PLANT-BASED FEED SUPPLEMENTS WHICH INCREASE ANTIBIOTIC SUSCEPTIBILITY OF ZOONOTIC PATHOGENS AND REDUCE RESISTANCE DEVELOPMENT

By

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DEDICATION

To my family and friends. To my son: Kavin Adithiya, Thank you for your love and understanding. You mean everything to me. My parents: Thank you for raising me and supporting me all the time

ORGANIZATION OF THE THESIS

This thesis is divided into six chapters which includes two manuscripts.

Chapter one gives a brief introduction about the risks associated with the use of antibiotics as growth promoters in animal feed and possible alternatives to solve this problem. It describes briefly the objectives of this study.

Chapter two gives a literature review about the antibiotics used in agriculture and antibiotic resistance problems associated with their use. The need to find alternatives for growth promoters and their current use in animals are also dicussed in this chapter.

Chapter three is a manuscript published in the International Journal of Food Microbiology and is entitled "Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria" (Palaniappan and Holley, 2010).

Chapter four is a manuscript which describes the second part of the work with chicken caecal content and poultry feed. It also discusses the use of a formaldehyde-based feed additive as another alternative to antibiotic growth promoters. It is entitled "*In vitro* evaluation of Termin 8 and thymol use in chicken caecal content and poultry feed against antibiotic resistant bacteria."

Chapter five gives the conclusions obtained from these studies. Finally chapter six summarizes some recommendations for future study.

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ABSTRACT

Bacterial isolates from animals, foods and clinical samples with resistances to one or more antibiotics are being found frequently each year. Selective pressure exerted by antibiotic growth promoters in food animals has been considered a main cause for the development of antibiotic resistance and antibiotic use has been strongly criticized as a serious public health threat. The gastrointestinal tract of animals not only serves as a reservoir of zoonotic agents but also as a spot for exchange of genetic information between pathogenic and commensal bacteria. Humans get infections from resistant bacteria either through the food chain, contaminated water or by direct contact with animals.

In this situation much of the concern has been directed against the use of antibiotic growth promoters in animals. The removal of synthetic antibiotics from animal diets created other problems such as a decline in animal welfare and an increase in the use of therapeutic antibiotics. So there is a need for new alternatives to antimicrobial drugs to overcome resistance development and related problems.

Plants and plant-derived compounds have long been considered to posess antimicrobial activity since they were frequently used in ancient medicine as natural remedies to treat human infections. Identifying new sources of natural antimicrobials and inhibitors of resistance development will yield novel therapeutic drugs and extend the useful life of existing antibiotics.

In the present work, individual and combined effects of five essential oils (eugenol, thymol, carvacrol, cinnamaldehyde, allyl isothiocyanate (AIT)) and a formaldehyde-based feed additive, Termin 8, with antibiotics against 4 antibiotic resistant bacteria with known determinants for resistance were tested using broth microdilution and the checker board

assay. The bacteria showed considerable susceptibility towards these antimicrobials and a significant reduction in the minimum inhibitory concentrations (MICs) of antibiotics was noted when paired combinations of antibiotic and antimicrobial were used. The synergistic interaction was further confirmed by the extent of decrease in logarithmic count or viable population (Log DP). Although most of the combinations were synergistic by fractional inhibitory concentration (FIC) values, fewer combinations showed synergistic interaction when Log DP was considered. Gram-positive bacteria were more sensitive to the antimicrobials than Gram-negative bacteria. In combination studies, carvacrol was more effective and showed synergistic interaction with at least three antibiotics. When used alone, AIT was more effective and the concentration needed to exhibit antimicrobial action was much lower when compared to other compounds.

An *in vitro* study was conducted to assess the antibacterial effects of Termin 8 and thymol in chicken caecal digesta and poultry feed samples by using a thin agar layer (TAL) method. Concentrations greater than the MIC of both the compounds was required to exert antimicrobial activity in the feed and digesta samples.

The natural antimicrobials and Termin 8 had significant inhibitory effects on the drug resistant bacteria and synergistically enhanced the efficacy of antibiotics when used in combination. Further studies are needed to test their effectiveness in animal models.

CHAPTER 1

Introduction

In spite of increasing efforts to improve slaughter hygiene and food protection techniques, food safety is still a signficant public health issue (Burt, 2004). There is growing concern about the increased prevalence of antibiotic resistance throughout the world (Bogaard and Stobberingh, 2000). Drugs may be used either therapeutically or prophylactically, or for growth promotion (as feed additives) (Threlfall et al., 2000). The continuous use of antibiotics in human medicine, in animal practice as antibiotic therapy and at sub-lethal levels as growth promoters are prone to select and enrich for resistant bacteria. Antibiotics were introduced into animal feeds about 20 years ago and their use as growth promoters in farming is thought to be the main reason for the development of resistance among certain strains of animal gut bacteria to some antibiotics (Jukes, 1972; Wallace, 2005). There is a close association between the quantities of antibiotics used and the rate of development of resistance (Sengelov et al., 2003; Barton, 2000; Teale, 2002). The use of antimicrobial agents in food animals has resulted in development of antimicrobial resistance among pathogenic and commensal bacteria in exposed animals, and the resistant bacteria may then be transferred to humans through food, contaminated water or by direct contact with animals. Humans become infected with zoonotic enteric pathogens by the ingestion of food contaminated with animal feces (contamination often occurs during processing). This resistance development in zoonotic bacteria constitutes a public health risk, primarily through the increased risk of treatment failure (Angulo et al., 2004a; Aarestrup and Wegener, 1999). Extensive antimicrobial gene transfer has been demonstrated among enteric bacteria including *Bacteroides* and Gram-positive bacteria in the human colon. These

organisms can serve as reservoirs of resistance genes that can be transferred to other members of the microflora or to pathogenic bacteria (Shoemaker et al., 2001).

There has been increasing research interest in the development of alternatives to address this problem. The pharmaceutical industry has tried to limit the consequences of resistance development by the search for and discovery of new antibiotics. In spite of that, in recent years bacteria resistant to an increasing number of antimicrobial agents have emerged world-wide (Aarestrup and Wegener, 1999). The animal feed industry is under increasing consumer pressure to reduce the use of antibiotics as feed additives and find substitutes for antibiotics in the diet (Alcicek et al., 2003). One area of research has been the development of alternative therapies and the therapeutic use of natural products, especially those derived from plants. The latter have an advantage because of their long-term use by humans, often hundreds or thousands of years. For a long time, mineral, plant and animal products have been the main sources of drugs (Rates, 2001; Fabricant and Farnsworth, 2001).

Spices and herbs have been added to foods since ancient times, not only as flavoring agents, but also as folk medicine and food preservatives. Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including food-borne pathogens (Shan et al., 2007). Essential (volatile) plant oils occur in edible, medicinal and herbal plants and are significant sources of natural ingredients for producing new antimicrobial drugs. It was also recently discovered that essential oils have a stimulating effect on the animal digestive system (Alcicek et al., 2003). The antimicrobial activities of essential oils are attributed to a number of small terpenoid and phenolic compounds (Helander et al., 1998). Many studies have reported that the phenolic compounds in many plants contribute to their antimicrobial and antioxidant activities (Shan et al., 2007).

Where animal waste from carcass processing has been used as a feed ingredient, formaldehyde treatment has been an important tool for disinfection (Koenig et al., 1978). Chemical products such as formaldehyde-based antibacterial feed additives have been reported to decrease both pathogen numbers and prevent recontamination of bacteria in feeds (DeRouchey et al., 2004).

Some studies have explored the combination of synthetic drugs plus essential oils with a view to evaluating and enhancing antimicrobial efficacy (Rosato et al., 2007). In antimicrobial therapy, synergism is a well known fact and was used to describe the supra-additive activity of antibiotics. When drugs are used in combination, the synergistic effect is believed to be brought about by concerted action at one or more different targets involved in drug uptake or metabolism (Hemaiswarya and Doble, 2009).

The aims of this study were to determine the effect of natural antimicrobials and a formaldehyde-based feed additive, Termin 8, against antibiotic resistant pathogenic bacteria commonly found in the animal environment, and to study the level of reduction in MIC's of antibiotics in the presence of several natural antimicrobials and Termin 8. The main objectives of this research were:

1. To screen natural antimicrobials and Termin 8 against antibiotic resistant bacteria and determine whether the pathogens show any resistance to natural antimicrobials.
2. To assess the natural antimicrobial and antibiotic combinations for antimicrobial action using previously described procedures.
3. To study the antimicrobial effects of Termin 8 and thymol in chicken gut digesta and poultry feed.

