)	7.5 (4.1,13.8)	**0.8132**
Bac 303	13.7 (8.0,23.7)	19.8 (12.6,31.0)	**0.4035**
Cperf 191	6.5 (4.1,10.1)	9.9 (5.4,18.2)	**0.4441**

B

ET						
Time point	**Wk 1**	**Wk 2**	**Wk 4**	**Wk 7**	**Wk 9**	**p value**
Probe						
Bif 164	11.5 (8.0,16.4)	8.6 (5.3,13.9)	7.7 (6.2,9.5)	6.7 (5.4,8.4)	5.4 (3.9,7.6)	**0.1711**
Lab 158	13.6 (9.2,20.3)	10.6 (6.2,18.1)	7.2 (4.8,11,0)	7.8 (5.0,12.2)	5.1 (3.5,7.2)	**0.0836**
Clit 135	12.0 (7.8,18.5)	8.4 (4.5,15.9)	6.2 (4.4,8.7)	8.3 (6.0,11,7)	8.6 (6.4,11.6)	**0.0713**
Erec 482	14.4 (9.2,22.5)	19.0 (13.5,26.6)	19.1 (13.6,26.7)	11.1 (8.3,14.9)	8.6 (6.4,11.5)	**0.0089**^{dfghi}
Chis 150	11.1 (7.0,17.9)	7.9 (4.3,14.5)	9.1 (7.3,11.5)	5.8 (3.4,9.9)	4.5 (3.4,5.6)	**0.0935**
Bac 303	24.8 (14.4,42.7)	15.1 (9.7,23.7)	14.6 (11.2,19.0)	15.3 (11.8,19.8)	19.5 (9.9,38.5)	**0.4694**
Cperf 191	11.9 (7.6,18.5)	10.7 (5.8,19.7)	5.0 (3.6,6.9)	7.8 (5.8,10.4)	4.8 (3.4,6.6)	**0.0031**^{bdeg}

C

C				
Time point	**Wk 1**	**Wk 2**	**Wk 4**	**p value**
Probe				
Bif 164	5.8 (4.1,8.3)	7.5 (4.6,12.2)	7.6 (6.1,9.4)	**0.0653**
Lab 158	5.2 (3.5,7.7)	6.0 (3.5,10.3)	8.6 (5.7,13.1)	**0.0566**
Clit 135	4.9 (3.2,7.6)	4.6 (2.5,8.7)	6.6 (4.7,9.2)	**0.4055**
Chis 150	5.1 (3.2,8.3)	8.0 (4.3,14.7)	7.7 (6.1,9.6)	**0.1389**
Bac 303	9.3 (5.4,16.1)	13.4 (8.5,21.0)	14.6 (11.2,19.1)	**0.1093**
Cperf 191	5.0 (3.2,7.8)	9.7 (5.2,17.8)	7.9 (5.7,10.9)	**0.0080**^{ab}

D

CT						
Time point	**Wk 1**	**Wk 2**	**Wk 4**	**Wk 7**	**Wk 9**	**p value**
Probe						
Bif 164	11.4 (8.0,16.2)	16.9 (10.4,27.3)	7.9 (6.3,9.8)	6.8 (5.4,8.4)	7.2 (5.2,10.1)	**0.0662**
Lab 158	11.7 (7.8,17.3)	21.0 (12.3,35.8)	7.8 (5.2,12.0)	5.9 (3.8,9.3)	6.3 (4.4,9.1)	**0.0441**^{cfg}
Clit 135	9.7 (6.3,15.0)	12.6 (6.7,23.8)	6.2 (4.4,8.7)	8.01 (5.9,10.8)	8.01 (5.9,10.8)	**0.4297**
Erec 482	15.9 (10.1,24.9)	22.2 (15.8,31.2)	16.6 (11.8,23.2)	11.0 (8.2,14.6)	9.8 (7.3,13.0)	**0.0583**
Chis 150	9.3 (5.8,15.0)	10.7 (5.8,19.8)	8.7(7.0,11.0)	8.2 (4.8,14.0)	6.0 (4.6,7.7)	**0.3513**
Bac 303	17.7 (10.3,30,6)	20.3 (13.0,31.9)	16.9 (13.0,22.1)	12.6 (9.7,16.4)	14.9 (7.6,29.5)	**0.4383**
Cperf 191	7.7(5.0,12.0)	13.6 (7.4,25.1)	6.5 (4.7,9.0)	6.0(4.5,8.0)	5.8 (4.2,8.1)	**0.4668**

The group receiving only tylosin supplementing their diet, CT (Table 5.2C), showed a significant decrease in proportions of *Lactobacillus* species (p = 0.0441) from week one to week seven, after an initial increase at week two. The same decreasing trend is seen for treatment ET, although it was not significant (p = 0.0836). This may imply a sensitivity to tylosin as this decrease was observed in treatment CT too. In contrast, treatment C showed an increasing trend in this bacterial group (close to significant, p = 0.0566) up to week four. Treatment CT also showed a decrease over time in the *C. coccoides/E. rectale* group which was almost significant (p = 0.0583), perhaps due to the high proportion of this group at week two and the decrease observed thereafter. Treatment ET shows a similar decrease in this group, although statistically significant (p = 0.0089), indicating the effect of tylosin. However, the control treatment also shows a decreasing trend from week two to week four, but not a significant difference.

The group, named ET, received the probiotic mix 10^9 CFU/day) and tylosin (Table 5.2D). This group showed significant decreases in the proportions of *C. coccoides/E. rectale* group (p = 0.0089) as well as for the *C. perfringens* group (p = 0.0031) over the feeding period. This infers a role of tylosin in decreasing this bacterial phylum, as well as the potential pathogen, *C. perfringens*. Treatment CT also showed a decreasing trend in *C. perfringens* after week two, although this was not statistically relevant. In contrast, the levels for this pathogen in treatment C were still significantly higher at weeks two and four than at week one. Young and Schmidt (2004) found that during the administration of a broad spectrum antibiotic in humans suffering from antibiotic-associated diarrhea, there was a significant decrease in butyrate-producing bacteria, most of which belong to *Clostridum* cluster XIVa, including *C. coccoides/E. rectale*. Treatment ET showed decreasing trends in the *Bifidobacterium* and *Lactobacillus* populations, but again these were not as statistically significant as the decrease in group CT, and may be a result of probiotic administration.

Since all the ostrich chicks in group E died after week two and by week four, this time frame was studied for all treatments to see if there were any trends, which may elucidate the cause of this mortality. At two weeks, Group E showed no significant differences for any of the bacteria tested, and was found to be predominated by *Bacteroides* (19.8%) whereas the other three treatments, C, CT and ET, were dominated by *C. coccoides* and *E. rectale* (22.4%, 22.2% and 19%, respectively) at this time point. In addition, Groups C, E and CT all showed an increase in *C. perfringens* at week two while treatment ET did not. By week four, both

treatments ET (p = 0.0065) and CT (p = 0.07) showed a significant decrease in *C. perfringens*, 10.7% to 5.0% and 13.6% to 6.5% respectively, half the amount seen at week two (Table 5.2D). This indicates that the administration of tylosin in the feed of these groups has a significant effect on the numbers of the pathogenic bacterium, *C. perfringens.* The incidence of the *C. perfringens* group is of particular interest given that this pathogen is a main cause of mortality in ostrich chicks (Huchzermeyer, 2002). Tylosin is well known for being an effective treatment against *C. perfringens* infection (Collier *et al.*, 2003; Martel *et al.*, 2004), but recent work has suggested that the overuse of this antibiotic as a prophylactic has lead to chronic resistance in this pathogen (Gharaibeh *et al.*, 2010).

