# 5 *Ginkgo biloba* and *Hypericum perforatum* extracts used as growth promoters in broilers

## 5.1 Introduction

Antibiotic growth promoters (AGPs) are substances that are added to feed in sub-therapeutic levels in intensive animal production to improve weight gain and conversion of feed into body mass (Dibner and Richards, 2005). Since January 2006, legislation has been in place in Europe to prohibit the use of antibiotics as growth promoters due to concerns that the use of antibiotics in feedstuffs contribute to the spread of antibiotic-resistance genes by promoting the selection of antibiotic-resistance bacteria in animals (Anonymous, 2003; Dibner and Richards, 2005; Castanon, 2007). As a result, economically important diseases, such as necrotic enteritis of chickens have become more prevalent in these countries.

Necrotic enteritis caused by the virulent net B producing strains of *Clostridium perfringens* is clinically characterized by inappetence, depression and increased mortality (Helmboldt and Bryant, 1971; Long, 1973). The chronic damage to the intestinal mucosa leads to decreased digestion and absorption reduced weight gain and increased feed-conversion ratios (Elwinger *et al.,* 1992; Kaldhusdal *et al.,* 2001). The disease is therefore associated with important economic losses in the poultry industry (Dahiya *et al.,* 2006).

The number of publications on the efficacy of possible replacements of AGPs to control the occurrence of NE has been steadily growing. The mode of action of these supplements can be divided into four basic groups with distinct strategies: 1) improvement of nutrient utilization by the host, 2) stimulation/modulation of the immune system, 3) stimulation of introduction of beneficial bacteria and 4) direct reduction of pathogens (Kocher and Choct, 2008). The antimicrobial effect of plants/plant extracts is well known (Rios and Recio, 2005) and their potential as an alternative to AGPs due to the direct effect on pathogenic bacteria has been investigated (Mitsch *et al.,* 2004). A beneficial effect on production traits has also been illustrated. Examples for chickens or quail include an increase in average daily weight gain (ADG) of 7% with oregano (Florou-Paneri *et al.,* 2006), 6% with thyme (Denli *et al.,* 2004) and between 1 and 8% with coriander (Güler *et al.,* 2005). Chikoto (2006) illustrated a significant increase of 4.8% in mean live body mass of broilers with *G. biloba* extract (included into feed at 14 ppm) in combination with grapeseed extract. A non-significant increase of 3% was obtained when *H. perforatum* extract was added to that mixture. These results were achieved in spite of the fact that grape seed extract on its own had a deleterious effect on growth promotion (Chikoto, 2006).

The current investigation is a continuation of the work of Chikoto (2006). Chikoto (2006) established that the acetone extracts of *G. biloba* and *H. perforatum* could be tolerated by broilers at a rate of 2 and 10 mg/kg respectively. Furthermore, the extracts were found to be stable in feed and water and antimicrobial activity was

found to be unaffected under varying pH conditions. It was thus decided to continue with the acetone extracts due to the absence of adverse *in vitro* and *in vivo* effects. It was also decided to increase the concentration of *H. perforatum* from the 14ppm used in Chikoto's (2006) experiment to 28ppm as this dose was found to be safe in *in vivo* tolerance studies. Grape seed was excluded due to the deleterious effects previously observed (Chikoto, 2006). The objective of the study was to determine the effect of a combination of extracts of *G. biloba* (14ppm) and *H. perforatum* (28ppm) on the performance of broiler chickens over a 35 day period. The effect on *C. perfringens* in the intestine was also investigated.

## 5.2 Materials and methods

### 5.2.1 Experimental design

The trial ran in the form of a 2 X 5 factorial design (Table 5.1). Treatments consisted of the following:

- Treatment 1: Broiler ration without growth promoter/ antibiotic (Not challenged with *C. perfringens* i.e. the negative control)
- Treatment 2 Broiler ration with growth promoter/ antibiotic Zn-bacitracin (75ppm) (challenged with *C. perfringens* i.e. the positive control)
- Treatment 3: Broiler ration without growth promoter/ antibiotic (*C. perfringens* challenged negative control)
- Treatment 4: Treatment 3 supplemented with *G. biloba* (14ppm)
- Treatment 5: Treatment 3 supplemented with a combination of extracts of *G. biloba* (14ppm) and *H. perforatum* (28ppm)

Table 5.1. Summary of the experimental design

| *C. perfringens* challenge (days) | Lesion /microbial scoring (days) | Diet | Weight monitoring (days) | Duration of trial (days) |
|---|---|---|---|---|
| 15,23,24, 27 | 14,21,29 | Maize-soya | 13, 20, 27, 35 | 35 |

### 5.2.2 Experimental animals and housing

The total of 700 (600 + 100 replacement birds) sexed (female) Ross 788 one day old, non-vaccinated chicks were obtained from a commercial hatchery from a single parent flock and randomly allocated to the 20 pens (initially 35 chickens/pen, 4 pens/treatment) (Figure 5.6). All birds were individually tagged on day 13 prior to oral

administration of *C. perfringens*. Birds were weighed on a per pen basis on day 1 and weekly on individual basis (from 14 days) until 35 days of age after which the birds were sent to a slaughterhouse.

The protocol for the project was approved by the University of Pretoria's Research committee and Animal Use and Care Committee (Protocol nr. V038-08) before commencement of the experiment. The experiment was conducted at the Poultry Researdh unit, Poultry Reference Centre of the Faculty of Veterinary Science, Onderstepoort. The part of the facility used consisted of 2 broiler houses, one with 4 pens (treatment 1) and the other with 16 pens (treatment 2 – 5) (Figure 5.1). Treatment 1 was kept separately to minimize contamination with *C. perfringens.* All birds were housed in pens bedded with wood shavings (Figure 5.6). The maximum stocking density of birds was 23 kg/$m^2$. Stocking densities were above those recommended by the South African National Standard: The use of animals for scientific purposes (10 386), but well below the minimum standards of 33 kg/$m^2$ (or 39 kg/$m^2$ with good ventilation and a temperature control system) agreed on by the European Commission (European Commission, 2007). With these stocking densities we were able to imitate commercial conditions where AGPs are normally required, without unnecessarily sacrificing the welfare of the chickens.

Environmental control was facilitated by means of electrical heaters (Figure 5.3) and extractor fans. On day one the house temperature control was set to 32°C, but decreased after 3 days to 28°C. The temperature was then kept constant for the remainder of the growing period. Records were kept in terms of daily min-max temperatures and daily procedures.

Pens were checked twice daily and morbidity or mortality noted. Sick birds were removed and euthanized. All mortalities were submitted for necropsy.

### 5.2.3 Feed and feeding

On day one, chicks' beaks were dipped in water from the fountain drinker to ensure that the swallow reflex was intact (Figure 5.2). Each pen was equipped with a feeding tray and chicken font drinker for the first 7 days (Figure 5.4) after which tube feeders (Figure 5.1) and nipple drinkers were supplied. All feed consisted of two phases i.e. a starter and a grower ration and were supplied *ad libitum*. Birds were fed on a commercial maize-soya diet from day 1 until three weeks (starter) after which a grower mix was supplied (Table 5.2). The feed contained no (other than mentioned) commercial antimicrobial growth promoters or coccidiostats.