CHAPTER 2

2. Literature review

2.1 Antibiotics and animal use

Antibiotics are defined as compounds used in both human and veterinary medicine which are naturally occurring, semi-synthetic or synthetic compounds with antimicrobial activity; they may be administered orally, parenterally or topically (Phillips et al., 2004). Antibiotics were introduced into human chemotherapy in the 1940's. Antimicrobial agents have been used in agriculture, including livestock and poultry, since the early 1950's to treat infections and improve growth and feed efficiency (Witte, 1999; Angulo et al., 2004b). In veterinary practice, antibiotics are used on pets, farm animals and in aquaculture (Teuber, 2001). Antimicrobial agents are used for therapeutic, metaphylactic and prophylactic reasons, and they may be used to promote growth. Drugs are given to animals by injection (intravenously, intramuscularly and subcutaneously), orally in food or water, topically on the skin, and by intramammary and intrauterine infusions (Johnston, 1998).

Therapeutic use of antibiotics in veterinary medicine involves treatment of an individual animal or a group of sick animals with one or more antibiotics during a defined period of time. These actions protect animal welfare and prevent the epidemic spread of animal disease and zoonotic disease transfer from animals to man (Teale, 2002). Antibiotics are used only upon prescription from veterinarians in western countries (Bogaard and Stobberingh, 2000). Among food animals, individual treatment is common in dairy cows and calves. The drug and dose chosen are appropriate for the animal and the nature and state of the disease. Adequate MIC concentrations are usually used and these are comparable to the use of antimicrobials in human medicine. As a result, therapeutic antibiotic use should lead

to maximum eradication and prevention of the emergence of resistant microorganisms because the antibiotic concentration used is high relative to the MIC of the organism (Schwarz et al., 2001; Phillips et al., 2004).

For poultry and animals kept in larger groups, group treatment is preferred where individual animal treatment is almost never practical. Sometimes the entire group must be treated at times when only a single animal of the group presents symptoms of the disease. This approach is taken when it is expected that most of the group will become affected and it is referred to as metaphylaxis.

When antibiotics are administered to the entire herd before the development of clinical signs to prevent illness, it is called prophylaxis (Johnston, 1998). Here, a wide range of antibiotics can be used and the dose rates and treatment times are subject to manipulation. Often it is difficult to distinguish between growth promotion and prophylactic application of antibiotics.

2.1.1 Antibiotic growth promoters and antibiotic resistance

Apart from veterinary use, antibiotics can be added continuously to animal feeds at non-therapeutic levels to enhance animal performance by increasing growth rate. In this application antibiotics act as antimicrobial growth promoters and this constitutes a large proportion of total antibiotic use. The term growth promoter is used for feed additives other than dietary nutrients which increase growth rate and improve feed efficiency in healthy animals fed a balanced diet (Bogaard and Stobberingh, 2000; Barton, 2000). Antibiotics were first used in commercial animal feed about 20 years ago. The impetus for the use of antibiotics in feeding farm animals began in 1949 when it was observed that animals fed

dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues had improved growth (Castanon, 2007; Jukes, 1972). The use of growth promoting antibiotics occured in conjunction with intensive animal rearing and as a result average growth improvement was found to be between 4 and 8%, while feed utilization was improved by 2 to 5 %. For example, broilers are typically raised under confinement in barns containing 10,000-20,000 birds and turkeys are raised in groups of 5,000-10,000. This integrated farming approach led to standardized management practices like drug treatment policies and many problematic infectious diseases were controlled with antibiotics (McEwen and Fedorka-Cray, 2002).

The exact mechanism behind growth promotion by antibiotics is still not clear, in part because the concentrations used are lower than therapeutic levels. The agents are presumed to exert their beneficial effects by: protecting nutrients against bacterial destruction; causing lethal or sub-lethal damage to the pathogens; decreasing the production of toxins by intestinal bacteria; allowing increased synthesis of vitamins and other growth factors; reducing the microbial destruction of essential nutrients in the gastrointestinal tract; or improving the absorption of nutrients by reducing the thickness of the intestinal epithelium (Barton, 2000; Butaye et al., 2003; Feighner and Dashkevicz, 1987; Visek, 1978).

At least 17 classes of antibiotics are approved for growth promotion in the United States. This includes tetracyclines, penicillins, macrolides, lincomycin (an analog of clindamycin), and virginiamycin (an analog of quinopristin/dalfopristin) (Angulo et al., 2004a; 2004b). Nearly 40% of the 2.1-2.5 million kilograms of antimicrobial agents used in the United States annually goes for animal feed supplementation. Among the animals raised in United States, 80% of poultry, 75% of swine, 60% of feed lot cattle, and 75% of dairy calves were estimated to have been fed an antimicrobial agent at some time during their life. The dose

range for growth-promoting purposes at subtherapeutic levels range from 1.9 g to 197 g/tonne of feed in the United States. The concentration used for prophylactic purposes is 98-394 g/tonne and this range is increased to 197-984 g/tonne for therapeutic treatment (Dupont and Steele, 1987).

2.1.2 Problems associated with the use of antibiotic growth promoters

Continuous use of antibiotics as growth promotants at subtherapeutic levels can create selective pressure for the emergence and dissemination of antimicrobially resistant bacteria. But the extent to which antibiotic use in animals contributes to the antibiotic resistance of bacteria in man is still uncertain (Teale, 2002). Antibiotic resistance among commensal and pathogenic bacteria in treated animals is a daunting public health threat. Resistant bacteria may be transmitted to humans through the food or water supply or by direct contact with animals. Though commensal bacteria are a less obvious threat, they may also transfer resistance from animals to humans. These bacteria may serve as a reservoir of resistance genes for pathogenic bacteria by carrying transferrable genetic determinants of resistance (Angulo et al., 2004b). It is possible that the application of veterinary antibiotics to food animals enhances the selection of strains resistant to antibiotics used in human medicine (Kemper, 2008). The transfer of genetic determinants for resistance can lead to diseases which cannot be treated with conventional antibiotics, yielding adverse health consequences in humans, including increased potential for treatment failure. Concerns about antibiotic residues in the tissues of treated animals has been the focus of some studies, but there is little scientific information on the effect of antibiotic residues on the bacterial flora of the human intestinal tract (Barton, 2000).

Antibiotics that elicit allergic reactions in humans include the β-Lactams such as penicillins, cephalosporins and to a lesser extent the macrolides, tetracyclines, sulphonamides and aminoglycosides. Among these penicillin appears to be more strongly allergenic than the other antimicrobials (Dewdney et al., 1991). Allergic reactions in sensitized individuals by penicillin residues in milk have been reported, but several studies concluded that there were no other adverse effects associated with antibiotic residues (Dyan, 1993; Dewdney and Edwards, 1984). The use of antimicrobial agents in aquaculture presents a risk to public health because some bacteria that cause infections in fish belong to the same genera as bacteria causing infections in humans. Their use in aquaculture was considered likely to increase the probability of spread of antimicrobial resistance from farmed fish to humans (Heuer et al., 2009).

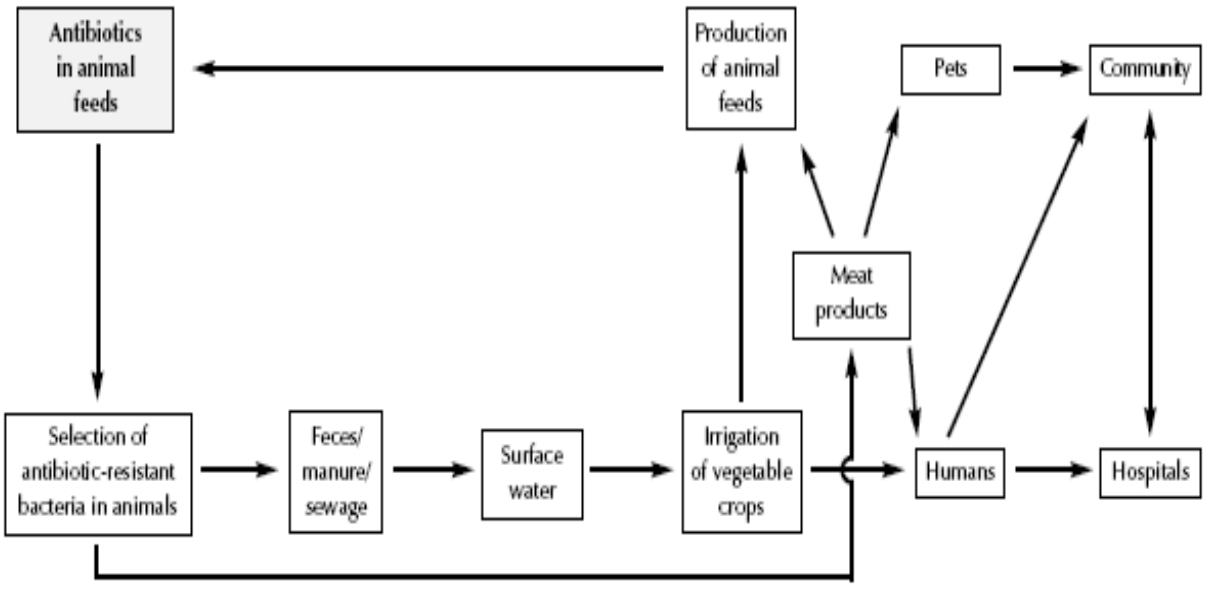


Fig.2.1 Routes of dissemination of antibiotic resistant bacteria in the environment (Khachatourians, 1998).