The significant decrease in the *C. perfringens* levels in treatment ET, as opposed to those seen in CT, may suggest a protective role of the administered probiotics to the host against this pathogen, although the *Lactobacillus* numbers remained relatively constant in group ET. Gérad *et al.*, (2008) also found that feeding a *Lactobacillus* strain excluded *C. perfringens* from inhabiting the ceca of chickens. Adhesion studies performed on our specific probiotic mix showed it to be able to inhibit and displace *C. perfringens*, but unable to out compete it (Chapter 2). From our FISH results it appears this pathogen was present from the start of the feeding trial. This data suggests that feeding the probiotics alone did not offer sufficient protection against gut pathogens, resulting in the death of the chicks in treatment E after week two and those in group C after four weeks. It also reinforces the important role of tylosin in pathogen control. It appears that feeding chicks with the probiotics alone (group E) enhanced pathogen invasion (as also shown by adhesion-competition studies in Chapter 3). The adhesion studies suggest that if the probiotic mix could be administered before or early in *C. perfringens* infection, they would be able to inhibit or displace the pathogen. It appears in this case that the pathogens had already been acquired by the time the probiotics were fed to the chicks. The close proximity of the pens may have contributed to the spread of these pathogenic bacteria.

The effect of tylosin on the autochthonous microbiota (group CT) only showed a significant decrease in *Lactobacillus* over the feeding trial. The effects of tylosin do not appear immediately. Only by week four is there a decrease in *Bifidobacterium* and *Lactobacillus* spp., as well as *Bacteroides* and *C. perfringens.* Wagner and Cerniglia (2005) showed L. *salivarius* and *L. ruminus* of human origin to be sensitive to tylosin, but Collier *et al.* (2003a) showed that tylosin treatment of pigs increased the percentage of lactobacilli present

after two weeks, specifically *L. gasseri*, found to be particularly resistant to tylosin. In our study, tylosin fed together with the probiotic mix initially has a detrimental effect on the *Bifidobacterium* spp., but over the nine weeks, significantly decreased *C. perfringens* and the *C. coccoides*/*E. rectale* group, agreeing with work by Collier *et al.* (2003b).

In conclusion, tylosin fed together with the five strain probiotic mix (three *Lactobacillus plantarum*, one *L. reuteri* and one *Bifidobacterium pseudolongum* subsp. *pseudolongum*), successfully decreased the *C. perfringens* levels over time in faecal samples. The probiotic/antibiotic combination significantly decreased *C. coccoides/E. rectale* numbers with time, and when the antibiotic was fed alone, it had a decreasing effect on the *Lactobacillus* population. The number of *C. perfringens* and *C. coccoides/E. rectale* increased in the control group with time and the birds in this control group died at week four, with the chicks showing similar lesions in the gut to those for group E. Due to the nature of the observed symptoms the death of the chicks were attributed to *C. perfringens* infection.

5.4.3. FISH enumeration of faecal samples between treatments

Comparing the relative proportions of each bacterial group between treatments (C, E, CT and ET) at week one (Table 5.3A), we found that ET and CT treatments showed significantly higher *Bifidobacterium* species compared to control group C ($p = 0.011$ and $p = 0.0117$, respectively), indicating that tylosin may be involved in selectively enriching for *Bifidobacterium* during early colonisation. Hereafter, the frequency of *Bifidobacterium* does not differ significantly between groups. Week one also displayed a significant increase in *Lactobacillus* for groups E, ET and CT when compared to C ($p = 0.0375$, $p = 0.0018$ and $p = 0.0067$, respectively) suggesting that either the probiotics or tylosin, or both, play a role in increasing these bacterial proportions. A higher percentage of the *Clostridium cluster* XI was observed for ET and CT when compared to C ($p = 0.0067$ and $p = 0.032$, respectively). Treatment ET shows a close to significant increase in this bacterial group when compared to E ($p = 0.0523$). *C. difficile* is an important pathogen which is included in this cluster and it is interesting to note that tylosin appears to increase the prevalence of this group. However, the probe was not specifically designed to detect *C. difficile.*

In week two (Table 5.3B), bifidobacteria were present at a higher frequency in treatment CT than in both C and ET ($p = 0.0224$ and $p = 0.052$), again suggesting the *Bifidobacterium* enriching role of tylosin. The *Lactobacillus* group was more common in CT than groups E ($p = 0.0226$) and C ($p = 0.0026$), again inferring a role of tylosin on this phylum, agreeing

with previous work showing that it increases the number of *Lactobacillus* when administered to pigs (Collier *et al.*, 2003a). Although no overall significant differences were observed for the *Clostridium* cluster XI, CT harboured a significantly higher prevalence of this group than C and E (p = 0.0304 and p = 0.0278), as in week one. At this time point, treatment E also showed a significantly lower prevalence of the *C. coccoides*/*E. rectale* group than C and CT (p = 0.0157 and 0.0174, respectively), showing that the probiotics had a detrimental effect on this beneficial, mostly butyrate producing bacterial group.

Table 5.3. (A to E) Proportions of *Bifidobacterium* (Bif164), *Lactobacillus* (Lab158), *Clostridium* cluster XI including *C. difficile* (Clit135), *Clostridium* cluster XIVa including *C. coccoides* and *E. rectale* (Erec482), *Clostridium* cluster I and II including *C. perfringens* (Chis150), *Bacteroides* (Bac303) and specifically *C. perfringens* (C.perf 191)in faecal samples in the group fed the five strain probiotic mix (E), the group fed the five strain probiotic mix and the antibiotic Tylosin (ET), the control group (C) and the control group fed Tylosin (CT). The relative proportions were calculated using the universal probe Eub 338 and the respective group specific probe. Results are reported as medians of cell numbers per gram of faecal sample; interquartile ranges in parentheses. Significant differences over time were calculated using mixed models, $P < 0.05$, $n = 15$. Key: a. C vs CT; b. C vs E; c. C vs ET; d. CT vs E; e. CT vs ET; f. E vs ET.