*Ginkgo biloba* extract was incorporated into a calcium carbonate premix at a rate of 14 mg per kg (14 ppm) and *H. perforatum* extract at a rate of 28 ppm (in combination with the *G. biloba* extract) of both the starter and the finisher feed.

All feed was weighed daily and the weight of left over feed subtracted from the total feed administered in order to determine feed intake per pen. Feed intake together with average mass gain was used to determine the feed conversion ratio (FCR) and the European Performance Efficiency Factor index (EPEF). The data were corrected for mortality.

$$\text{FCR} = \frac{\text{Feed consumed per bird}}{\text{Weight gain per bird}}$$

$$\text{EPEF} = \frac{\text{Mean live mass on specific day of trial} \times \text{\% survivors} \times 100}{\text{FCR} \times \text{number of days}}$$

Table 5.2. Composition of broiler grower feed

| Broilers grower ingredients | Percentage |
|---|---|
| Yellow maize | 64.0 |
| Soya | 24.5 |
| Fish meal | 5.0 |
| Fat | 2.5 |
| Limestone | 1.2 |
| Salt | 0.25 |
| Dicalcium phosphate | 1.4 |
| Lysine HCL | 0.03 |
| Methionine | 0.17 |
| Threonine | 0.05 |
| Vitamin and Minerals | 0.6 |
| Sodium bicarbonate | 0.25 |

### 5.2.4 Challenge with *C. perfringens*

#### 5.2.4.1 Preparation of inoculum

*Clostridium perfringens* type A culture was obtained from Dr J. Picard from Department of Veterinary Tropical Diseases at the Faculty of Veterinary Science, University of Pretoria. This strain had been originally isolated from a bird that died whilst suffering from necrotic enteritis and had been used for the experimental induction of necrotic enteritis on several occasions including the experiments of Chikoto (2006). Frozen isolates were thawed, streaked out on Horse Blood Columbia agar (BCA) (Selecta Media) and incubated at 37°C overnight in an anaerobic chamber. Cultures were checked for purity and inoculated into Erlenmeyer flasks containing sterilised BHI - broth (brain-heart-infusion broth) (Oxoid) augmented with 0.04% cysteine and incubated aerobically for 24 h at 37°C. Cultures were resuspended at a rate of 1 x $10^8$ cells/ml each (optical density (OD) of 1 measure on the spectrophotometer at 540 nm) in new sterilised BHI-broth augmented with 0.04% cysteine and distributed in 16 bottles containing 50 ml cultured broth each. Four clearly marked bottles containing sterile broth were kept separately for the non challenged controls. One bottle per pen was used. Cultures were kept at room temperature at the broiler facility and used within 6 h of preparation.

#### 5.2.4.2 Inoculation of chickens

Feed was withdrawn from all the birds (including the non challenged treatment) for 24 h prior to oral administration of *C. perfringens* broth culture to minimize the occurrence of regurgitation (adapted from Collier *et al*., 2003). On days 15, 23, 24 and 27, all chickens received 1ml of either broth containing 1 x $10^8$ cells/ml *C. perfringens* (challenged group) or uninoculated sterile BHI broth (non challenged control) by oral gavage using a sterile syringe (Figure 5.7). Feeding was resumed as soon as administration of *C. perfringens* was completed.

### 5.2.5 Collection of samples

#### 5.2.5.1 Necrotic enteritis lesion scoring

On days 14, 21 and 29 three birds per pen (all treatments) were randomly selected and euthanized by $CO_2$. The abdomen was opened and the gastro-intestinal tract exposed and examined for lesions and scored. Lesion scoring was performed as follows (Chalmers *et al.,* 2007):

0 = Small intestine grossly normal

1 = Small intestine wall grossly thinner than normal and break or tear easily under mild tension but with no evidence of mucosal necrosis or other abnormalities

2 = One or more focal round or oval areas of acute full thickness mucosal necroses of the small intestine. These foci can vary in diameter from approximately 1 – 5 mm. The surface of these lesions is raised above the surrounding tissue and consists of grey or white necrotic debris. Alternatively, when superficial necrotic material is removed, the lesions are slightly depressed with grey or white colour.

3 = Irregularly shaped confluent areas of full thickness mucosal necrosis of the small intestine >5mm in diameter but affecting <25% of the small intestine surface area. The surface of these lesions appears to be raised above the surrounding tissue and consist of orange/brown necrotic debris. When portions of the superficial necrotic material are removed, the lesions can be slightly depressed from the surrounding tissue.

4 = Large confluent areas of full thickness mucosal necrosis of the small intestine affecting 25% or more of the small intestinal surface area and involving the entire internal circumference of the affected small bowel. The surface of these lesions is generally raised above the surrounding tissue and consists of orange/brown necrotic debris.

#### 5.2.5.2 Microbial scoring

##### 5.2.5.2.1 *C. perfringens* culture

A 5 cm portion of the duodenum (Figure 5.8) was opened aseptically and an area 0.5 by 2cm (by using a new plastic template for each sample) was abraded with a sterile bacteriological swab and placed in 1ml sterile BHI broth augmented with 0.4g/l cysteine. The sample was cultured anaerobically on BCA augmented with 0.1g/l neomycin by streaking into 4 quadrants, sterilizing the loop between quadrants. Plates were incubated at 37°C for 24 h.

Colonies on blood agar plates were identified as *C. perfringens* if they exhibited a double-zone β-haemolysis (Chalmers, 2007). The number of colonies meeting these criteria were counted within each quadrant, and the density scored as 4+, 1 or more colonies in the fourth quadrant; 3+, 0 colonies in the fourth quadrant, 1-10 in the third quadrant; 2+, 0 colonies in the fourth and third quadrants,1-10 colonies in the second quadrant; 1+, 0 colonies in the second, third and fourth quadrant, 1-10 colonies in the first quadrant; 0, no colonies in any quadrant (Chalmers, 2007).

#### 5.2.5.2.2 Identification and quantification of *C. perfringens*

A volume of 0.01 ml from the above mentioned BHI broth was spread on a designated area (using a circular template with a diameter of 16 mm) on a microscopic slide, air dried and stained using Gram's stain. The number of Gram-positive rods (magnification 1000X) was counted in 10 microscopic fields and graded as 0, no Gram-positive rods; 1, 1-10 gram positive rods (Figure 5.11); 2, 11-20 Gram-positive rods; 3, 21-30 Gram-positive rods; 4, >30 Gram-positive rods (Figure 5.12) (Chalmers, 2007).

### 5.2.6 Statistical analysis

Statistical analysis of the data was performed using the SPSS 17 programme. For all parameters (FCR, live body mass, % survival and EPEF) significant differences between groups were detected using a one-way analysis of variance (ANOVA). Post-hoc significance was determined using Bonferroni. Significance was set at P = 0.05. For the microbial scoring system significant differences between the groups were determined using the non-parametric Kruskal-Wallis (non-challenged and challenged control, *G. biloba-*, *G. biloba + H. perforatum-* and Zn-bacitracin treatments). Post-hoc comparison to the non-challenged control group was determined using a Mann-Witney (P = 0.05). In tables and figures, values differing at a P = 0.05 level are indicated with different superscripts.