The agricultural use of animal feed containing antibiotics can result in the selection and transmission of antibiotic resistant bacteria. These bacteria move through the environment by variety of routes, and their presence ultimately can have consequences to human health (Fig. 2.1).

2.1.3 Impact of discontinuing the use of antimicrobial growth promoters

As a result of concerns about bacterial resistance to antibiotics used in human medicine, the World Health Organization recommended discontinuing the use of antimicrobial growth promoters that belong to the same classes as those used for humans (WHO, 1997). In 1999, farmers in Denmark voluntarily stopped using antimicrobial agents as growth promoters (Angulo et al., 2004a). The quantity of antibiotics used in food animals in Denmark declined 54% from a peak of 205,686 kg in 1994 to 94,200 kg in 2001. This resulted in increased demand for therapeutic use in animals of some antimicrobials used in humans like tetracycline, penicillin and the macrolides (WHO, 2002). The banning of growth promoting antibiotics might have adverse consequences for animal health and welfare and economic consequences for farmers.

In Sweden, the loss in production of pigs resulting from antibiotic withdrawl from feed has not yet fully recovered, even 16 years after the ban on growth promoters. Increased mortality and morbidity among pigs, mostly associated with enteric infections, has been reported in Denmark. Also 11% of finishing herds experienced permanent problems with increased frequency of diarrhea and reduced weight gain. The Danish National Department of Poultry Production points out that since the late 1990's, the broiler industry has been struggling with leg and skin problems in birds (Casewell et al., 2003). McEwen and Fedorka-Cray (2002)

reported possible consequences of withdrawal of antibiotic growth promoters, which included the following: decreased incentive for new drug development, poorer production efficiency, compensatory increases in antibiotic prophylaxis or therapy, increased incidence of infectious diseases in animals and limitations on the ability of veterinarians and farmers to treat and prevent disease.

2.2 Alternatives to antibiotics

The above issues have stimulated investigation to find alternative treatments for vulnerable human and animal populations in the event that antibiotic availability is substantially reduced. There is a necessity to explore new ways to protect and improve animal health, to guarantee animal performance and to protect the environment. These concerns have driven the search for new alternatives that are broadly effective and less likely to induce antimicrobial resistance (Sang and Blecha, 2008)

A number of strategies for improvements in animal health, productivity and microbial food safety that do not involve antibiotics have been recently explored (Joerger, 2003). Outcomes can be improved through use of clean housing and optimum climate conditions along with the best possible combination of pro-nutrients (Wenk, 2002).

In poultry production, probiotics and competitive exclusion have been studied for their ability to reduce pathogen colonization of the birds (Pattersson and Buckholder, 2003). Probiotics are live cultures of a single bacterial strain or a mixture of different strains which are fed to animals to improve health and growth. Often lactic acid bacteria or sometimes species of other genera are used, and they are all believed to produce beneficial effects by altering the intestinal microbial balance (Doyle, 2001; Griggs and Jacob, 2005).

The addition of enzymes has been found to be beneficial depending on the feed ingredients used. Dietary enzymes may enhance the animals own digestive enzyme activity or enable the animal to utilize the energy in complex carbohydrates which would otherwise pass unchanged through the gastrointestinal tract (Doyle, 2001).

Organic acids have been used as a tool to reduce undesirable bacteria and have been added to drinking water or to feed (Griggs and Jacob, 2005). A significant decrease in the colonization of *Salmonella* Enteritidis in the caeca and internal organs of chickens fed diets containing 0.3 % caproic acid was reported by Immerseel et al. (2004).

Bacteriocins are antibacterial proteins or peptides produced by bacteria which kill or inhibit the growth of other bacteria (Cleveland et al., 2001). Their minimal inhibitory concentration (MIC) is often in the nanomolar range, but activity is frequently restricted to related Gram-positive bacteria or Enterobacteriaceae (Sang and Blecha, 2008).

Bacteriophages are viruses that kill bacteria by causing their lysis (bacteriolysis) through disruption of the cell wall. Phage therapies are highly efficient, highly specific, and relatively cost effective. However, resistance may develop and since phages may carry harmful genes, precautions are required in the selection of phages for therapeutic applications (Parisien et al., 2008).

2.2.1 Plant derived compounds

"Eat leeks in March and wild garlic in May,

And all the year after the physicians may play."

Traditional Welsh rhyme

"An apple a day keeps the doctor away."

Traditional American rhyme (Cowan, 1999)

Plants and their compounds have been noted for a long time to be a valuable source of natural products, particularly as rich sources of anti-infective agents for maintaining human health (Nascimento et al., 2000). It is estimated that there are 250,000 to 500,000 species of plants on Earth, but only 1 to 10 % of these are used for food by both humans and animals (Cowan, 1999). Commonly available herbs, spices and aromatic plants that exhibit antimicrobial activity could provide sources of acceptable, natural alternatives to antibiotics (Delaquis et al., 2002). They are potential alternatives because they do not have any significant medical or environmental impact, but some have sensory characteristics that limit their use in food/feed. The active compounds available in medicinal plants have been a dependable source of therapeutics for the treatment of various ailments since before recorded history. Plant extracts can beneficially affect feed intake, secretion of digestive juices and the immune system of animals. They can have antibacterial, coccidiostatic, anti-helminthic, antiviral or anti-inflammatory activity and some posess antioxidant properties (Cimanga et al., 2002; Alcicek et al., 2003).

Plants have an almost limitless ability to synthesize aromatic substances, and yield useful medicinal compounds. Many compounds are responsible for flavor and humans use some of them to season food. Antimicrobial phytochemicals in plants can be divided into several categories which include simple phenolic and polyphenolic acids, quinones, flavones, flavonoids, flavonols, tannins, coumarins, terpenoids, essential oils, alkaloids, lectins, polypeptides and can be used alone or in mixtures. The best example of a mixture is latex (a milky sap) in papaya (*Carica papaya*) which is a complex combination of papain, carpaine plus terpenoids and was reported to have anti-helminthic activity against *Ascaris suum* in

pigs and was also fungistatic towards *Candida albicans.* The seed and fruit extracts of papaya inhibited the growth of *S. aureus, B. cereus E. coli, P. aeruginosa* and *Shigella flexneri* (Scartezzini and Speroni, 2000; Cowan, 1999; Krishna et al., 2008; Emeruwa, 1982). The chewing sticks widely used in African countries for dental and oral hygiene are usually formulated from different species of plants, but active compounds present are heterogeneous and are inhibitory toward oral bacteria (Rotimi and Mosadomi, 1987).

Plant essential oils as well as similar compounds have been reported to have antimicrobial activity against a wide range of spoilage and pathogenic bacteria (Holley and Patel, 2005). Plant essential oils are usually mixtures of several components and compounds with phenolic groups are most effective. Phenolic compounds have been reported to have multiple biological effects and are commonly found in both edible and non-edible plants. Flavonoids and other phenolics have been suggested to have protective effects against cancer and coronary heart disease development (Kahkonen et al., 1999). An estimated 3,000 essential oils are known, of which about 300 are commercially important for flavors and fragrances in the market (Burt, 2004). Many studies report that phenolic compounds in spices and herbs significantly contribute to the antioxidant and pharmaceutical properties of plant extracts and might also play a major role in their antimicrobial activity. Limited data have been reported on the antioxidant and antimicrobial effects of spices and herbs (Shan et al., 2007).

2.2.2 Antibacterial properties of plant essential oils

In addition to spices and their derivatives being used for food and beverage as well as flavoring, to extend the shelf life of foods and for medication, they have also been highly

valued for their use as antimicrobials (Baydar et al., 2004). Many studies have demonstrated activity against bacteria, fungi and viruses. More recently, due to increased antimicrobial resistance, the potential use of essential oils for the prevention and treatment of infection has been the focus of several studies (Karpanen et al., 2008).