A

	Week 1				
	E	**ET**	**C**	**CT**	**p value**
Probe					
Bif 164	8.4 (5.9,11.9)	11.5 (8.0,16.4)	5.8 (4.1,8.3)	11.4 (8.0,16.2)	**0.0338**[ac]
Lab 158	9.4 (6.3,14.0)	13.6 (9.2,20.3)	5.2 (3.5,7.7)	11.7 (7.8,17.3)	**0.0095**[abc]
Clit 135	6.5 (4.2,10.1)	12.0 (7.8,18.5)	5.0 (3.2,7.6)	9.7 (6.3,15.0)	**0.0298**[ac]
Erec 482	13.1 (8.4,20.5)	14.4 (9.2,22.5)	12.1 (7.7,18.9)	15.9 (10.1,24.9)	**0.8229**
Chis 150	7.1 (4.4,11.4)	11.1 (6.9,17.9)	5.1 (3.2,8.3)	9.3 (5.8,15.0)	**0.1217**
Bac 303	13.7 (8.0,23.7)	24.8 (14.4,42,7)	9.3 (5.4,16.1)	17.7 (10.3,30.6)	**0.0906**
Cperf 191	6.5 (4.1,10.1)	11.9 (7.6,18.5)	5.0 (3.2,7.8)	7.7 (5.0,12.0)	**0.0601**

B

	Week 2				
	E	**ET**	**C**	**CT**	**p value**
Probe					
Bif 164	10.6 (6.5,17.1)	8.6 (5.3,13,9)	7.5 (4.6,12.2)	16.9 (10.4,27.3)	**0.1028**
Lab 158	8.6 (5.0,14.6)	10.6 (6.2,18.1)	6.0 (3.5,10.3)	21.0 (12.3,35.7)	**0.0186**[ad]
Clit 135	4.5 (2.4,8.6)	8.4 (4.5,15.9)	4.6 (2.5,8.7)	12.6 (6.7,23.8)	**0.0762**
Erec 482	12.2 (8.7,17.2)	19.0 (13.5,26.6)	22.4 (16.0,31.5)	22.2 (15.8,31.2)	**0.0519**
Chis 150	7.5 (4.1,13.8)	7.9 (4.3,14.5)	8.0 (4.3,14.7)	10.7 (5.8,19.8)	**0.8175**
Bac 303	19.8 (12.6,31.0)	15.1 (9.7,23.7)	13.4 (8.5,21.0)	20.3 (13.0,31.8)	**0.4575**
Cperf 191	9.9 (5.4,18.2)	10.7 (5.8,19.7)	9.7 (5.2,17.8)	13.6 (7.4,25.1)	**0.8378**

C

	Week 4			
	ET	**C**	**CT**	**p value**
Probe				
Bif 164	7.7 (6.2,9.5)	7.6 (6.1,9.4)	7.9 (6.3,9.8)	**0.9619**
Lab 158	7.2 (4.8,11.0)	8.6 (5.7,13.1)	7.8 (5.2,12.0)	**0.8232**
Clit 135	6.2 (4.4,8.7)	6.6 (4.7,9.2)	6.2 (4.4,8.7)	**0.9548**
Erec 482	19.1 (13.6,26.7)	17.3 (12.4,24.2)	16.6 (11.8,23.2)	**0.8124**
Chis 150	9.1 (7.3,11.5)	7.7 (6.1,9.6)	8.7 (7.0,11.0)	**0.4849**
Bac 303	14.6 (11.2,19.0)	14.6 (11.2,19.1)	16.9 (13.0,22.1)	**0.6387**
Cperf 191	5.0 (3.6,6.9)	7.9 (5.7,10.9)	6.5 (4.7,9.0)	**0.1364**

D

	Week 7		
	ET	**CT**	**p value**
Probe			
Bif 164	6.7 (5.4,8.4)	6.8 (5.4,8.4)	**0.9624**
Lab 158	7.8 (5.0,12.2)	5.9 (3.8,9.3)	**0.3569**
Clit 135	8.4 (6.0,11.7)	8.8 (6.3,12.2)	**0.8331**
Erec 482	11.1 (8.3,14.9)	10.9 (8.2,14.6)	**0.9170**
Chis 150	5.8 (3.4,9.9)	8.2 (4.8,14.0)	**0.3188**
Bac 303	15.3 (11.8,19.8)	12.6 (9.7,16.4)	**0.2745**
Cperf 191	7.8 (5.8,10.4)	6.0 (4.5,8.0)	**0.1867**

E

	Week 9		
	ET	**CT**	**p value**
Probe			
Bif 164	5.5 (3.9,7.6)	7.2 (5.2,10.1)	**0.2062**
Lab 158	5.1 (3.5,7.2)	6.3 (4.4,9.1)	**0.3419**
Clit 135	8.6 (6.4,11.6)	8.0 (5.9,10.8)	**0.7085**
Erec 482	8.6 (6.4,11.5)	9.8 (7.3,13.0)	**0.5026**
Chis 150	4.4 (3.4,5.6)	6.0 (4.6,7.7)	**0.0798**
Bac 303	19.5 (9.9,38.5)	14.9 (7.6,29.5)	**0.5500**
Cperf 191	4.8 (3.4,6.6)	5.8 (4.2,8.1)	**0.3658**

Initially, chicks in treatments E, ET and CT are dominated by *Bacteroides* (13.7; 24.8 and 17.7%, respectively) and treatment C by the *C. coccoides/E. rectale* group (12.1%). By week two, treatments ET, C and CT, were dominated by the *C. coccoides/E. rectale* group (19.0; 22.4 and 22.2% respectively), with treatment E still being dominated by *Bacteroides*. From week seven onwards, the *Bacteroides* group was the dominant gut bacteria of those tested in this study, suggesting that this may be the dominant group of bacteria in ostriches. Work by Zhu and Joerger (2003) found a similar trend in broiler chicken caeca, where the highest percentage of bacteria hybridised to the *C. coccoides/E. rectale* probe at two weeks, and decreased thereafter. Similar to this study, they also found that *Bacteroides* and *Bifidobacterium* groups proportions did not change from one week old onwards, although the proportions of these bacteria were much lower (3 – 6% for the chicken vs 14.4 – 39.9% for the ostrich chick and 3 – 8% for chickens vs 9.8 – 13.4% for ostrich chicks) respectively.

Although there were few significant differences seen between treatments, it was always treatment C which showed the lowest proportions of the tested bacterial group, except in week two where both E and C showed significantly lower levels of *Lactobacillus* than CT. In week one, ET and CT show higher proportions of *Bifidobacterium* and *Lactobacillus.* Again, this suggests that tylosin may select *Bifidobacterium* and *Lactobacillus,* agreeing with the theory that this antibiotic enriches potentially beneficial bacteria (Suchodolski *et al.*, 2009), and that feeding probiotics containing these two species may help to maintain the *Lactobacillus* level. From week four onwards, the microbiota in both surviving groups, ET and CT, appears to have stabilised. It is unknown whether it was the tylosin which stabilised the microbiota or if it occurred naturally as the control groups E and C had both died by this stage.

5.4.4. FISH enumeration of gut mucosal samples between two slaughter times

Mucosal scrapings from three areas of the ostrich GIT, namely, the duodenum, the caecum and the colon, were collected at two time points (week two and week five), when one chick from each pen was slaughtered by cervical dislocation and the mucosal scrapings were analysed using FISH and the probes listed in Table 5.1. This aimed to get an insight into the autochthonous microbiota as well as to test the adhesion capabilities of the probiotics, and to see if the ingested probiotics were transient or adhesive to the gut mucosa. Treatment E could not be included in this section of work since the group had died after week two. Also, the results over time and between treatments are reported, excluding the location on which the

FISH was performed. This is because when the effect of location, together with the slaughter time was compared, no significant differences were seen.