Figure 5.1. Broiler facility where challenged treatments were housed

Figure 5.2. Day old chicks' beaks are being dipped in water to ensure swallow reflex is intact

Figure 5.3. Heater hanging from the roof

Figure 5.4. Day old chicks in and around the feeding tray and water fountain

Figure 5. 5. Nine day old chicks around tube feeder

Figure 5.6. Cages (each cage represent one treatment replicate)

Figure 5.7. Oral gavage of 15 day old chicken with *C. perfringens*

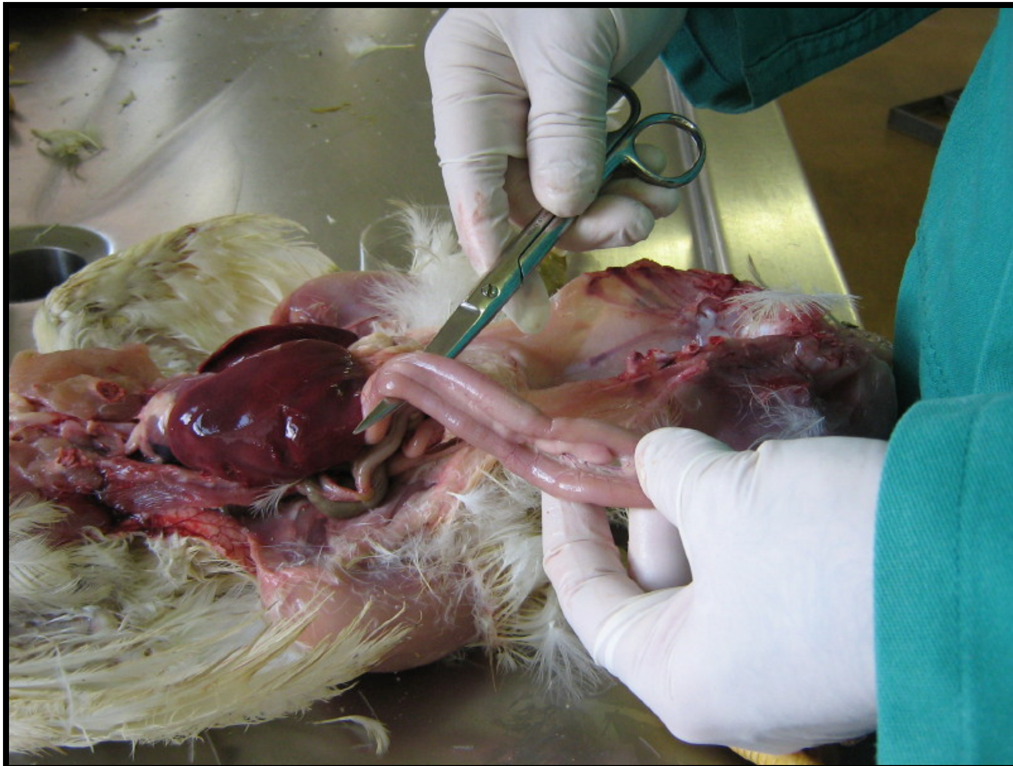

Figure 5.8. Section of intestine of chick where a sample were taken for smears and culturing of *C. perfringens*

## 5.3 Results and discussion

### 5.3.1 Mortality

Each pen initially contained 35 broilers and a total of 9 broilers per pen were sacrificed for the purpose of lesion scoring on days 14, 21 and 29. Mortality of 1.3% (results not shown) did occur before the *C. perfringens* challenge on day 13 but necropsy analysis revealed that this was unrelated to treatment. No significant differences were found between challenged and non-challenged treatments on the different days (Table 5.3) although a trend towards higher survival rates in the Zn-bacitracin group could be observed. Similarly, this trend was also observed with respect to cumulative daily mortality after challenge with *C. perfringens* i.e. treatment with Zn-bacitracin resulted in lower mortality (similar to non-challenged negative control) compared to other treatments (Figure 5.9). Survival rate (on day 35) that ranged between 92.1 and 94.8% for the non-challenged and challenged broilers was within the normal ranges and provides evidence of the safety of the feed additives administered. No lesions were observed in any of the broilers that died during the experiment ruling out necrotic enteritis due to *C. perfringens* as a cause of death.

Table 5.3. Percentage survivors of broiler chickens fed different combinations of feed additives

| Treatments | % Survivors | | |
|---|---|---|---|
| | Day 20 | Day 27 | Day 35 |
| Neg control (non challenged) | 100[a] | 98.2 [a] | 94.8 [a] |
| Neg control (challenged) | 98.4 [a] | 95.5 [a] | 92.1 [a] |
| Zn-bacitracin* | 99.2 [a] | 97.3 [a] | 97.1 [a] |
| *G. biloba** | 99.2 [a] | 94.8 [a] | 92.3 [a] |
| *G. biloba + H. perforatum** | 97.6 [a] | 93.9 [a] | 93.2 [a] |
| P-value | 0.234 | 0.502 | 0.660 |

*Challenged

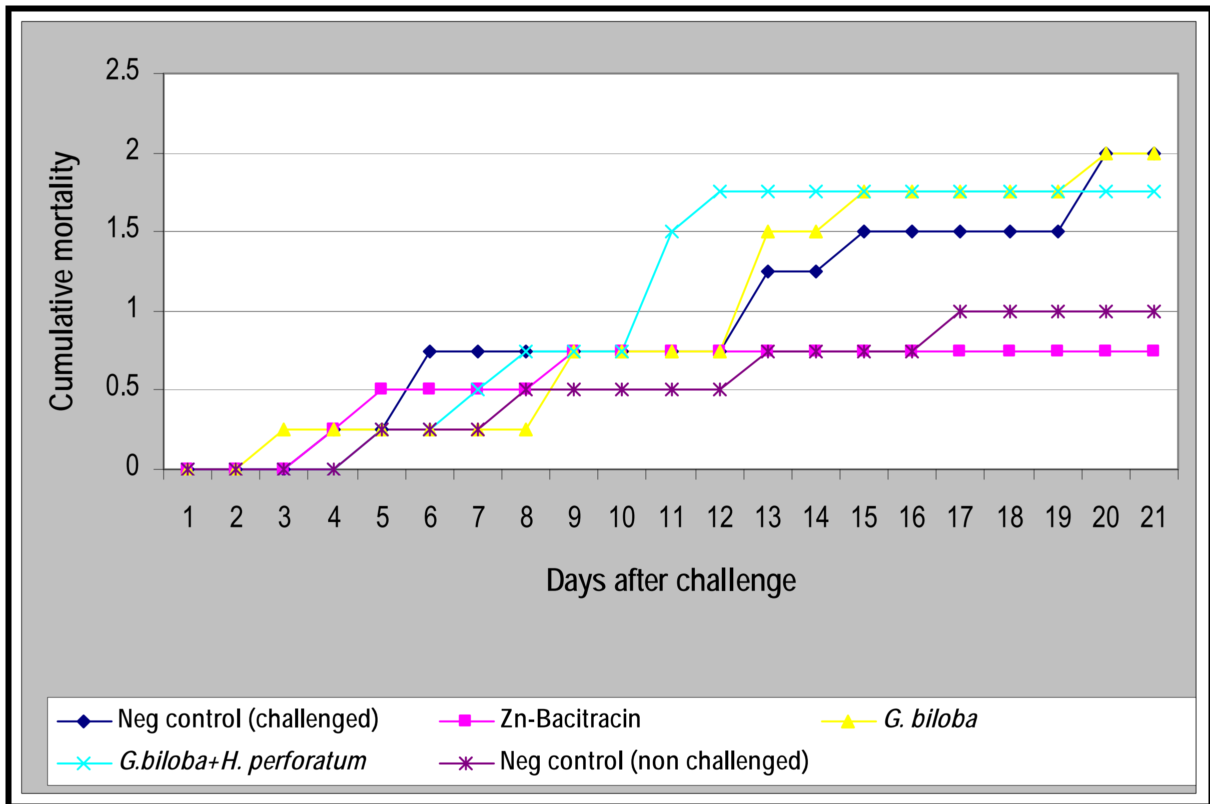


Figure 5.9. Cumulative mortality associated with different treatments after challenge with *C. perfringens*