Essential oils are a rich source of biologically active compounds. These are aromatic oily liquids obtained from different parts of plants like flowers, buds, seeds, leaves, twings, bark, wood, fruits and roots. They can be obtained by different methods such as expression, fermentation or extraction (Prabuseenivasan et al., 2006). Since these oils are bactericidal, they are more frequently used in pharmaceuticals and foods as alternatives to synthetic chemical products. For these applications, extraction by steam distillation is most common for commercial production and products are chemotyped by gas chromatography and mass spectrometry analyses. The extracted products can vary in quality, quantity, as well as composition, and these may produce great variation in activity (Bakkali et al., 2008). Other factors can influence the variation in antimicrobial activity and these include the culture medium, the technique of testing, the botanical source of the plant, its age, the state of the plant material used (whether dried or fresh), the quantity of the oil used for the test and the isolation techniques (Cimanga et al., 2002).

Shan et al. (2007) reported a strong correlation between the concentration of phenolic compounds and antibacterial activity of plant extracts and thus to antioxidant capacity. The wide spectra of antimicrobial activity of extracts of many spices are mainly due to constituent active aromatic phenolic compound, such as thymol and carvacrol in oregano and thyme, eugenol in clove and cinnamon, and cinnamaldehyde in cinnamon (Karapinar and Aktug, 1987; Beuchat and Golden, 1989). High levels of phenolics (thymol and carvacrol) in thyme and winter savory oils were found to be most active and exhibited the

greatest inhibition against *Brevibacterium linens*, *Brochothrix thermosphacta* and *Lactobacillus plantarum* (Piccaglia et al., 1993). Baydar et al. (2004) concluded that the antibacterial properties of the essences of wild oregano (*Origanum minutiflorum*), oregano (*Origanum onites*), black thyme (*Thymbra spicata*) and wild savory (*Satureja cuneifolia*) were mostly attributed to the phenolic compound carvacrol and the hydrocarbons γ-terpinene and p-cymene.

Prabuseenivasan et al. (2006) found cinnamon, clove, geranium, lemon, lime, orange and rosemary oils exhibited strong antibacterial activity against selected bacterial strains. Among these they reported that the essential oil of cinnamon was most effective as an antibacterial agent. Cinamaldehyde was the predominant active compound found in cinnamon oil (Simic et al., 2004; Baratta et al., 1998). Hili et al. (1997) demonstrated that the *Cinnamomum zeylanicum* leaf oil completely inhibited the growth of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The main constituents of *C. zeylanicum* leaf oils are cinnamaldehyde and eugenol (Chang et al., 2001).

Polyphenols, such as tannins and flavonoids are also considered to be important antibacterial substances. Ellagitannin (punicalagin) in pomegranate (*Puninca granatum*) is the active compound responsible for its antimicrobial activity (Machado et al., 2002). Mathabe et al. (2006) reported that water soluble tannins like gallotannins and ellagitannins in *Puninca granatum* fruit rind are the principal components responsible for its antimicrobial action. Plant extracts from species of *Hypericum*, *Capsella* and *Chromolaena* are rich in flavonoids and have been reported to posess antibacterial activity (Cushine and Lamb, 2005). Tereschuk et al. (1997) reported that the growth of *E. coli* was inhibited by the flavonoid extract (total) from the plant, *Tagetes minuta,* at lower concentrations than by chloramphenicol. In addition, the absence of antimicrobial activity against the non-

pathogenic human bacteria *Zymomonas mobilis*, the yeast *Saccharomyces cerevisiae* and all *Lactobacillus* species tested was also noted, which could be beneficial for intestinal disease treatments where the intestinal flora must be preserved. Many plant flavonoids such as epigallocatechin and catechin in green tea, myricetin and quercetin in yellow onions, apples, tomatoes, green and black teas have been reported to exhibit antimicrobial activity (Cushine and Lamb, 2005; Lakhanpal and Rai, 2007).

Garlic was found to exhibit antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria including multi-drug resistant enterotoxigenic strains of *E. coli*. It was inhibitory toward *Candida albicans*, and showed antiparasitic and antiviral activity (Ankri amd Mirelman, 1999). The antibacterial action of garlic is mainly due to the oxygenated sulfur compound allicin (Cavallito and Bailey, 1944; Block, 1985).The sensitivity of *E. coli*, *S. aureus* and *S.* Typhi to an aqueous extract of garlic was reported by Arora and Kaur, (1999).

Essential oils of *Eucalyptus camadulensis* and *Eucalyptus terticornis* exhibited pronounced activity against the most resistant clinical bacteria *Pseudomonas aeruginosa*. The antibacterial activity of some essential oils, particularly that of *Eucalyptus* species are related to the presence of beneficial compounds such as alcohols, aldehydes, alkenes, esters and ethers (Cimanga et al., 2002). Allyl isothiocyanate (AIT) has potential for use as an antimicrobial agent in variety of foods. It is a component of mustard oil and of cabbage, cauliflower and horseradish (Shelef, 1983; Delaquis and Sholberg, 1997; Lin et al., 2000). A substantial reduction in the numbers of *E. coli* O157:H7 was found in fresh ground beef during refrigerated or frozen storage when the meat was treated with AIT (Nadarajah et al., 2005). The efficacy of antimicrobial agent action is greatly influenced by the target microorganism (Shan et al., 2007). It has been often reported that Gram-negative organisms

are less susceptible to natural antimicrobials than Gram-positive bacteria (Burt, 2004; Smith-Palmer et al., 1998; Blaszyk and Holley, 1998; Shelef, 1983; Shan et al., 2007).

2.2.3 Mechanism of antimicrobial action

It is very clear that essential oils and their components posess antimicrobial activity, but the mechanism behind this activity is not completely understood and may involve multiple modes of action (Shan et al., 2007). Essential oils degrade the bacterial cell wall by interacting with the components of the cytoplasmic membrane causing its degradation (Helander et al., 1998; Lambert et al., 2001). They damage membrane protein, and interfere with membrane bound enzymes as well as the synthesis of DNA and RNA and interrupt protein translocation and the function of the mitochondrion in eukaryotes (Raccach, 1984). These actions are facilitated because essential oils and their components are hydrophobic, creating changes in the fatty acid and phospholipid content of mitochondria and bacterial cell membranes, which impair enzymatic action responsible for energy production, and cause altered nutrient uptake and electron transport. Cell membrane structure is disturbed and it becomes more permeable which causes extensive leakage from cells and loss of critical molecules and ions which leads to cell death. A diagrammatic representation of sites in bacteria where these natural antimicrobials act is shown in Fig. 2.2.

Aromatic and phenolic compounds also exert their antimicrobial effects by altering the structure and function of the cytoplasmic membrane (Sikkema et al., 1994), which causes efflux of cytoplasmic constituents (Cowan, 1999). The loss of the differential permeability character of the cytoplasmic membrane is frequently identified as a cause of cell death (Holley and Patel, 2005). The flavonoids quercetin and naringenin cause depletion of the

proton motive force which inhibits bacterial motility (Sikkema et al., 1994; Ultee et al., 2002). Bacterial motility and chemotaxis are important for the virulence of bacteria as they facilitate bacterial access to sites of adherence and invasion. Propolis and some of its cinnamic and flavonoid components were also reported to inhibit bacterial motility. Propolis is a natural bee product and was found to uncouple the energy transducing reactions in the cytoplasmic membrane, cause dissipation of the membrane potential and to yield bactericidal activity (Orsi et al., 2006; Mirzoeva et al., 1997; Stepanovic et al., 2003).

Catechins, a group of flavonoids, also have greater activity against Gram-positive than Gram-negative bacteria. Epigallocatechin gallate (EGCg), a strong antibacterial catechin found in green tea induced leakage of small molecules from the intraliposomal space when liposomes were used to model bacterial membrane function. Aggregation of liposomes was also noted and it was concluded that catechins primarily act on and damage bacterial membranes. A reduction in leakage induced by epigallocatechin was noted when liposome membranes were prepared containing negatively charged lipids (Ikigai et al., 1993; Kajiya et al., 2004). These studies suggested that the presence of lipopolysaccharide in Gram-negative bacteria may act as a barrier and contribute to the lower susceptibility of these organisms to catechins.

AIT at concentrations that inhibit microbial growth alters protein structures (Cushine and Lamb, 2005). AIT mainly reacts with free amino acids and disulfide bonds of proteins and it affects the metabolic functions of yeast by inhibition of oxygen uptake. AIT inhibits specific carriers in the electron transport chain, notably cytochrome oxidase, and acts as an uncoupler of oxidative phosphorylation. Disruption of aerobic respiration could be the main cause for acute sensitivity of aerobic microorganisms to AIT (Delaquis and Sholberg, 1997; Lin et al., 2000). The ability of allicin, the active compound in garlic, to react with the thiol-containing

compound L-cysteine in microorganisms to form an S-thiolation product, S-allylmericaptocysteine, was assumed to be the main mechanism involved in its inhibitory action (Cavallito and Bailey, 1944; Rabinkov et al., 1998).