At slaughter one (two weeks of age), the colon and caecum samples were shown to harbour 10^8 cells/g mucosa compared to the duodenum, having 10^7 cells/g mucosa. At slaughter two (five weeks of age), the colon and caecum had 10^9 cells/g mucosa and the duodenum had 10^8 cells/g mucosa. The enumeration of bacterial species between slaughters (at week two and week five; Table 5.4A) showed a significant increase in the *Clostridium* cluster XI group for treatments C and ET ($p = 0.01$ and 0.002, respectively), and the *C. coccoides/E. rectale* group for C ($p = 0.001$), ET ($p < 0.001$) and CT ($p = 0.002$). By week five, this phylum was now the dominant group in the gut, followed by the *Bacteroides* group, which significantly increased for treatments C and ET ($p = 0.02$ and 0.03, respectively). *C. perfringens* increased significantly for treatments C ($p = 0.01$) and ET ($p = 0.02$), and almost significantly for treatment C ($p = 0.06$). It is interesting to note that birds from treatments C and ET showed an increase in proportions of both important pathogens, i.e. *C. difficile* and *C. perfringens.* Although treatment CT did not show a significant increase in *C. perfringens*, it showed an increasing trend ($p = 0.0582$). This does not correspond with the previous findings in this study where tylosin was found to decrease the number of *C. perfringens* in the faeces and may suggest that *C. perfringens* was less prevalent in the faecal samples as they adhere well to the gut mucosa. Gérard *et al.*, (2008) fed broiler chickens a *Lactobacillus* strain and gut samples were taken four and 19 days after the start of the trial. At 19 days a significant decrease ($p < 0.05$) was observed for the *C. coccoides/E. rectale* group, a significant increase in the *Faecalibacterium prausnitzii* group, and a significant increase in *Bacteroides*, between the experimental and the control group were observed. In the case of the ostrich chicks, *Bacteroides* also increased significantly by week five, becoming one of the predominant species. In chickens, *F. prausnitzii* is the dominant species present at this time point, with the *C. coccoides/E. rectale* group decreasing over time, as opposed to the increase seen in ostrich chicks. The increase in the presence of *C. coccoides/E. rectale* for groups CT and ET may be due to the tylosin, and in treatment C due to the lack of feeding probiotics. Gérard *et al.* (2008) fed *Lactobacillus* to broiler chickens and found they decreased the counts of *C.* coccoides/*E. rectale* in the gut.

Table 5.4 (A and B). Proportions of *Bifidobacterium* (Bif164), *Lactobacillus* (Lab158), *Clostridium* cluster XI including *C. difficile* (Clit135), most of *Clostridium* cluster XIVa including *C. coccoides* and *E. rectale* (Erec482), most of *Clostridium* cluster I and II including *C. perfringens* (Chis150), *Bacteroides* (Bac303) and specifically *C. perfringens* (C.perf 191)in gut biopsies in the group fed the five strain probiotic mix (E), where applicable, the group fed the five strain probiotic mix and the antibiotic tylosin (ET), the control group (C) and the control group fed tylosin (CT). The relative proportions were calculated using the universal probe Eub 338 and the respective group specific probe. Results are reported as medians of cell numbers per gram of faecal sample; interquartile ranges in parentheses . Significant differences between treatments were calculated using mixed models, $P < 0.05$, $n = 18$.

A

Between slaughters				
Probe	**Treatment**	**Slaughter 1**	**Slaughter 2**	**p value**
Bif 164	**C**	10.5 (6.8, 16.2)	11.4 (7.4, 17.6)	**0.78**
	CT	13.4 (8.7, 20.7)	12.1 (7.9, 18.6)	**0.72**
	ET	9.8 (6.4, 15.2)	12.1 (7.9, 18.6)	**0.49**
Lab 158	**C**	13.2 (8.8, 19.8)	13.8 (9.2, 20.7)	**0.88**
	CT	10.5 (7.0, 15.8)	15.5 (10.0, 23.3)	**0.20**
	ET	10.7 (7.1, 16.0)	15 (7.1, 16.0)	**0.26**
Clit 135	**C**	17.3 (12.0, 25.0)	36.3 (25.1, 52.6)	**0.01**
	CT	23.2 (16.1, 33.6)	35.4 (24.4, 51.2)	**0.14**
	ET	13.7 (9.4, 19.8)	35.9 (24.8, 51.9)	**0.002**
Erec 482	**C**	19.8 (14.3, 27.2)	45.9 (33.3, 63.3)	**0.001**
	CT	20.3 (14.8, 28.0)	44.4 (32.2, 61.2)	**0.002**
	ET	16.5 (12.0, 22.7)	46.9 (34.0, 61.6)	**<0.001**
Chis 150	**C**	9.3 (6.5, 13.2)	10.1 (7.0, 14.3)	**0.74**
	CT	8.5 (5.9, 12.1)	12.4 (8.7, 17.8)	**0.13**
	ET	6.5 (4.6, 9.3)	10.1 (7.1, 14.5)	**0.09**
		14.4 (9.0, 23.1)	35.6 (22.2, 57.1)	
Bac 303	**C**			**0.02**
	CT	21.1 (13.2, 33.9)	37.6 (23.4, 60.2)	**0.12**
	ET	17.7 (11., 28.4)	39.9 (24.9, 64.0)	**0.03**
Cperf		10 (6.4, 15.8)	24.5 (15.6, 38.7)	
191	**C**			**0.01**
	CT	13.6 (8.6, 21.5)	26.0 (16.5, 41.0)	**0.06**
		10.6 (6.7, 16.7)	24 (15.2, 37.8)	
	ET			**0.02**

B

Between treatments			
Probe	**Treatment**	**Slaughter 1**	**Slaughter 2**
Bif 164	C	10.5 (6.0, 18.5)	11.4 (8.6, 15.1)
	CT	13.4 (7.7, 23.6)	12.1 (9.2, 16.0)
	E	14.4 (8.3, 25.4)	
	ET	9.8 (5.6, 17.3)	12.1 (9.2, 16.0)
	p value	**0.71**	**0.94**
Lab 158	C	13.2 (7.9, 22.1)	13.8 (10.4, 18.2)
	CT	10.5 (6.3, 17.6)	15.5 (11.7, 20.6)
	E	13.6 (8.1, 22.7)	
	ET	10.7 (6.4, 17.9)	15.0 (11.3, 19.8)
	p value	**0.84**	**0.82**
Clit 135	C	17.3 (8.8, 33.9)	36.3 (28.5, 46.3)
	CT	23.2 (11.8, 45.6)	35.4 (27.8, 45.1)
	E	26.4 (13.5, 51.9)	
	ET	13.7 (7.0, 26.8)	35.9 (28.1, 45.7)
	p value	**0.43**	**0.99**
Erec		19.8 (12.9, 30.3)	45.9 (36.8, 57.2)
482	C		
	CT	20.3 (13.3, 31.2)	44.4 (35.7, 55.4)
	E	22.1 (14.4, 33.9)	
		16.5 (10.8, 25.2)	46.9 (37.6, 58.4)
	ET		
	p value	**0.79**	**0.94**
Chis		9.3 (6.3, 13.6)	10.1 (6.4, 15.8)
150	C		
	CT	8.5 (5.8, 12.4)	12.4 (7.9, 19.5)
	E	9.0 (6.1, 13.3)	
	ET	6.5 (4.4, 9.6)	10.1 (6.5, 15.9)
	p value	**0.55**	**0.67**
Bac 303	C	14.4 (8.1, 25.8)	35.6 (25.8, 49.1)
	CT	21.1 (11.8, 37.7)	37.6 (27.2, 51.8)
	E	19.6 (11.0, 34.9)	
	ET	17.7 (9.9, 31.6)	39.9 (28.9, 55.1)
	p value	**0.80**	**0.87**
Cperf		10.0 (5.3, 19.0)	24.5 (15.6, 38.6)
191	C		
	CT	13.6 (7.2, 25.8)	26.0 (16.5, 40.9)
	E	10.1 (5.4, 19.1)	
	ET	10.6 (5.6, 20.0)	24.0 (15.3, 37.7)
	p value	**0.88**	**0.95**