### 5.3.2 Lesion scores

No lesions (lesion score zero) were observed in the intestines (jejunum, duodenum, ileum or colorectum) of broilers examined on the different days, indicating that the disease was not successfully induced. Necrotic

enteritis of broilers is characterized by gross lesions in the jejunum (Parish, 1961a; Kaldhusdal and Hofshagen, 1992), sometimes extending to the duodenum (Helmboldt and Bryant, 1971; Kaldhusdal and Hofshagen, 1992) or the ileum (Parish, 1961a; Broussard *et al.,* 1986). Rarely, lesions also occur in the colorectum or the caecal tonsils or necks (Long *et al.,* 1973). Typical focal lesions are sharply demarcated from the surrounding mucosa (Parish, 1961a; Kaldhusdal and Hofshagen, 1992). In severe cases, lesions are longitudinally expanded along the intestines, and the epithelium is eroded and detached (Williams, 2005).

Several researchers have reported difficulties in inducing necrotic enteritis under experimental conditions (Gholamiandehkordi *et al.,* 2007; Kocher and Choct, 2008). The model in this study was based on that of Chikoto (2006) who observed a significant increase in broilers with lesions in the challenged groups compared to those in the non-challenged controls using an identical *C. perfringens* isolate. Other researchers used different infection protocols, most of which use predisposing factors to induce disease. These predisposing factors include coccidial infection, immunosuppression and manipulation of the diet with regards to type of cereal and protein content (Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996). Intestinal damage caused by coccidial infection can cause leakage of plasma proteins and thus provide growth factors for the proliferation of *C. perfringens* (van Immerseel *et al.,* 2004). Diets based on wheat, rye, oats or barley increase digestra viscosity, which increases the intestinal passage time (van der Klis and van Voorst, 1993) which in turn may allow anaerobic bacteria to proliferate and produce enterotoxins (Parish, 1961a; Vahjen *et al.,* 1998). Immunosuppression by exposure to infectious bursal disease, chicken infectious anaemia virus and Marek's disease as well as non-specific stress, may predispose birds to necrotic enteritis (Schuring & van Gills, 2001). The presence of one or more predisposing factors such as a natural coccidia infection or other non-specific stress factors could be the reason why Chikoto's (2006) model worked at the different location where the experiment was performed. This is supported by the fact that up to 80% of broilers had lesions even before the *C. perfringens* challenge (Chikoto, 2006).

### 5.3.3 Microbial scores

Although no lesions were observed, *C. perfringens* were more frequently isolated from the intestines of broilers of the challenged treatments compared to the non challenged control as indicated by the microbial scores on the cultured plates (Table 5.4 and Figure 5.10). The presence of *C. perfringens* in the intestine of the non-challenged chickens can be explained by the fact that this organism occurs naturally in a healthy chicken's intestine (Barnes *et al.,* 1972). Spores of *C. perfringens* are ubiquitous in the environment (van Immerseel *et al.,* 2008) and can also be present in feed especially when fish-meal is included in the diet (Wijewanta and Seneviratna, 1971). It is exremely difficult to ensure that poultry feed is spore-free, because spores are resistant to heat and can tolerate 100°C for 2 h (Parish, 1961b).

Necrotic enteritis results from the high frequency of adhesion by *C. perfringens* to the damaged mucosa (Baba *et al.,* 1992) facilitating bacterial proliferation and toxin production. The pathogenicity of the *C. perfringens* strain is however also an important factor in disease development. Chalmers *et al* (2007) found considerable variances in the ability of *C. perfringens* stains from field outbreaks to reproduce the disease in an experimental model (no coccidial challenge were used). Only one out of five field isolates was able to cause 33.7% necrotic enteritis-associate mortalities and produced an average lesion score of 1.83 while the other isolates resulted in mortality of 0-3.8% and lesion scores of between 0.33 and 1.17 in the sacrificed birds. Treatment with the virulent isolate also resulted in higher culture and smear scores (3.6) in mortalities compared to the scores from mortalities from treatment with avirulent strains (average 0-1.3) (Chalmers *et al.,* 2007). It is thus possible that the *C. perfringens* strain used in my study did not display sufficient virulence to induce disease. Chikoto (2006) did not perform any quantitative or qualitative studies on *C. perfringens* in the intestines of broilers challenged with the same *C. perfringens* strain used in my study. It is therefore not possible to comment on the association between *C. perfringens* colonization and severity of the disease (lesions in 79% of the challenged birds vs. 67% in the non-challenge birds 9 days after *C. perfringens* challenge) (Chikoto, 2006). Chalmers (2007) found that a high degree of genetic relatedness or the presence of virulent genes does not necessarily imply analogous virulence in the challenge model. It is thus not possible to predict the virulence level of the strain based on genetic relatedness to known virulent strains.

There was a general trend (not on a statistical significant level) towards a reduction in microbial scores in the feed supplemented with *G. biloba* and *H. perforatum*. Extracts of *G. biloba* and *H. perforatum* have an *in vitro* antimicrobial effect on *C. perfringens* (Chapter 2-unpublished data; Ahn *et al.,* 1994; Lee and Kim, 2002) and could therefore have a effect on the reduction of *C. perfringens* in the intestine of broiler chickens. Direct effects of an AGP on the microflora have the potential to decrease competition for nutrients and to reduce microbial metabolites that depress growth (Visek, 1978; Anderson *et al.,* 1999). Mitsch *et al.* (2004) were able to illustrate a reduction in the *C. perfringens* concentrations in the intestines and faeces of broilers in field trials using essential oil blends containing a combination of thymol, eugenol, curcumin and peperin or a combination of thymol, carvacrol, eugenol, curcumin and piperin.