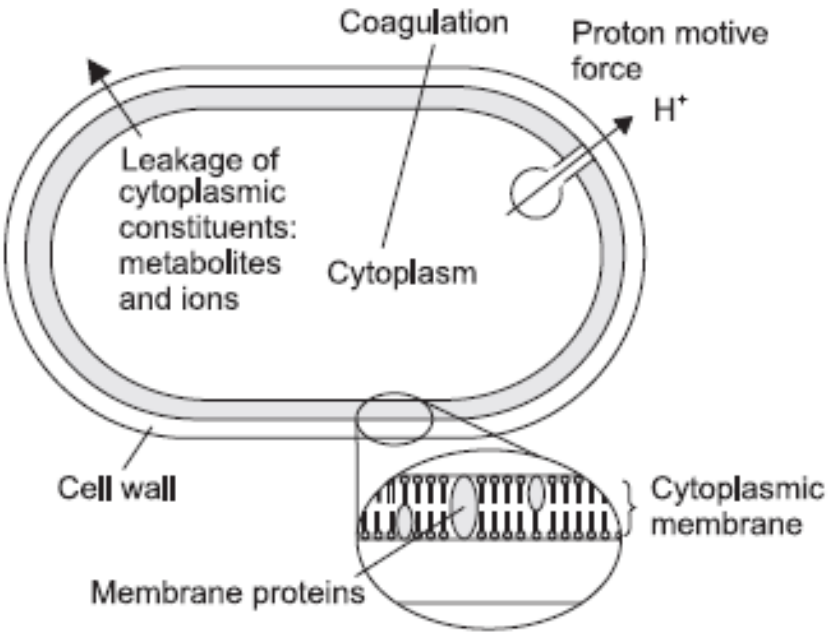


Fig. 2.2 Locations in the bacterial cell and mechanisms thought to be sites of action for essential oil components (Burt, 2004).

The difference in susceptibility of Gram-positive and Gram-negative bacteria to drugs may be due to the presence of an outer membrane and periplasmic space in Gram-negative bacteria. The outer membrane is known to act as a barrier to drug penetration and some enzymes present in the periplasmic space are capable of breaking down foreign molecules introduced from outside (Duffy and Power, 2001).

2.3 Natural antimicrobial and antibiotic combination

The development of antibiotic resistance can be natural or acquired and can be overcome by the use of a combination of drugs (Hemaiswarya et al., 2008). Since the introduction of penicillin, many compounds have been developed for use as antibiotics, but loss of their effectiveness due to resistance development by microorganisms is problematic (Jacoby and Archer, 1991). The effectiveness of these compounds can be maintained by using agents that interfere with antibiotic resistance development. Studies have been done with herb materials used in combination with antibiotics against some resistant strains and a significant reduction in the MIC's of the antibiotics was found (Kim et al., 1995; Park et al., 1997).

Kim et al. (1998) found that some essential oil components from extracts of *Anetheum graveolens* L. and *Acorus gramineus* Soland showed inhibitory activities against the multi-drug resistant bacterium *Staphylococcus aureus* SA2 when combined with ampicillin or chloramphenicol. The strain *S. aureus* SA2 has resistance to 10 common antibiotics. Kim et al. (1998) identified acetone as the active principle in the extract which reduced drug resistance when used at 5 μg/ml and combined with 50 μg/ml of chloramphenicol. Similarly, the greatest reduction of resistance to chloramphenicol was noted when chloramphenicol was combined with a hexane fraction of the edible plant *Aster scaber* (commonly called Chiwinmool in Korea) against multidrug resistant *Staphylococcus aureus* SA2 (Park et al., 1997).

Aburjai et al. (2001) studied the combined effect of 19 Jordanian plants with seven different antibiotics to determine whether combinations could reduce the drug resistance of *Pseudomonas aeruginosa.* A significant improvement in the activity of chloramphenicol, gentamicin and cephalosporin was found with almost all plant materials with few exceptions. Synergistic interaction of β-lactam antibiotics (BLA) with natural compounds to

overcome drug resistant microorganism have been reported in many studies (Hemaiswarya et al., 2008). Baicalin, an active compound from the Chinese herb Xi-nan Huangqin (*Scutellaria amoena*), was found to have the potential to restore the effectiveness of BLA against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains of β-Lactam resistant *S. aureus* (Liu et al., 2000). Liu et al. (2000) found that the minimum inhibitory concentration of benzylpenicillin was reduced from 125 μg/ml to 4 μg/ml against MRSA and from 250 μg/ml to 16 μg/ml against penicillin-resistant *S. aureus* when combined with baicalin (16 μg/ml).

Enterococcci, components of the normal bowel flora of humans, are the second leading cause of nosocomial infections in the USA. Extensive use of vancomycin was believed responsible for the rapid emergence of vancomycin-resistant enterococci (VRE). Vancomycin was also used as the first choice antibiotic for severe MRSA infections (Moellering, 1998; Schouten et al., 2000). Sato et al. (2004) noted significant inhibition in the growth of VRE and MRSA when phytochemicals isolated from the roots of *Erythrina zeyheri* (*Leguminosae*) were tested against these microbes, and the interactions were found to be synergistic or additive with vancomycin. Similarly a reversal of vancomycin resistance in clinical vancomycin-resistant strains of *Enterococcus faecalis* and *Enterococcus faecium* by flavonoids was reported by Liu et al. (2001). A combination of vancomycin with the flavonoid galangin significantly lowered the colony forming unit (CFU) values of the culture, and the inhibition was maintained for over a period of 24 h. Two to four fold potentiation of tetracycline activity against resistant strains of *Streptococcus sanguis* TH-13, *Streptococcus oralis* Sh-2, and *Fusobacterium nucleatum* were found when the antibiotic and aqueous crude khat (*Cantha edulis*) extracts of Yemen were used in paired combination (Al-hebshi et al., 2006).

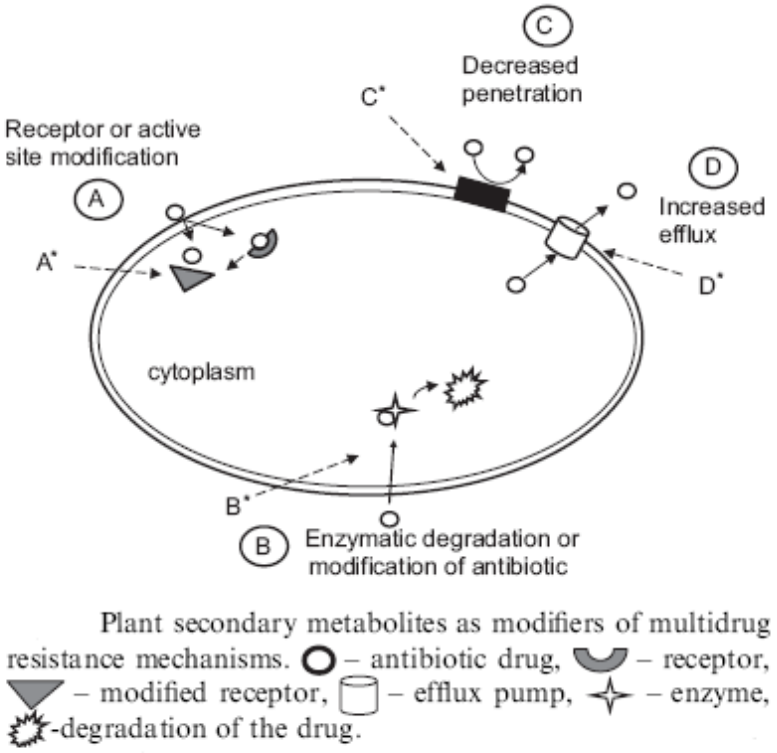
Crude extracts of the Indian medicinal plants, *Acorus calamus*, *Hemidesmus indicus*, *Holarrhena antidysenterica* and *Plumbago Zeylanica* showed synergistic interactions with tetracycline and ciprofloxacin against extended spectrum β-Lactamase (ESBL) producing multidrug-resistant enteric bacteria (Ahmad and Aqil, 2007). The synergism was greater with ciprofloxacin than with tetracycline. In another study, Tiwari et al. (2005) observed that growth of *Shigella dysenteriae* was inhibited more efficiently at low combined concentrations of chloramphenicol (2.5 μg/ml) and tea extract (5.09 mg/ml) than by the individual compounds (The individual MIC was 5 μg/ml for chloramphenicol and 9.09 mg/ml for black tea extract). A synergistic reaction between Japanese green tea extract and the antibiotic levofloxacin against enterohemorrhagic *Escherichia coli* O157 was reported by Isogai et al. (2001). Allicin, one of the active principles of freshly crushed garlic was noted to have a synergistic effect with antibiotics such as streptomycin or chloramphenicol against *Mycobacterium tuberculosis*. (Ankri and Mirelman, 1999).