The control group C, as well as treatment ET, showed significant increases in the same bacterial taxa (*Clostridia* cluster XI, *C. coccoides* and *E. rectale*, *Bacteroides* and *C. perfringens*; Table 5.4A). The reason that treatment C died soon after the second slaughter point is therefore unclear. All three treatments showed increases in *C. coccoides/E. rectale*

suggesting this bacterial group is not controlled by tylosin or the combination of antibiotic and probiotic. Most members of this bacterial group (*Clostridium* cluster XIVa) are butyrate producers and are therefore assumed to be important members of the ostrich microbiota, given the fact that ostriches derive approximately 76% of their metabolisable energy from volatile fatty acids such as butyrate (Swart *et al.*, 1993b).

At two weeks, the gut mucosal samples were dominated by *C. coccoides/E. rectale* (treatment C), the *Clostridium* cluster XI (treatment CT) and *Bacteroides* (treatment ET; Table 5.4A). By week five, all three treatments were dominated by *C. coccoides/E. rectale* (Table 5.4A). *Bacteroides* and *Clostridium* cluster XI were found to be the second predominant group of bacteria in the gut of ostrich chicks by week five. This differs from the faecal samples (Table 5.3), week two and four dominated by *C. coccoides/E. rectale* with the exception of treatment CT which shows an equal prevalence of *Bacteroides* and *C. coccoides/E. rectale* at week four. This initial difference could be due to the adhesion capabilities of *C. coccoides/E. rectale,* being flushed out to begin with, but adhering well by week four/week five. It would have been useful to take another gut mucosal scraping at week nine to truly compare the patterns observed between the gut and faecal counts.

5.4.5. FISH enumeration of gut mucosal samples between treatments at each slaughter time

In an attempt to monitor the change in microbiota of ostrich chicks with age, ie week two versus week five (Table 5.4B), the effect of each treatment at the two slaughter times was compared. No significant differences were observed for any bacterial phyla tested. This may suggest that the microbiota of the ostrich chick is settled by week two or that the ingested probiotics were transient in the ostrich gut, not adhering to the gut mucosa. This is unlikely as DGGE analysis combined with 16S rRNA sequencing of excised bands on faecal samples in the previous chapter, did not detect either of the two *Lactobacillus* species included in the probiotic mix. Previous work by Poole *et al.* (2003) suggested that once the bacterial population has equilibrated, it becomes very difficult for exogenous strains to colonise. This may be due to the diversity of inhibitory products such as volatile fatty acids or bacteriocins which could act on exogenous bacteria faster than they can adapt. It may also imply that tylosin has no effect on the bacteria which are adhered to the gut wall.

Although at week two, there were no clear differences between proportions of the bacteria in the gut scrapings compared to the faecal counts, with the exception of the group that

hybridised to probe Clit 135 showed a higher prevalence in the gut samples (Table 5.4B) than in the faecal samples (Table 5.3B), but by week five, all bacterial groups showed higher prevalence in the gut samples than in the faecal samples. This may suggest that all the bacterial groups studied here, adhere well to the gut mucosa as fewer counts were found in the faecal samples. Zoetendal *et al.,* (2002) also found that mucosa-associated bacterial communities differed significantly from faecal samples. They propose that since biopsy samples are small and therefore more easily exposed to oxygen during sampling, a highly reduced number of strict anaerobes may be found in the gut than in the faecal samples, which are larger and offer more protection to the inside of the sample from oxygen exposure. True to this, Zoetendal *et al.,* (2002) found more facultative anaerobes in the biopsy samples than in the faecal samples. In this current study this is not so as most of the bacterial groups tested here are strict anaerobes, and higher proportions of each taxon were detected in mucosal samples than in faecal samples. This may be due to efficient sampling, with mucosal samples being rapidly transferred into a mixture of PBS and paraformaldehyde, immediately after scraping the GIT. The faecal samples were exposed to oxygen for longer periods of time before collection, and although samples were obtained from the centre of the faecal matter, oxygen damage may have occurred throughout the faecal sample. Marteau *et al.,* (2001) also found that the caecal microbiota differs qualitatively and quantitatively from the faecal microbiota, harbouring fewer strict anaerobes such as *Bacteroides* and *Bifidobacterium.* They also found that the facultative anaerobes did not differ significantly between the faeces and biopsies.

5.4.6. FISH enumeration of gut mucosal samples between different areas of the ostrich gastrointestinal tract

The ostrich has an unusually long hindgut (approximately 11m in length) with well developed, sacculated ceca, and a capacious, haustrated colon (Cho *et al.,* 1984; Swart *et al.,* 1993), and although the hindgut is known to be the site of fibre digestion (Matsui *et al.,* 2010), little is known about the microbial diversity present. In this study, the differences in microbiota were studied between three different areas of the ostrich gut to investigate the predominance of each bacterial group tested in the caecum, the colon and the duodenum (Table 5.5). Interestingly, the proportions of each tested bacterial group in the duodenum were significantly higher than in the colon and caecum. In the case of the groups hybridising to probes Clit 135 and Erec 482, higher values were also obtained in the duodenum, but not

by significant amounts. Also, the *Bacteroides* group only differed significantly between the colon and duodenum. This is surprising as the duodenum would be expected to harbour more facultative anaerobes than strict anaerobes such as the bacteria hybridising to the probes Bac 303, Clit 135, Cperf 191 and Erec 482 and Chis 150. The caecum and colon were both predominantly *C. coccoides/E. rectale* group (24.4 and 30.4%, respectively), followed by the *Clostridium* cluster XI (25.5% and 20.8%, respectively). Since the hindgut is the site of fibre digestion (Matsui *et al.*, 2010), it would be expected to be dominated by fibre-digesting and butyrate-producing bacteria. Most of the strains belonging to *C. coccoides/E. rectale* perform both these functions. The duodenum, however, was dominated by the *Bacteroides* group (34.7%) followed by the *C. coccoides/E. rectale* group (31.7%) and *Clostridium* cluster XI (30.3%). The actual numbers of each bacterial group tested are significantly lower in the duodenum than in the caecum and colon, in accordance with previous work which shows that the pig and chicken duodenum harbours fewer bacteria than the large intestine (Collado and Sanz, 2007).

Table 5.5. Proportions of *Bifidobacterium* (Bif164), *Lactobacillus* (Lab158), *Clostridium* cluster XI including *C. difficile* (Clit135), *Clostridium* cluster XIVa including *C. coccoides* and *E. rectale* (Erec482), *Clostridium* cluster I and II including *C. perfringens* (Chis150), *Bacteroides* (Bac303) and specifically *C. perfringens* (C.perf 191) in gut biopsies of ostrich chicks from all treatments at both slaughter times (week two and week five). The relative proportions were calculated using the universal probe Eub 338 and the respective group specific probe. Results are reported as medians of cell numbers per gram of faecal sample; interquartile ranges in parentheses. Significant differences between treatments were calculated using mixed models, $P < 0.05$, $n = 18$. Key a. caecum vs colon; b. caecum vs duodenum; c. colon vs duodenum.