Table 5.4. Mean microbial scores of *C. perfringens* on plates (cultures) and slides (smears) from the intestines of challenged broilers fed different feed additives

| Treatments | Day 14* | | Day 21 | | Day 29 | |
|---|---|---|---|---|---|---|
| | Plate | Slide | Plate | Slide | Plate | Slide |
| Neg control (non-challenged) | 0 | 0.06 [a] | 0.08 [a] | 0.13 [a] | 0.08[b] | 0.48 [a] |
| Neg control (challenged) | 0 | 0.12 [a] | 0 [a] | 0.09 [a] | 0.58[a] | 0.95 [a] |
| Zn-bacitracin | 0 | 0.04 [a] | 0 [a] | 0.06 [a] | 0.75[a] | 0.93 [a] |
| *G. biloba* | 0 | 0.03 [a] | 0.08 [a] | 0.08 [a] | 0.75[a] | 1.03 [a] |
| *G. biloba + H. perforatum* | 0 | 0.09 [a] | 0 [a] | 0.17 [a] | 0.42[ab] | 0.65 [a] |
| P-value[1] | - | 0.860 | 0.549 | 0.922 | 0.034 | 0.447 |

*Before challenge; [1]F-probability value; (Scoring system: Slides: 0, no Gram-positive rods; 1, 1-10 Gram positive rods; 2, 11-20 Gram positive rods; 3, 21-30 Gram positive rods; 4, >30 Gram positive rods : Plates: 4+, 1 or more colonies in the fourth quadrant; 3+, 0 colonies in the fourth quadrant, 1-10 in the third quadrant; 2+, 0 colonies in the fourth and third quadrants,1-10 colonies in the second quadrant; 1+, 0 colonies in the second, third and fourth quadrant, 1-10 colonies in the first quadrant; 0, no colonies in any quadrant (Chalmers, 2007).

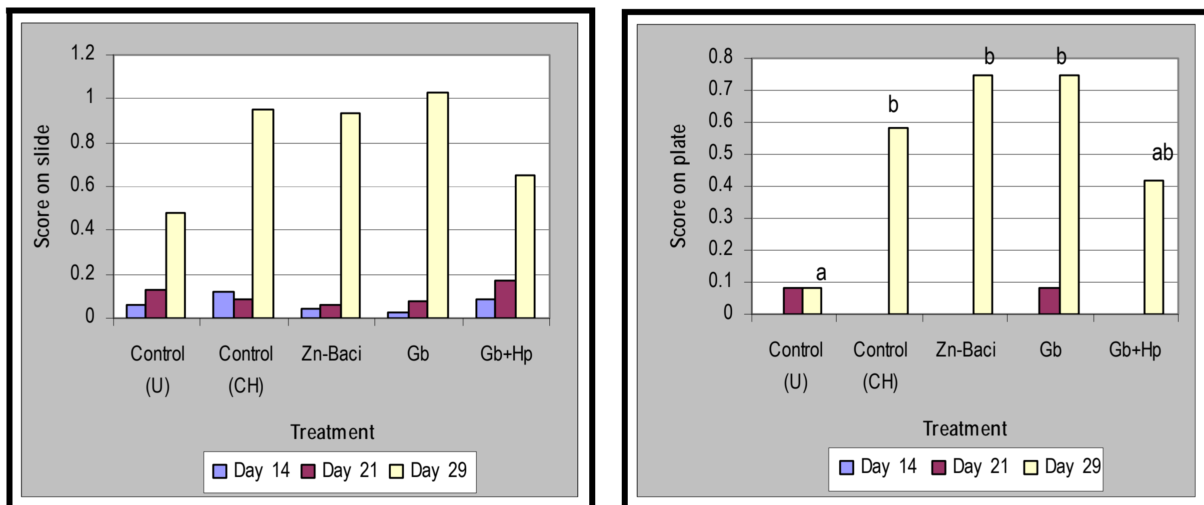


Figure 5.10. Smear (left) and culture (right) scores of C. perfringens indicating colonization of intestines of broiler fed different feed additives. U = non challenged control; CH = challenged control; Zn-Baci= Zn-bacitracin; Gb = *G. biloba*; Gb+Hp = *G.biloba* + *H. perforatum*. n = 12 for each treatment group

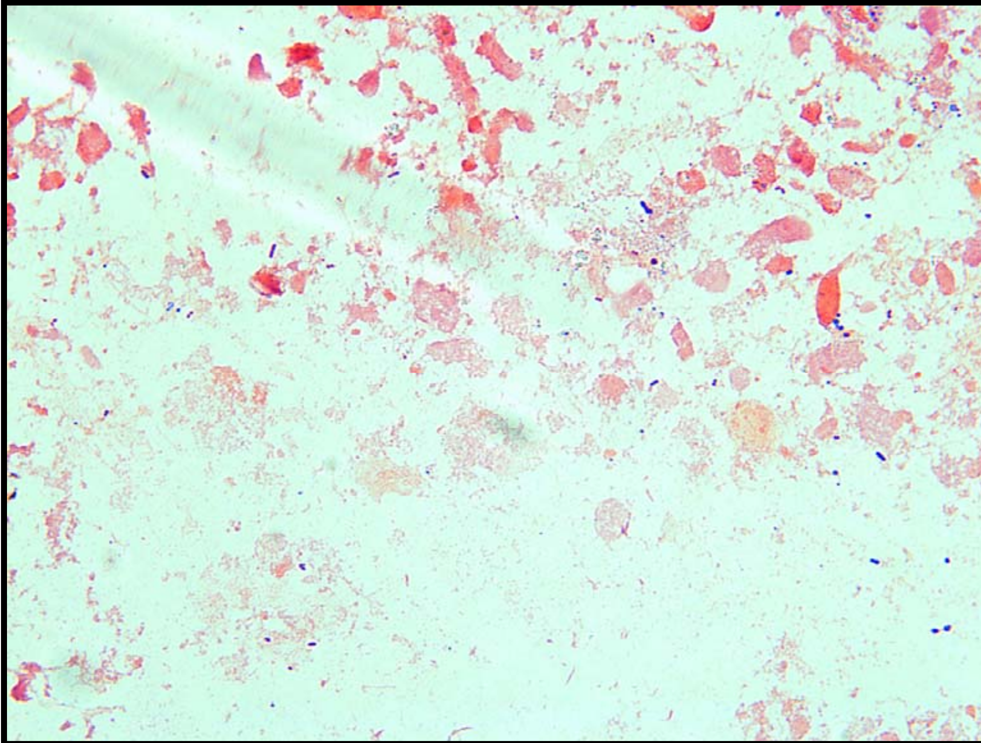

Figure 5.11. Slide showing a low concentration of *C. perfringens* (score = 1)