2.3.1 Mechanism of action involved in synergy

The ability of plant extracts to potentiate susceptibility to antibiotics has not been well explained. The positive effects of plant extracts when combined with antibiotics seem to be due to resistance modifying or modulating activities of the extracts (Gibbons, 2004). More specifically, is assumed that they act by inhibiting one or more drug resistance mechanisms in bacteria such as drug efflux, enzymatic degradation, or cell wall permeability (Lewis and Ausubel, 2006; Zhoa et al., 2002). Subsequently, there has been work which has identified and isolated potential drug resistance modifiers of natural antimicrobial orgin which showed synergistic interactions when used in combination with antibiotics (Sibanda and Okoh, 2007).

The combined action of EGCg in tea catechins and oxacillin produced a synergistic interaction against *S. aureus* (Zhoa et al., 2001). This was attributed to the combined action of the compounds on the biosynthesis of the cell wall, thereby neutralizing the normal resistance mechanism which involved the reduced affinity of penicillin binding proteins (PBP) for oxacillin. Two possibilities were suggested to explain the reduction of β-Lactam MIC's in MRSA by corilagin, a polyphenol from *Asctostaphylos uva-ursi*. Corilagin may act by inhibition of PBP2a activity or inhibit its production (Shimizu et al., 2001). Shiota et al. (2004) later reported that the PBP2a of MRSA cells grown in the presence of corilagin lost their ability to bind to Bocillin FL, a fluorescently-labeled benzyl penicillin.

The plant alkaloid reserpine, isolated from the roots of *Rauwolfia vomitoria,* was tested in combination with tetracycline against *Bacillus subtilis*. Reserpine inhibited the Bmr efflux pump in *Bacillus subtilis* which mediated tetracycline efflux (Stavri et al., 2007; Neyfakh et al., 1991). Polyphenolic compounds have been shown to exert their antimicrobial activity through membrane perturbations. The enhanced activity of antibiotics in combination with polyphenols could be due to this perturbation of the cell membrane coupled with the action of β-Lactams to inhibit transpeptidation of the cell wall mucopeptide (Esimone et al., 2006). It is likely that in most cases of synergism, the mechanisms of interaction involve multiple targets and the action of various molecules present in the antimicrobial compounds. There is a possibility that the minor molecules often present in the natural antimicrobials may modulate the activity of the main components (Bakkali et al., 2008; Fig. 2.3).



Plant secondary metabolites as modifiers of multidrug resistance mechanisms. – antibiotic drug, – receptor, – modified receptor, – efflux pump, – enzyme, -degradation of the drug.

Fig 2.3 Sites of action of natural antimicrobials as resistance modifiers (Hemaiswarya et al., 2008)

2.4 Natural antimicrobials in animal diets

The use of natural plant extracts in animal diets could be one of the alternatives to the use of antibiotic growth promoters. Many studies have reported the bactericidal and bacteriostatic activities of plants and plant extracts. Essential oils and purified compounds derived from spices and herbs have been shown to have antimicrobial actions *in vitro* (Cowan, 1999; Cross et al., 2003; Lewis et al., 2003). In addition, it was reported that the essential oils have a stimulating effect on the animal digestive system. The effect was believed due to increased production of digestive enzymes and the improved utilization of digestive products through enhanced liver function (Alcicek et al., 2003; Williams and Losa, 2001; Hernandez et al., 2004).

2.4.1 In bird diets

Herbs and plant extracts stimulate the growth of beneficial bacteria and minimize pathogenic bacterial activity in the gastrointestinal tract (Wenk, 2002). Botsoglou et al. (2003) noticed improved oxidative stability of raw and cooked muscle tissues during refrigerated and long-term frozen storage when the essential oil of oregano was incorporated in turkey diets. In another study Govaries et al. (2005) found that inclusion of 100 mg/g of oregano essential oil in turkey diets exerted an inhibitory effect on microbial growth on the breast fillets during refrigerated storage. It was thought that dietary supplementation allowed more uniform incorporation of compounds into the cellular membranes than direct addition to the muscle food.

Cross et al. (2003) reported that supplementation of thyme essential oil in the broiler diet at 1 g/kg with enzymes acted synergistically and improved the performance of the birds. This may have been due to the beneficial effects of the combination on the microflora within the gastrointestinal (GIT) tract. Even though it was initially unpalatable to chickens in diets for several weeks due to the phenolic terpene content, later the chicks adapted to thyme oil which yielded a considerable increase in growth. Thyme oil has a protective effect; a one log reduction of coliforms occured in birds on diets supplemented with thyme oil (Cross et al., 2002).

Alcicek et al. (2004) observed a significant improvement in body weight gain, feed conversion ratio and carcass yield in broilers fed diets supplemented with a mixture of herbal essential oils. They suggested that the herbal essential oil mixture might be considered of value as a growth promoter in these animals. Subsequently, Alcicek et al. (2003) reported that inclusion of 48 mg of a commercial essential oil compound (EOC)/kg broiler diet for 42 d significantly improved the body weight, feed conversion ratio and

carcass yield. The EOC (Herbromix^{TM}) contained six different essential oils derived from oregano oil (*Origanum* sp.), laurel leaf oil (*Laurus nobilis* L.), sage leaf oil (*Salvia triloba* L.), myrtle leaf oil (*Myrtus communis*), fennel seed oil (*Foeniculum vulgare*) and citrus peel oil (*Citrus* sp.). No significant effect was noticed by Sarica et al. (2005) on the growth performance, total plasma cholesterol concentration, dry matter content of excreta, the relative weights of some internal organs, hot and cold carcass yields, or the concentration of total aerobic bacteria and *E. coli* in the small intestine of broilers fed a wheat-based diet supplemented with two herbal feed additives (thyme and garlic) with or without a xylanase-based enzyme complex. However, the garlic-supplemented diet significantly increased the length of the small intestine.

A study with chicken demonstrated that a commercial blend of essential oil (EO), CRINA® Poultry (includes thymol), stimulated activities and secretion of digestive enzymes including amylase and triggered pancreatic enzyme secretion (Jang et al., 2007; William and Losa, 2001). The increased weight gain of birds fed diets supplemented with EO might be associated with enhanced nutrient digestion in the intestine and was thought to have a positive impact on starch digestion in the small intestine (Xu et al., 2003). In addition to stimulating enzyme activity, essential oils had inhibitory effects on pathogens in the digestive system (Alcicek et al., 2003). Supplementation of 2% coriander seed in Japanese quail diets over a growing period of 6 weeks significantly improved body weight, feed conversion ratio and carcass yield (Guler et al., 2005). The positive impacts could have been due to the essential oil components in the coriander seeds. It was reported that coriander seed contains up to 1 % essential oils with 60-70% being linalool and other monoterpenoids. Linalool in the diet appears to be appetizing to poultry and has stimulating effects on the digestive process. The essential oils derived from coriander seeds negatively affected

pathogenic microorganisms in the digestive system (Delaquis et al., 2002; Guler et al., 2005). Sengul et al. (2008) reported that supplementation of thyme extract products in the diet of Japanese quail did not have a negative impact on the growth performance of the birds. During the study, decreased oxidative stress and DNA damage was also identified and reported. Improved digestibility in chickens receiving feed supplemented with a blend of essential oils of cinnamon, pepper and oregano was noted compared to chickens fed a control diet without the blend (Hernandez et al., 2004).

2.4.2 In swine diets

Plants and their extracts have been fed to swine to investigate their *in vivo* potential as alternatives to antibiotics. Dietary inclusion of *Quillaja saponaria* as a saponin source showed beneficial effects on growth performance and immune response in weaning pigs regardless of disease status (Turner, 2001). No positive effect was noticed on feed intake, weight gain and feed efficiency by dietary inclusion of garlic in weaning pigs (Horton et al., 1991). However, decreased mortality (1.2% to 3.9%) of piglets normally caused by intestinal diarrhea was reported when enteroguard (a combination of freeze-dried cinnamon and garlic) was included in the diets at 980 g/tonne (Peet-Schwering and Swinkels, 2000). Savoini et al. (2000) observed an increase in total leucocyte counts in weaning pigs fed an extract containing *Echinacea*, genzian-root, essential oils of juniper and thyme, tannins, and salicic acid. After *in vitro* evaluation of a large number of essential oils and their components in caecal digesta, Si et al. (2006b) identified candidate EO's and their components for *in vivo* studies to reduce swine gastro-intestinal pathogens. But most of the essential oils lost their activity after being mixed with diets in the animal trial. The study hypothesized that the essential oils and their components were bound to fats and other

hydrophobic materials in the pig diets and hence became unavailable for action against the target pathogens. Si et al. (2006a) reported that a higher concentration of cinnamaldehyde essential oil (at least three times its minimum bactericidal concentration, MBC) was required to retain its antimicrobial activity against *Salmonella* in pig diets (Si et al., 2006a).