Between gut locations				
Probe	Caecum	Colon	Duodenum	**p value**
Bif 164	9.6 (7.6, 12.3)	9.1 (7.1, 11.6)	19.0 (14.9, 24.2)	$<0.001^{bc}$
Lab 158	11.2 (8.8, 14.2)	11.1 (8.7, 14.1)	17.9 (14.0, 22.8)	0.01^{bc}
Clit 135	25.5 (19.9, 32.8)	20.8 (16.2, 26.7)	30.3 (23.6, 38.9)	0.11
Erec 482	30.4 (24.5, 37.6)	24.4 (19.7, 30.3)	31.7 (25.6, 39.3)	0.19
Chis 150	8.0 (6.6, 9.7)	7.9 (6.5, 9.6)	12.6 (10.4, 15.4)	0.002^{bc}
Bac 303	24.2 (18.7, 31.5)	18.7 (14.4, 24.2)	34.7 (26.8, 45.1)	0.008^{c}
Cperf 191	14.2 (10.6, 18.9)	11.9 (8.9, 15.9)	24.1 (18.0, 32.2)	0.004^{bc}

5.4.7. Comparison of microbiota from the GIT of various animals

The upper intestinal tract is seen as a transition zone for microorganisms because of motility, the presence of bile salts and low pH (Collado and Sanz, 2007). Their work also showed the small intestine of pigs and chickens to have similar numbers of total bacteria to the large intestine (approximately 10^8 cells/cm^2). Studies on chicken microbiota have shown that the gut bacterial numbers are highest in faeces, reaching 10^{11} or 10^{12} per g faecal contents but the microbial contents of faecal samples may not be representative of the numbers present in different GIT sections (Sghir *et al.*, 2000; Zoetendal *et al.*, 2002). In this study, faecal counts contained 10^8cells/g faeces (Figure 5.1), while slaughter one showed colon and caecum samples to harbour 10^8 cells/g mucosa compared to the duodenum, having 10^7 cells/g mucosa. At slaughter two, at five weeks of age, the colon and caecum had 10^9 cells/g mucosa and the duodenum had 10^8 cells/g mucosa. Although these numbers are lower than expected (the faecal counts in particular), similar results have been found in the chicken and pig intestines using FCM-FISH (Collado and Sanz, 2007). It could, however, also be due to the technique used. FCM-FISH relies on single cells and therefore any clumps of cells would be detected as a single cell. The permeability of the cells to probes is another limiting factor. Collado and Sanz (2007) reported that the probe Bif 164 did not hybridise with all the particular *Bifidobacterium* species previously reported to be present in pigs and chickens. These factors can all contribute to lower counts. Collado and Sanz (2007) studied mucosa-associated microbiota of pigs and chickens and found the total eubacterial counts to be similar throughout the GIT, from the crop to the large intestine. They also found that the dominant bacteria in all chicken samples at 2 months old were *E. coli, Bacteroides, Bifidobacterium, Lactobacillus* and *Atopobium*, whereas in the ostrich gut *Bacteroides*, the *C. coccoides/E. rectale* group and *Clostridium* cluster XI were the predominant bacteria throughout the gut. The same study by Collado and Sanz (2007) found lactic acid bacteria to be one of the predominant mucosa-associated bacteria in pigs and chickens and suggested a possible role as a health index status of these animals.

Zhu and Joerger (2003) studied the microbial composition of broiler chickens using FISH and compared the microbial numbers of the cecal contents and the cecal mucosa. They found no significant differences between these two samples for any probes used except for the general enteric bacteria probe, which showed a higher proportion of these bacteria in the mucosa than

in the cecal contents. They suggested this may be due to these bacteria's ability to utilize oxygen transported to the cecal tissue through the blood supply.

In this study, gut mucosal scrapings were taken at two time points. When comparing the average bacterial proportions of the ostrich chick gut and faecal samples at two weeks (slaughter one), all the bacterial group proportions were similar, except *Clostridum* cluster XI where gut mucosa contained 23.8% and faecal samples show 12.4%, and *C. perfringens* where gut mucosal samples show 10.7% and faecal samples 17.6%.

At the second slaughter (week five) in this study, gut biopsies showed higher proportions of *Clostridum* cluster XI (34.5% vs 10.7%), *C. coccoides/E. rectale* (46.1% vs 11.8%), *Bacteroides* (39.2% vs 16.6%) and *C. perfringens* (23.7% vs 6.2%) than faecal counts. This confirms earlier findings in this study and work done by Torok *et al.*, (2008) that suggested that the microbiota had been established and settled by this stage, and that the above mentioned taxa form the basic endogenous microbiota in ostrich chicks.

Two probes were used in this study for the detection of *C. perfringens.* They are Chis 150 (Franks *et al.,* 1998), aimed at targeting the *C. histolyticum* group (*C. perfringens* being a member) and Cperf 191 (Fallani *et al.,* 2006), validated to hybridise specifically to *C. perfringens*. Since *C. perfringens* is one of the most important pathogens in ostriches, Chis 150 may not have given sufficiently specific data on the pathogens abundance in faecal samples, and therefore the more specific Cperf 191 was also used in this study. It would be expected that the Chis 150 probe would hybridise to more cells than the Cperf 191 probe, since Chis 150 would detect several members of the *C. histolyticum* group, for example *C. histolyticum, C. butyricum, C. putrificum* and *C. cadaveris* (Franks *et al.,* 1998). However, in this study, the Cperf 191 probe detected more targets than those for the Chis 150 probe. Fallani *et al.,* (2006) reported the Cperf 191 probe to be suitable for detection of dominant populations of *C. perfringens* but less suitable for detecting sub-dominant populations of this species. This inconsistency may also be due to the fact that *C. perfringens* may have been the main or only species present in the *C. histolyticum* cluster in some of these samples.

In studies such as this, it is considered preferable to use gut mucosal samples rather than faecal samples, as faecal samples may be contaminated by floor litter, and do not give information on bacteria in separate gut locations. Tracking the faecal matter to the donor was also difficult.

The microbiota of animals is thought to change constantly due to host and environmental factors, especially over the development stage of the host (Zhou *et al.,* 2007). Animals also show a considerable intra-individual variation as seen in broiler chickens (Gong *et al.,* 2005) and pigs (Richard *et al.,* 2005). The use of in-feed antibiotic is a common practice and is thought to affect the microbiota considerably. Knarreborg *et al.* (2002) showed that treatment of broiler chickens with subtherapeutic levels of avilamycin and salinomycin decreased the levels of *C. perfringens* and lactobacilli specifically. Collier *et al.* (2003) showed that the use of tylosin greatly reduced the number of mucolytic bacteria, *C. perfringens* particularly. They showed that birds receiving tylosin had a more homogenous and distinct microbiota than the control birds, as well as an increase in *Lactobacillus gasseri.* In South Africa, tylosin is a commonly used antibiotic in animal farming, including ostrich rearing. This study has shown that tylosin can effectively protect the ostrich chicks from pathogens especially *C. perfringens,* which appears to be a major pathogen of ostrich chicks.