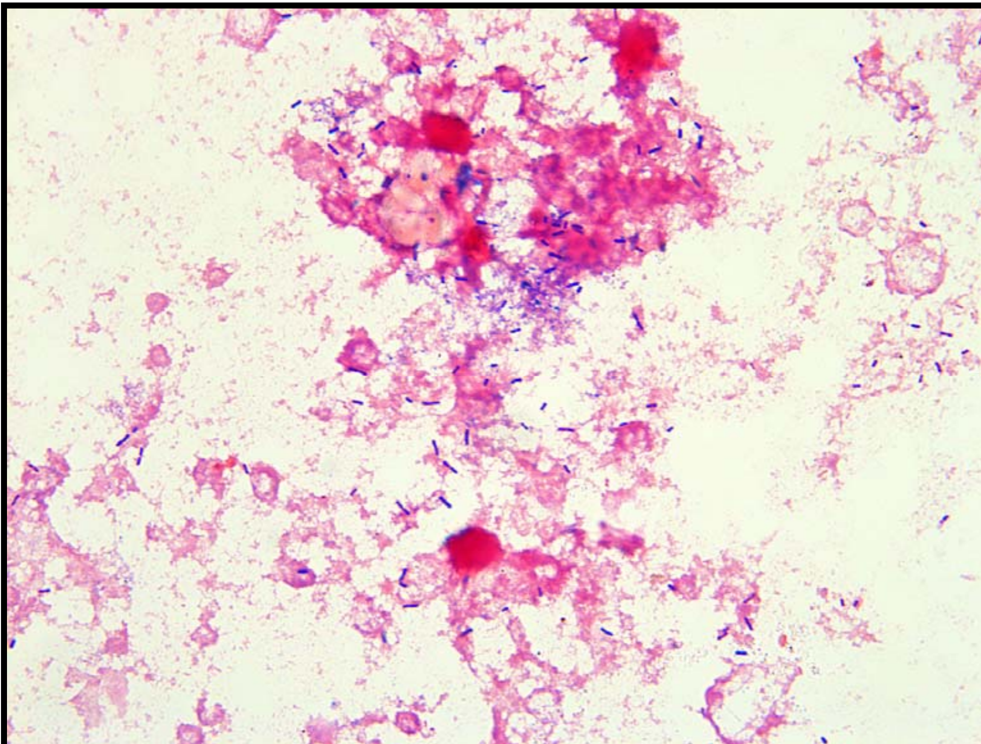

Figure 5.12. Slide showing high concentrations of *C. perfringens* (score = 4)

### 5.3.4 Live body mass

No differences were observed in body mass of *C. perfringens* challenged broiler chickens fed different feed additives or Zn-Bacitracin (Table 5.5). Oral antibiotics do not have growth-promoting effects in healthy animals (Coates *et al.,* 1955, 1963) This probably indicates that disease was not succesfully induced in this experiment. The non-challenged birds were kept in a different growth room under apparently similar conditions to inhibit cross infection. There was however a slower growth rate up to day 27 which is not easy to explain. The average live body masses of the non-challenged group were 263 g (SEM 3.03), 593 g (6.67), 915 g (8.97) and 1479 g (15.81) on day 13, 20, 27 and 35 respectively. These values were not included in the statistical analysis.

Chikoto (2006) previously illustrated a growth stimulatory effect of 4.9% (on a statistically significant basis) in broilers with experimentally induced necrotic enterits and fed with grapeseed extract in combination with *G. biloba* extract. Grapeseed extract in combination with *G. biloba* and *H. perforatum* extract also resulted in a 2.9% (not statististically significant) increase in body mass compared to the untreated control. In contrast with these findings, no differences in live body mass were observed in the same experiment after a 42 day period in broilers fed different combinations of these extracts (as well as with the Zn-bacitracin control) not challenged with *C. perfringens*. It can however be said that inclusion of any of the feed additives in this experiment did not have any negative effect on production. Çabuk *et al* (2006) were also unable to demonstrate any growth stimulatory response in healthy broilers treated with essential oil extract from a combination of oregano, laurel leaf, sage leaf, myrtle leaf, fennel seed and citrus peel. Similarly, Madrid *et al.* (2003) found that the oral administration of a blend of plant extracts containing oregano, cinnamon and pepper oil did not have any effext on body weight of the broilers. In contrast, positive effects of dietary essential oils on body weight were observed by Alcicek *et al* (2003) and Denli *et al* (2004). Hernandez *et al* (2004) also found that the addition of two plant extracts to a broiler diet significantly improved body weight at 35 days of age. Moreover, Jamroz *et al.* (2003) found that the inclusion of 150 or 300mg/kg of a plant extract containing capsaicin, carvacrol and cinnamicaldehyde in a diet improved body weight by 5.4 and 8.1% respectively.

Table 5.5. Mean live body mass (g) on days 13, 20, 27 and 35 (each chicken analysed as an experimental unit) of *C. perfringens* challenged broiler chickens fed different feed additives

| Treatments | Mean live body mass (g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 13* | | Day 20 | | Day 27 | | Day 35 | |
| | | n | | n | | n | | n |
| Neg control (challenged) | 289.7 (2.94)[a] | 136 | 616.4 (6.27) [a] | 122 | 910.0 (8.59) [a] | 106 | 1490.4 (13.14 [a]) | 91 |
| Zn-bacitracin | 291.9 (3.36) [a] | 138 | 624.3 (6.59) [a] | 125 | 911.4 (8.88) [a] | 111 | 1502.3 (13.56) [a] | 98 |
| *G. biloba* | 292.7 (2.80) [a] | 141 | 627.1 (5.42) [a] | 128 | 916.0 (7.60) [a] | 111 | 1497.0 (11.75) [a] | 97 |
| *G. biloba + H. perforatum* | 295.2 (2.72) [a] | 141 | 630.0 (6.18) [a] | 126 | 921.9 (8.41) [a] | 109 | 1494.5 (15.07) [a] | 96 |
| P-value | 0.633 | | 0.438 | | 0.748 | | 0.939 | |

*Before challenge

### 5.3.5 Feed conversion ratios

There were no statistical difference in FCRs in the different challenged treatments except on day 13 (before challenge) where treatment with Zn-bacitracin resulted in better FCRs (Table 5.6). These differences can not be attibuted to differences in live body mass (Table 5.5) and are thus due to differences in rate of consumption i.e. higher rates of consumption (but less effective conversion into body mass) in the untreated, *G. biloba* and combination of *G. biloba* and *H. perforatum* treatments only up to day 13. FCRs were lower for the non-challenged group which were kept in a separate facility room. This could be ascribed to food wastage because a ring was not placed on the tube feeder. These values were not included in the statistical analysis.

Chikoto (2006) found no differences in FCRs of non-challenged broilers after treatment with combinations of grapeseed, *G. biloba*- or *H. perforatum* extract as well as the Zn-bacitracin control. Treatment with a combination of grapeseed- and *G. biloba* extract resulted in a significantly better FCR (4.3% improvement relative to the untreated control) in broiler chickens with experimentally induced necrotic enteritis. This improvement was achieved in spite of the fact that grapeseed extract on its own had a negative effect on FCR (Chikoto, 2006).

The current findings on FCR are in agreement with those of Lee *et al.* (2003), Botseglou *et al.* (2004) and Hernandez *et al.* (2004) who reported that addition of plant extracts or essential oils to the diet had no beneficial effect on feed intake or FCR. In contrast, Lee *et al.* (2003), who studied carvacrol from oregano, Madrid *et al.* (2003) who studied the effect of plant extracts and Alcicek *et al.* (2004) who used 48 mg/kg of an essential oil mixture in the diet of the broiler, found an improvenent in FCR when these were included in the diet of broilers.