2.4.2 In ruminant diets

Natural plant extracts can also be used as an alternative to ruminal modifiers to improve energy yield or protein use in the rumen since microbial fermentation in the rumen may cause considerable energy and protein losses in the form of methane and ammonia. The control of ammonia production will improve energy or protein use and reduce the fecal aroma (Cardozo et al., 2004). Sarsaponins, secondary metabolites in *Yucca schidigera* have been reported to decrease ammonia N concentration and alter the acetate and propionate proportions in ruminal fluid (Cardozo et al., 2005). Similarly, a secondary component of *Origanum vulgare*, thymol was found to decrease the acetate and propionate concentrations and increase the acetate/propionate ratio in *in vitro* mixed rumen fluid incubations (Evans and Martin, 2000). Cowan (1999) reported that high doses of plant extracts in the diet of ruminants had antibacterial effects and reduced volatile fatty acid concentration in the rumen.

The antimicrobial activity of plant extracts and their components against microbial pathogens has been proven by many studies. Most of the research done in this area has been performed *in vitro*. However, the number of published studies regarding the use of these compounds in animal diets is very limited.

2.5 Methods to evaluate the efficacy of natural antimicrobials

2.5.1 Methods to determine antimicrobial efficacy

Assay methods for antimicrobial activity should be simple, rapid, inexpensive, accurate, reproducible and easy to conduct. The antimicrobial effectiveness of a compound is most often described in terms of its minimum inhibitory concentration (MIC). Methods used for MIC determination can involve the disc diffusion assay or the dilution or plate methods (Bauer et al., 1966; Haltalin et al., 1973). Agar dilution and broth dilution are the most commonly used techniques to determine the MIC of antimicrobial agents (Wiegand et al., 2008). The disc diffusion assay assesses the size of the zone of inhibition produced on a paper disc impregnated with an antibiotic compound placed over a test culture inoculated on an agar surface in a petri dish. This assay often does not give sharp demarcation since the incubation time neccessary may be unsuitable for some unstable antimicrobial agents (Chand et al., 1994).

In the agar dilution technique, the agar is mixed with different concentrations of the test antimicrobial substance. Then solutions with a defined number of bacterial cells are spotted or streaked directly onto the agar plate. The MIC is measured by monitoring growth after incubation (Wiegand et al., 2008; Fernandes et al., 2007). A liquid growth medium is used in the broth dilution method. These methods are laborious and vulnerable to contamination (Chand et al., 1994).

The most widely used technique is broth microdilution since standard serial dilution requires large amounts of extract (Eloff, 1998; Langfield et al., 2004; Kreander et al., 2005; Rodriguez-Tudela et al., 2002; Scorzoni et al., 2007; Olasupo et al., 2003; Takahata et al., 1999). The broth microdilution method gave reproducible results and required only 10- 25 µl

of extract to determine MIC values (Eloff, 1998; Langfield et al., 2004). This method can be performed in sterile plastic, disposable 96 well microtitration plates. The inhibitors and target organism are added to the wells in broth and incubated. Inhibitor effectiveness after incubation is measured by using absorbance or by using redox sensitive indicator dyes (Chand et al., 1994; Eloff, 1998; Kreander, 2005). This method is easy to perform, rapid, economic and gives reproducible results. It is compatible with microtitration plate readers which allows for direct transfer, storage and manipulation of data by computer and produces results consistent with requirements of the American National Committee for Clinical and Laboratory Standards (NCCLS) (Rodriguez-Tudela et al., 2002; Eloff, 1998).

2.5.2 Methods to estimate combined action

The study of interactive inhibitory effects includes the use of paired and triple combinations of agents to show interactions upon the growth of target microorganisms (Odds, 2003). A number of methods have been developed to detect *in vitro* synergy between/among antibiotics. The most commonly used techniques are the checker board and time-kill curve methods (White et al., 1996). The time-kill method gives a dynamic picture of antimicrobial action and interactions over time, and curves can be interpreted graphically (Vigil et al., 2005; Pillai et al., 2005). But it has limitations since this technique uses a fixed concentration of each antimicrobial; also, repetitive sampling is necessary when the concentration altered. Moreover this method is time-consuming, labor intensive and it is difficult to interpret results because relatively few antibiotic concentrations are examined (White et al., 1996; Vigil et al., 2005; Pillai et al., 2005).

The checker board method combined with the fractional inhibitory concentration (FIC) index is commonly used to test the antimicrobial activity of drugs in medicine and has been one of the traditional methods used to measure antibiotic synergy (Pei et al., 2009; Rand et al., 1993; Si et al., 2008). This is one of the best known and most frequently used methods to assess antimicrobial combinations because: (1) its rationale is easy to understand; (2) the mathematics necessary to calculate and interpret the results are simple; (3) it can be readily performed in the laboratory using microdilution systems; (4) and it is relatively simple to perform (Vigil et al., 2005). Often researchers choose different methods to perform multiple agent assays and adopt various indices to evaluate the results, and this creates difficulty when comparing results. Sometimes two indices, the fractional inhibitory concentration (FIC) and the effect of combination (EC), are used to evaluate combined inhibitor effects (Shin and Kang, 2003; Zhou et al., 2007a, 2007b; Pei et al., 2009). All three possible effects of two or more agents (additive, synergistic or antagonistic) are evaluated using the FIC index. Synergistic effects on viability can be determined using the EC index. The EC index is based on the log decrease in population viability (log DP), which represents how strong the antimicrobial activity is in combination. In conjunction with ANOVA it is used to indicate the relationship between a single component and the combination, and therefore, it characterizes component contributions as well as validates that the synergism observed is meaningful (Pei et al., 2009).

The Log DP is expressed by the following formula:

$$\text{Log DP} = \text{Log}\,(N / N_0) = (\text{Log}\,N) - (\text{Log}\,N_0)$$

Where N is the population after an incubation period and N_0 is the initial population.

The effect of combination (EC) is calculated using the formula:

$$EC = \text{Log } DP_I - \text{Log } DP_{II}$$

The Log DP_I and Log DP_{II} represent the Log DP of the combinated agents and the single agent respectively (Pei et al., 2009; Zhou et al., 2007a).

The synergistic effect of the combinations is determined based on three principles:

(1) The decrease in population (>90%): it is considered that only when the DP < 0.1 (log DP < -1) does the combination of various agents have significant anti-bacterial activity.

(2) The combination is more effective when there is a significant difference, analyzed by one way analysis of variance (ANOVA) between the antibacterial activity of the combination and the individual agents, respectively.

(3) Synergy is defined as a 2 log decrease of population in the agent combination group when compared with the most effective single agent (Tan et al., 1993).

2.5.3 Methods to study inhibitory efficacy in chicken feed and caecal digesta

Numerous studies have been done to develop methods for isolation and identification of target bacteria in food and other contaminated samples (Humbert et al., 1989; Beumer et al., 1991). These involve enrichment, isolation and confirmation and are based on traditional microbiological techniques (Doyle and Schoeni et al., 1986, 1987; McCarthy et al., 1990). Enrichment techniques are time-consuming and require several days to weeks to obtain confirmation. Direct plating to enumerate a target pathogen is often difficult when the sample is heavily contaminated with other microbes (Kang and Fung, 1999). Addition of chemicals with selective properties to the growth media may facilitate detection of specific microorganisms in food. However, some of these agents inhibit repair of injured cells. Cole

et al. (1993) reported that in a non-selective medium such as tryptic soy agar (TSA), substantial repair of injured cells can occur. Injured cells may fail to resuscitate on media containing selective agents when samples are plated directly on the selective media.

Speck et al. (1975) and Hartman et al. (1975) developed a special plating procedure involving two steps to allow for recovery from injury and subsequent enumeration. First the cells were plated on non-selective medium such as TSA and incubated for 2 to 4 h, then a layer of selective agar was overlayed on the top of the resuscitated cells. Thus, the recovered cells are not affected by the selective agents, which will inhibit undesired bacteria in the sample. This procedure is termed the overlay resuscitation method. Although the procedure is useful for enumeration of injured cells, it is cumbersome and results are variable. The overlay agar sometimes resuspends sample organisms, obscuring descrete colony development.