Gérard *et al.* (2008) found no effect of feeding *Lactobacillus* species on the composition of the cecal microbiota of chickens. This is consistent with previous work (Tannock *et al.,* 2000), where FISH showed that probiotic supplementation did not change the proportion of dominant phylogenetic groups in the human microbiota, which could be regarded as an advantage. However, the FISH technique does not account for alteration at the species level. In this work, no significant findings were seen for the group receiving probiotics, and the five strain probiotic mix alone did not provide sufficient protection to the chicks, with group E dying after two weeks.

5.5. Conclusion

The use of FISH in this study was to obtain more accurate and specific enumeration of bacterial groups found in faecal and gut mucosal scrapings, and to be able to compare the prevalence of bacterial groups in these different samples. Since the work described in the two previous chapters used faecal matter as the template, comparing the bacteria found in the mucosal scrapings would give invaluable information on the commensal bacteria which adhere to the mucosal lining of the ostrich gut rather than pass through. It also allowed for more precise comparisons between the bacterial proportions found in the different areas of the gut, in order to evaluate the effect of tylosin and the probiotic mix on the specific taxa.

Although treatment C shows increasing trends in important beneficial bacteria, it also appears to manifest *C. perfringens*, thought to be one of the most prevalent pathogens and causes of mortality in this feeding trial. Feeding the chicks the probiotic mix only (group E) appears to be insufficient in protecting them from pathogenic invasion. The mix alone also does not seem to contribute to the microbiota, as no increase in lactic acid bacteria or *Bifidobacterium* spp. was observed in the faecal matter. Group ET, the combination of tylosin and the probiotic mix appeared to be the most beneficial, offering protection against common pathogens as well increasing the prevalence of *Bifidobacterium* and *Lactobacillus* in the mucosal samples. Treatment ET also shows a decrease in *C. coccoides/E. rectale* which may be detrimental to gut health since most of these members are butyrate producers. No obvious trends were observed here, and further trials are needed to confirm the effects of each new proposed probiotic.

Microbial enumeration between faecal and mucosal populations can vary, showing little difference or significant differences. Optimally, both locations should be studied in each separate trial as adhesion potential and effect of probiotics differ in each host. In this study, examining both was of great importance given the fact that ostriches are hindgut fermenters and fermentative bacteria are passed out with the faeces (as opposed to foregut fermenters where the microbes are digested as a protein source; Ley *et al.*, 2008). The mucosal scrapings also gave important information on the commensal microbiota of the ostrich on which there is very little known.

This chapter has shown the usefulness of FISH-FCM in analysing the microbiota using probes based on 16S rRNA, especially for those genera difficult to cultivate like *Bacteroides* and *Clostridium*. It also allows objective analysis of intra-individual, inter-treatment, and longitudinal data. This work has given the first in-depth data of the microbes associated with ostriches in a farmed environment obtained using FISH, and provides important information on the use of tylosin, showing that it is beneficial and promotes the health of the ostrich microbiota. The five strain probiotic mix used in this study with the intention of replacing tylosin, is not effective at protecting the chicks from fatal pathogen invasions. Previous work has shown probiotics to be promising candidates for an antibiotic replacement, beneficially changing pig, chicken and cattle faecal microbiota, improving weight gain and reducing the number of cases diarrhea (Reid and Friendship, 2002).

CHAPTER 6

General conclusions

The gut microbiota of humans and animals is an extremely diverse and complex environment and has been a main focus of ongoing research due to its vital role in health and disease. Due to this complexity, the study of this microbiome has been made possible by recent advances in molecular and bioinformatics techniques. Large metagenomics studies on the human microbiota have lead to aiding the definition of a healthy gut and monitoring the effects of shifts in this microbiota (Tap *et al.,* 2009; Candela *et al.,* 2010), and allow for an understanding of the phylogenetic composition and functional potential of the microbial community present. For example, Li *et al.* (2011) studied the rumen microbiome of pre-ruminant calves and reported that the rumen microbial communities of these calves maintained a stable function and metabolic potential while their phylogenetic composition fluctuated greatly. The work presented in this thesis was divided into three main objectives, each focusing on a particular step in the process involved in selecting probiotics for use in the ostrich farming industry in South Africa.

The first step involved in this process revolved around the identification and characterisation of ostrich isolates as probiotics. The ability to persist in the intestine is an added advantage in achieving maximal probiotic efficiency (Vaughan *et al.,* 2002) and in order to survive and proliferate in the gastrointestinal tract the probiotics must tolerate several harsh environmental hurdles including the low pH and high bile concentrations. The original 71 ostrich isolates were preliminarily screened for probiotic properties and of these, five were chosen based on their probiotic potential including acid and bile tolerance. All five strains could tolerate up to 2% bile and pH2, and also showed successful auto- and co-aggregation abilities with the tested pathogens. In mucous-binding experiments, the five strains and their various combinations could inhibit and displace these pathogens in most cases, however they were less successful at out-competing them. This highlights the importance of early colonisation of the gut by beneficial bacteria. The five strains were able to produce antimicrobial compounds active against pathogens *E. coli* and *S. typhimurium,* but only one strain was active against *C. perfringens.* In addition, the isolates produced enzymes important in the high plant fibre diet the ostrich chicks are fed. While bacterial strains can evolve to attain resistant properties and tolerances, variation exists between different strains. Processes

to improve physiological robustness of potential probiotics would be desirable (Sleator and Hill, 2006). Pathogenic strains have developed mechanisms whereby they ensure their continued survival in diverse ecological niches and may therefore act as useful reservoirs for stress survival mechanisms, termed "patho-biotechnology" (Sleator and Hill 2005, 2006). Shehaan *et al.* (2006) successfully improved the stress tolerance of *Lactobacillus salivarius* UCC118 by heterologous expression of the betaine uptake system BetL, isolated from *Listeria monocytogenes.* Similarly, Shehaan *et al.* (2007) introduced BetL into *Bifidobacterium breve* UCC2003, resulting in this probiotic bacterium showing improved gastric transit and intestinal persistence, as well as improving the clinical efficacy of the strain when fed to mice. Although recombinant DNA technology may not be accepted by the regulatory authorities, these studies prove the concept of developing more versatile strains is possible. The natural selection of probiotic expressing such homologues may discard the necessity of genetic engineering all together (Shehaan *et al.,* 2007).

The second stage of this work involved the *in-vivo* assessment of the effect of the selected probiotic mix on the microbiota of the ostrich chicks and the comparison to the effect of tylosin. For this, a suitable mode of delivery was developed, encapsulating the strains in calcium alginate, which maintained their viability. The five strains were fed together in a mix in equal amounts, with the intention of having a multi-species mix, often more effective than a single-strain probiotic (Timmerman *et al.,* 2004). During the feeding trial, important factors such as growth and mortality rates and feed conversion ratios were also measured. No significant differences were observed for the feed conversion ratio or the weight gain of the chicks between those fed probiotics or antibiotics. However, the mortality rate of the control and probiotic-fed birds was noticeably higher than those receiving tylosin. Although there is a lot of interest in the use of probiotics, there are relatively few strains which have shown efficacy *in-vivo* (Gardiner *et al.,* 2004) and it is important to test strains in *in-vivo* trials.