Table 5.6. Feed conversion ratios (FCR) of broilers challenged with *C. perfringens* and fed different feed additives

| Treatments | FCR | | | |
|---|---|---|---|---|
| | Day 13* | Day 20 | Day 27 | Day 35 |
| Neg control | 1.75 (0.018)[ac] | 1.68 (0.021)[a] | 1.85 (0.028)[a] | 1.68 (0.028)[a] |
| Zn-bacitracin | 1.52 (0.029)[bc] | 1.66 (0.022)[a] | 1.86 (0.011)[a] | 1.69 (0.005)[a] |
| *G. biloba* | 1.94 (0.019)[ab] | 1.64 (0.013)[a] | 1.84 (0.026)[a] | 1.62 (0.054)[a] |
| *G. biloba + H. perforatum* | 1.88 (0.043)[a] | 1.60 (0.039)[a] | 1.80 (0.036)[a] | 1.66 (0.018)[a] |
| P-value | 0.00 | 0.283 | 0.427 | 0.383 |

*Before challenge

### 5.3.6 European Performance Efficiency Factor index

Means of the EPEF index were not significantly different between any of the treatment of the non-challenged broilers although the trend observed on day 35 was an improvement compared to the negative control of 2.1% with *G. biloba + H. perforatum* , 5.0% with *G. biloba* and 5.5% with Zn-Bacitracin (Table 5.7). Chikoto (2006) also observed a trend in the C*. perfringens* challenged group that treatment with Zn-bacitracin and treatments containing *G. biloba* and/or *H. perforatum* resulted in higher EPEF values than in the negative control. His values of 234 for the negative control vs. 248.4 and 244 for the treatments containing *G. biloba* + grapeseed and a combination of *G. biloba* + *H.perforatum* + grapeseed respectively on day 42 are comparable to what was found in this study. It should be noted that the grapeseed treatment on its own in Chikoto (2006)'s experiment resulted in values lower than the untreated control (challenged and non-challenged groups).

Table 5.7. European Performance efficiency index (EPEF) of broilers challenged with *C. perfringens* and fed different feed additives

| Treatments | EPEF | | |
|---|---|---|---|
| | Day 20 | Day 27 | Day 35 |
| Neg control | 181.2[a] | 174.6[a] | 234.0[a] |
| Zn-bacitracin | 187.2[a] | 176.3[a] | 246.9[a] |
| *G. biloba* | 190.2[a] | 174.7[a] | 245.6[a] |
| *G. biloba + H. perforatum* | 192.2[a] | 178.7[a] | 239.6[a] |
| P-value | 0.416 | 0.942 | 0.839 |

## 5.4 Conclusions

The objective of the study was firstly to determine the effect of a combination of extracts of *G. biloba* and *H. perforatum* on the performance of broiler chickens over a 35 day period and secondly to investigate the effect on *C. perfringens* in the intestine of broilers.

No significant differences were found with relation to any of the production parameters studied although a trend towards more favourable EPEF values were observed for treatments containing *G. biloba* (5% improvement) or a combination of *G. biloba* and *H. perforatum* (2.1% improvement) compared to the untreated control. Similarly, Zn-bacitracin resulted in a 5.5% improvement compared to the untreated control. This trend was also previously observed by Chikoto (2006) with treatments containing the same extracts.

*C. perfringens* was more frequently isolated from the intestines of broilers of the challenged treatments compared to the non challenged control in spite of the fact that necrotic enteritis was not succesfully induced in this experiment. There was a general trend (not on a statistical significant level) towards a reduction in microbial scores in the feed supplemented with *G. biloba* and *H. perforatum* which can probably be ascribed to the direct antimicrobial effect. The rate of colonization was too low to cause infection and have an impact on production probably due to the absence of predisposing factors and the possibility that the challenge strain was insufficiently virulent. It is necessary for an effective disease model to be developed in order for the efficacy of any new treatment method to be properly evaluated. Such a model will require a much higher incidence of disease and reproducibility than was achieved in this project.

## 5.5 Acknowledgements

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# 6 General conclusions

The commercial rearing of broilers has to date depended on the inclusion of in-feed antibiotic growth promoters (AGP's). This practice is, however, disappearing due to the threat of development of antibiotic resistant microbes with public concern forcing the poultry industry to consider alternatives. Strategies to control necrotic enteritis in the absence of AGPs have focused on dietary and management practices and alternative feed additives. Among the candidate replacements for antibiotics are the inclusion of plant extracts into the diet. No single non-antibiotic measure has been identified yet, even though the *in vitro* antimicrobial effect of plant extracts against intestinal pathogens has been demonstrated (Kamel, 2000). Our study group joined this search for a commercially viable antibacterial phytogenic product from *Ginkgo biloba* and *Hypericum perforatum* with a low potential to develop resistance, as an alternative to AGPs in poultry production.

The objectives of the study were to:

- Evaluate the activity and potentize extracts from *G. biloba* and *H. perforatum* for optimal activity against selected bacterial pathogens (Chapter 2).
- Combine extracts or fractions of extracts of *G. biloba* and *H. perforatum* for optimising activity against selected bacterial pathogens (Chapter 2).
- Isolate and identify the major antibacterial compound in *G. biloba* (Chapter 3).
- Evaluate certain pharmacodynamic aspects of *G. biloba* extracts i.e. evaluate the potential of *E. faecalis* to develop *in vitro* resistance against a fraction/compound of *G. biloba* extracts and study the time-kill kinetics of this fraction using above mentioned pathogen over a 24 h period (Chapter 4).
- Determine the effect of extracts of *G. biloba* and/or *H. perforatum* on growth promotion and *Clostridium perfringens* in the intestines of broiler chickens challenged with *C. perfringens* (Chapter 5).

In order to prove the combination of *G. biloba* and *H. perforatum's* potential as an alternative to AGPs in poultry, the quantitative and qualitative aspects surrounding the *in vitro* antimicrobial effect needed to be investigated. Extracts of ethyl acetate (EA), hexane, dichloromethane (DCM) and acetone (in order of activity) from the direct extraction procedure of powdered *G. biloba* leaves were active against *E. faecalis, S. aureus* and *C. perfringens*. The EA, hexane and DCM extracts were 2 to 3 times more active than the acetone extract (average total activity 1728 ml/g dry extract for the 3 pathogens). The DCM, EA, acetone and hexane extracts (in order of activity) from the direct extraction procedure from *H. perforatum* were only active against *C. perfringens* with the first three extracts having a total activity of between 1 026 and 1 333 ml/g dry material and the hexane extract a total activity of 333 ml/g dry material. The spectrum of activity of *G. biloba* corresponds to that of Zn-Bacitracin which is commonly used an antibiotic growth promoter in the poultry industry.

Combination of the hexane fractions of *G. biloba* and *H. perforatum* from the solvent-solvent extraction procedure had a synergistic effect (activity enhanced 2.2 times when a 1:15 combination of *G. biloba: H. perforatum* were used) against *E. faecalis* but a neutral (neither synergistic nor antagonistic) effect against *C. perfringens.* An indifferent to additive effect was observed when combining the acetone extracts from the two plant species against *C. perfringens* or *E. faecalis*. Biological activity may thus be enhanced through the use of a combination of phytochemicals with different metabolic effects.

At least one compound was consistently present in above-mentioned active extracts of *G. biloba* as observed by using the bioautography technique. This compound was isolated and identified as ginkgolic acid, a 6-alkylsalicylic acid with $C_{17:1}$ alkyl substituent belonging to the alkylphenol group of compounds. Ginkgolic acid plays a role in the activity of these extracts against *E. faecalis* (12.5-50%) and *C. perfringens* (50-200%). The enhanced activity of ginkgolic acid against *C. perfringens* can be ascribed to proven synergistic interactions (2.4 times) between the hexane- and EA fractions of the acetone extract, with only the hexane extract containing ginkgolic acid. Combination of these two fractions resulted in a MIC 3.1 times lower against *E. faecalis* compared to the sum of the MIC of the individual fractions. The potential use of isolated active compounds as antimicrobial products proved not only expensive and time-consuming, but also exclude positive interactions between constituents in whole extracts which could considerably enhance activity. According to Yuan and Lin (2000), the National Cancer Institute of the USA and the USD Department of Agriculture screened 35 000 samples from different tissues from plant species and only three new drugs were discovered. Nevertheless, the ancient Oriental pharmacopoeias contain thousands of therapeutic formulations, indicating that the biological activity of these preparations might result from synergy of active compounds rather than a single chemical entity.

In chapter 4 the potential of *E. faecalis* to develop resistance against the hexane fraction (containing the highest concentration of ginkgolic acid) as well as ginkgolic acid from *G. biloba* was evaluated. No decreased susceptibility of the hexane fraction or ginkgolic acid was observed even after 10 and 9 passages respectively while an 8-fold increase was observed with the gentamycin control within 7 passages. This is not surprising as *G. biloba* has survived over 200 million years probably due to its ability to resist pests and diseases. Previous studies have indeed shown the presence of antibacterial compounds in *G. biloba* (chapter 2 – unpublished data; Mazzanti *et al.,* 2000; Pan, 2007) as well as synergistic interactions between constituents which enhancesantimicrobial activity (chapter 4 – unpublished data).

In addition to the emergence of resistance studies performed in chapter 4, another pharmacodynamic end point i.e. the extent of bacterial killing, was also investigated, as this is an important parameter to consider early in the development of a new antimicrobial agent. The bactericidal nature of the hexane extract from *G. biloba* as well the absence of decreased susceptibility in the resistance studies against *E. faecalis* indicated that this extract has potential to be exploited as a alternative to AGPs in the poultry industry.

Chapter 5 was a continuation of the work of Chikoto (2006). The objective was to determine the effect of a combination of *G. biloba* and *H. perforatum* on the performance of broiler chickens over a 35-day period. The effect on *C. perfringens* in the intestine was also investigated. In spite of the fact that the hexane, DCM and EA extracts from *G. biloba* yielded the best results in terms of antimicrobial activity against *E. faecalis* and *C. perfringens* (chapter 2), the acetone extract was used in this experiment. The reasons were three-fold: firstly Chikoto (2006) established that the acetone extracts of *G. biloba* and *H. perforatum* can be tolerated by broilers at a rate of 2 and 10mg/kg respectively and secondly the extracts were found to be stable in feed and water. Antimicrobial activity was also found to be stable under varying pH conditions. No significant differences were found after treatment with *G. biloba* and/or *H. perforatum* with relation to live body weight, feed conversion ratio (FCR), mortality or European Performance efficacy factor (EPEF) although a trend towards more favourable EPEF values were observed for treatments containing *G. biloba* (5% improvement) or a combination of *G. biloba* and *H. perforatum* (2.1% improvement) compared to the untreated control. Similarly, Zn-Bacitracin resulted in a 5.5% improvement compared to the untreated control. This trend was also previously observed by Chikoto (2006) with treatments containing the same extracts.

*C. perfringens* was more frequently isolated from the intestines of broilers of the challenged treatments compared to the non challenged control in spite of the fact that necrotic enteritis was not succesfully induced in this experiment. There was a general trend (not on a statistically significant level) towards a reduction in microbial scores in the feed supplemented with *G. biloba* and *H. perforatum,* which can probably be ascribed to the direct antimicrobial effect. The rate of colonization was too low to cause infection and have an impact on production probably due to the abence of predisposing factors. It is necessary for an effective disease model to be developed in order for the efficacy of any new treatment method to be properly evaluated. Such a model will require a much higher incidence of disease and reproducibility than was achieved in this project.

The use of a product containing ginkgolic acid as active ingredient raises some questions regarding safety as ginkgolic acids and related alkylphenols have been recognized as hazardous compounds with suspected cytotoxic and allergenic properties (Pan, 2007). The acetone extract from *G. biloba* was included into the chicken feed at a rate of 14 ppm. Considering that the chickens in this trial consumed up to 170 g of feed/kg live weight of chicken / day the maximum dose of the acetone extract from *G. biloba* extract was 2.4 mg/kg/day. The active ginkgolic acid constitutes up to 5% of the acetone extract (chapter 3), thus relating to 0.12 mg/kg/day (0.12 ppm). For reasons of drug safety a maximum concentration of ≤5 ppm of ginkgolic acids is recommended by the Monograph of the Commission E of the former German Federal Health Agency (Baron-Ruppert and Luepke, 2001). Therefore the effect of residues on consumers of chicken products would probably be negligible. During production of the standard *Ginkgo* extract EGb761®, alkylphenols are largely eliminated as water insoluble compounds (decanter sludge) from the primary acetone extract. Baron-Ruppert and Luepke (2001) evaluated different fractions from the decanter sludge for their embryotoxic effects in the hen's egg test (HET). A fraction enriched for ginkgolic acids (16%) and biflavones (6.7%) was found to induce death of 50% of the chick embryos

($LD_{50}$) at a dose of 1.8 mg/egg (≈33 ppm). A similar strong lethal effect ($LD_{50}$:3.5 mg/egg; 64 ppm) was observed for a fraction which contained 58% ginkgolic acids but less than 0.02% biflavones. Some scientists believe that the safety hazard of ginkgolic acids was overemphasized by the Schwabe company as part of a marketing exercise (personal communication: Prof. J.N. Eloff). Hausen (1998) for example found that though the guinea pigs in his study were sensitized successfully with pure ginkgolic acids, the animals could not be sensitized with the leaf extract. Leaf extracts of *G. biloba* taken orally or given by infusion to treat diffuse cerebral disturbances can be considered safe, even when they contain up to 1000 ppm of the sensitizing ginkgolic acids (Hausen, 1998). The absence of adverse effects when alkylphenols are taken orally are supported by the fact that no reports have been filed on adverse effects of *Ginkgo* mother tinctures in spite of the fact that such extracts contain 2.2% (22000 ppm) of ginkgolic acids and have been on the market for several years (van Beek, 2002).

The biological activities of the ginkgolic acid derivates have attracted considerable attention for their molluscicidal activity against *Oncomelania hupensis* (Yang *et al.,* 2008), anti-*Toxoplasma gondii* activity (Chen *et al.,* 2008) antitumor (Kubo *et al.,* 1993) antioxidant (Kubo *et al.,* 2005) and xanthine oxidase inhibitory action (Masuoka and Kubo, 2004). Ginkgolic acids or extracts containing high concentrations thereof can thus be exploited for it's benefits in many different fields such as the agricultural and pharmaceutical industry.

The combination of extracts of *G. biloba* and *H. perforatum* holds promise as a potential growth promoter in poultry production. Better results may be achieved if potentized extracts are used and compared with Zn-Bacitracin and a negative control under industrial growth conditions where the birds are stressed and natural infections would take place.

## 6.1 References

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