The final stage involved using a combination of both modern and classical techniques to monitor the effect of the probiotics and antibiotic tylosin on the mucosal and faecal microbiota of ostrich chicks over nine weeks. Lahtinen *et al.* (2006) suggest that the choice of method used for enumeration of bacteria may have significant effects on the results. The strain-specific properties and the objects of the analysis should be considered when choosing a method of enumeration as well as when analysing the results. For example, enumerating using the plate count method gives information on viability while PCR-DGGE does not. In

contrast, the FISH method has potential to be used for viability testing as the half-life of rRNA is shorter than that of DNA but depends on the rate of rRNA decay after cell death (Lahtinen *et al.*, 2006). Each method has benefits and downfalls, and therefore using more than one technique allows for a broader results base.

The work in this thesis used culture-dependent techniques on selective growth media, PCR-DGGE with species-specific primers, and FISH-FC, with species- and strain-specific probes, to investigate the initial colonisers of the ostrich chick gut and to monitor the maturation of these bacterial populations with age. Chicks are born with a sterile GIT and the immediate colonisation of the gut by beneficial bacteria would provide a barrier against ingested pathogens. Culturing of the faecal samples did not show many significant differences in the bacterial populations between the treatments, but most groups increased over time for each treatment. Chicks receiving the probiotic mix showed higher counts for LAB and *Bifidobacterium* spp., while the probiotic group not receiving tylosin also showed higher coliform and *C. perfringens* counts at week two. Culturing of the mucosal samples showed increasing trends for total bacteria, LAB and *Enterobacteriaceae,* and by slaughter two, *C. perfringens* was only detected in treatment ET in the colon and caecum. This pathogen was found to be present in all gut areas tested for chicks in treatment E. Post-mortems on the birds from all treatments, confirmed the death was due to this pathogen. *C. perfringens* has been suggested to be an important pathogen present in ostrich chicks (Huchzermeyer, 2002) but this is the first study to confirm that it is a major pathogen in ostrich chick mortality.

DGGE analysis of faecal samples indicated that the probiotics alone had no effect on the total bacteria or *Lactobacillus* spp. populations, but did alter the *Bifidobacterium* spp. populations. Together with tylosin, the probiotic mix did not change the diversity of the total bacterial population or the *Bifidobacterium* spp., but decreased the diversity of the *Lactobacillus* spp. population. In comparison to the culture-dependent technique, DGGE did not show the presence of *C. perfringens.* The identification of excised DGGE bands showed that the faeces were dominated by bacteria belonging to the *C. coccoides* and *C. leptum* group.

FISH-FC analysis of faecal and mucosal samples showed ostrich chicks to be colonised by 10^8 cfu/g of faeces by week one. The faecal samples were dominated by the *C. coccoides*/*E. rectale* and *Bacteroides* groups initially, but *Bacteroides* was the major group present by week seven. Studies on the gut samples showed the caecum and colon to be predominated by butyrate-producing *Clostridium* cluster XIVa and the duodenum mostly by *Bacteroides*. This

technique showed the presence of *C. perfringens* in all areas of the gut, but its proportions in the duodenum were highest.

Although these techniques all give an idea of the diversity of the species present in the samples tested, the results obtained from each method differed. The enumerating techniques (culturing and FISH-FC) showed similar results in some cases. For example, the culturing of samples on TSC agar used to enumerate *C. perfringens,* showed a decrease of this pathogen in faecal samples over time for treatment ET, as did the Cperf191 probe used in FISH-FC. The DGGE method was used primarily to get an indication of the diversity in the faecal samples, and the majority of excised bands sequenced did agree with the FISH-FC results which showed *C. coccoides/E. rectale* to be the dominant group present in the ostrich gut. Even though the culture-dependent techniques give important information on the microbiome, and the use of this method together with the molecular methods is invaluable, the difficulty associated with culturing anaerobic bacteria, and the possibility of the bacteria being dormant and not readily culturable should be taken into consideration. However, a recent study by Bomar *et al.* (2011) suggested using metatranscriptomics to growing bacteria in diverse habitats to enable the design of media allowing for their cultivation.

Overall, no significant effect was seen for either the probiotics or tylosin on the microbiota of the ostrich. Tylosin clearly allowed for the survival of the chicks in the presence of *C. perfringens,* where the probiotic mix did not, the chicks in this group dying prematurely, reportedly due to *C. perfringens*-like lesions. The combination of these two supplements appeared to be the most beneficial, not only increasing the survival rates but also increasing the beneficial bacterial groups in the mucosal samples.

Further investigations into the ostrich gut microbiota need to be carried out, as well as the testing of more effective probiotics or an alternative combination of bacterial strains. A probiotic which can more successfully out-compete *C. perfringens* would be optimal. As ostrich chicks are naturally coprophagous and in the wild obtain their first inoculums of beneficial bacteria almost immediately, spray-inoculating the hatcheries to aid this process of colonisation may be a strategy to prevent such high rates of mortality. In addition, the layout of the pens in this study was such that transfer of faecal matter between pens of different

treatments was possible. In future, the different treatments should be housed in separate buildings to make this less likely.

It would be beneficial to design species-specific probes to particularly detect the probiotic strains added to the mix to confirm if they persist in the gut and their location in the gut. Due to the limitations of both DGGE and FISH-FCM, detection to the species level was not possible. The addition of enumeration by quantitative PCR to this data would allow confirmation of results obtained with FISH-FCM. Studies of metagenomics and the microbiome could expand our knowledge of microbial communities hugely, aiding in the understanding of bacterial variations and genetic parameters and development of therapeutic approaches in the treatment of acute and chronic disorders, in humans and animals. Effectively assessing the total bacterial community is a challenge, and Holben *et al.* (2004) reported a novel strategy involving GC fractionation combined with DGGE to improve the accuracy of these studies. The fractionation step reduces the complexity of the community in each fraction which subsequently facilitates detection of diversity in these fractions and combines mechanistically different community analysis approaches (Holben *et al.,* 2004). Although the information of the diversity of the gut microbiota in humans and animals is highly important, knowledge of the functionality of the gut microbiota would be of indispensible use, giving an idea of why each taxon is present. Eeckhaut *et al.* (2011) used PCR to detect functional genes involved in the butyrate-production pathway from culturable *Firmicutes* isolated from the chicken gut. This allows enumeration as well as functionality of the microbiome, information which could later be used for improved gut health and treatment of disorders.

Next generation antibiotics, including antimicrobial peptides and new drug classes, will be identified as the study of the commensal gut bacteria increases, and instead of mining the soils for these products, it is predicted that the 21st century antimicrobial agents will be found by exploring the human and mammalian microbiomes (Preidis and Versalovic, 2009). These compounds may be evolutionarily tailored to preserve health-associated microbiomes while increasing defences against pathogens. Identification of key substrate and signalling mediators will help understand microbe-microbe and microbe-host interactions and therapeutic treatments will target specific microorganisms, as well as metabolites of entire microbial populations (Preidis and Versalovic, 2009). Further knowledge of the ostrich microbiome would therefore be important in this aspect.

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