Kang and Fung. (2000) developed a one-step thin agar layer (TAL) method to recover heat injured *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O157:H7. In the TAL method, after solidification of sterilized selective agar in a Petri dish, 7 ml of melted non-selective media (TSA) is overlaid, allowed to solidify and then another layer of 7 ml TSA is overlaid and allowed to solidify for few minutes. Cells in a test sample are inoculated directly on the upper layer of the TAL medium. The injured cells resuscitate and grow on the top layer. When selective agents from the bottom layer diffuse to the top thin layer of TSA, the resuscitated cells will form colonies typical of those grown on selective agar. At the same time the selective agents inhibit the growth of unwanted microorganisms (Wu et al., 2001).

CHAPTER 3

Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria

3.1 Abstract

Plant-derived antibacterial compounds may be of value as a novel means for controlling antibiotic resistant zoonotic pathogens which contaminate food animals and their products. Individual activity of natural antimicrobials (eugenol, thymol, carvacrol, cinnamaldehyde, allyl isothiocyanate (AIT) and activity when paired with an antibiotic was studied using broth microdilution and checkerboard methods. In the latter assays, fractional inhibitory concentration (FIC) values were calculated to characterize interactions between the inhibitors. Bacteria tested were chosen because of their resistance to at least one antibiotic which had a known genetic basis. Substantial susceptibility of these bacteria toward the natural antimicrobials and a considerable reduction in the minimum inhibitory concentrations (MIC's) of the antibiotics were noted when paired combinations of antimicrobial and antibiotic were used. In the interaction study, thymol and carvacrol were found to be highly effective in reducing the resistance of *Salmonella* Typhimurium SGI 1 (*tet A*) to ampicillin, tetracycline, penicillin, bacitracin, erythromycin and novobiocin (FIC<0.4) and resistance of *Streptococcus pyogenes ermB* to erythromycin (FIC<0.5). With *Escherichia coli* N00 666, thymol and cinnamaldehyde were found to have a similar effect (FIC<0.4) in reducing the MIC's of ampicillin, tetracycline, penicillin, erythromycin and novobiocin. Carvacrol, thymol (FIC<0.3) and cinnamaldehyde (FIC<0.4) were effective against *Staphylococcus aureus blaZ* and in reducing the MIC's of ampicillin, penicillin and bacitracin. Allyl isothiocyanate (AIT) was effective in reducing the MIC of erythromycin (FIC<0.3) when tested against *S. pyogenes.* Fewer combinations were found to be

synergistic when the decrease in viable population (log DP) was calculated. Together, fractional inhibitory concentrations ≤ 0.5 and log DP < -1 indicated synergistic action between four natural antimicrobials and as many as three antibiotics to which these bacteria were normally resistant.

3.2 Introduction

In recent years, there have been concerns about the greater frequencies of antibiotic resistance among bacteria isolated from food animals and from the environment (Jensen et al., 2001). To facilitate intensive animal production, antibiotics are used continuously as feed additives at sub-therapeutic levels to promote growth, increase feed efficiency and to prevent infection (Jindal et al., 2006; Wegener, 2003; Khachatourians, 1998). In the United States, 50% of all antibiotics produced are administered to animals, mostly for sub-therapeutic purposes (Anonymous, 2001). The Union of Concerned Scientists estimated that each year 11.2 million kg of antibiotics are given to animals in the US for non-therapeutic purposes and 900,000 kg are used for therapy (Mellon et al., 2001). The extensive use of antibiotics in human medicine, in animal practice for therapy and as growth promoters in agriculture are considered to be the major reasons for the development of bacterial resistance to antibiotics (Sengelov et al., 2003; Barton, 2000). Bacteria originating from food animals frequently carry resistance to a range of antimicrobial agents commonly used in humans and it is possible that these resistant organisms can be transferred to humans either directly via the food chain or indirectly as a result of spread of animal waste in fields (Ghosh and LaPara, 2007; Hammerum and Heuer, 2009). The presence of antimicrobially resistant bacteria in meat products may have important public health consequences, especially in

developing countries where there is widespread and uncontrolled use of antibiotics (Aslim and Yucel, 2008; Ahmad and Beg, 2001).

Humans can be colonized with antibiotic resistant bacteria of animal origin and these bacteria may cause infections for which limited therapeutic options are available, and which may lead to greater frequencies of treatment failure (Sengelov et al., 2003; Wegener, 2003; Aarestrup, 1999). Another concern is the horizontal spread of the resistance genes from these animal (zoonotic) bacteria to commensal strains in the intestinal microflora of humans (Anonymous, 2001). With the widespread development of resistance in zoonotic bacteria pathogenic to humans, new strategies are needed to control these organisms. There is a growing interest in using natural antibacterial compounds such as extracts of spices and herbs for food preservation (Shan et al., 2007) and these could be an alternative source of novel therapeutics. The relatively low frequency of infectious diseases in wild plants suggests that in many cases these natural defense mechanisms can be very effective (Hemaiswarya et al., 2008). In the present work, the effectiveness of plant-derived natural antimicrobials against antibiotic resistant bacteria was examined. It was also of interest to assess these agents for their synergistic interaction with antibiotics to which the target bacteria were resistant.

3.3 Materials and methods

3.3.1 Organisms

Bacterial strains used were selected from groups having known genetic determinants (genes) for resistance to some of the test antibiotics. Cultures were obtained from Dr. M. Mulvey, Public Health Agency of Canada, Winnipeg, MB. The tetracycline resistant Gram negative

bacteria used were *Salmonella* Typhimurium SGI1 (*tet A*) and *Escherichia coli* N00-666. The Gram positive bacteria used were *Staphylococcus aureus blaZ,* resistant to penicillin, and erythromycin resistant *Streptococcus pyogenes ermB*.

3.3.2 Antimicrobial agents

The antibiotics used were selected from those drugs that are permitted for use in animals in Canada as feed additives. The following antibiotics were included: tetracycline, ampicillin, penicillin G, erythromycin, bacitracin and novobiocin (Sigma-Aldrich Canada Ltd., Oakville, ON). Selected natural antimicrobials known to be active against the genera of bacterial pathogens of interest were used. The specific agents were eugenol (99% purity), carvacrol (99%), thymol (99.5%), cinnamaldehyde (>93%) and allyl isothiocyanate (AIT) (94%). These were analytical grade products (Sigma-Aldrich Canada Ltd., Oakville, ON).

3.3.3 MIC determination

The resistances of bacteria to the antibiotics and the MIC's of natural antimicrobials were determined by standard broth microdilution (Eloff, 1998; Wiegand et al., 2008; CLSI, 2002). Mueller-Hinton medium (MHB) (Oxoid, Fisher Scientific, Edmonton, AB, Canada) was used for *Salmonella* Typhimurium, *E. coli* and *S. aureus*. Brain Heart Infusion broth (BHIB) (Oxoid) was used for *S. pyogenes*. Bacterial suspensions for inocula were prepared by the dilution of pre-cultures, where the strains were cultured for 16 h at 37ºC and 100 µl of each suspension was re-cultured in 5 ml of fresh broth and incubated at 37 ºC for 1 to 2 h before dilution. The cultures were adjusted to the required bacterial density (1×10^8 cfu/ml) using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England) at 600nm.

The wells (370 µl capacity) of 96 well microplates (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, NJ, USA) were filled with 50 µl of medium. Antibiotic solutions and natural antimicrobials were prepared at different concentrations for each bacterium and serially diluted in the microwell plates. Each of 5 antibiotics was dissolved in distilled water and filter sterilized through 0.20 µm syringe filter units (Fisher Scientific, Edmonton, AB, Canada). Ethanol (25% v/v) was used as a carrier for tetracycline and the natural antimicrobials. The ethanol concentration in the starting well was < 2.5%. The effect of ethanol alone on the growth of bacteria was examined in preliminary tests and no effects were seen (the microdilution method was used to test concentrations of ethanol to 2.5% and the turbidity following incubation was compared with a control without ethanol). Test inocula were prepared from the fresh culture by serial dilution to yield 5×10^5 cfu/ml in the test wells (CLSI, 2002). Fifty microlitres of inocula were added to each well and incubated for 16 h at 37ºC. During natural antimicrobial tests the covered plates were sealed with parafilm to prevent evaporation (Berard et al., 2009) and placed in a shaker at 150 rpm (Titer plate shaker, model no. 4625, Barnstead/Lab-Line, Barnstead International, Dubuque, IA, USA). For AIT tests, screw-capped tubes were used. The MIC was defined as the lowest antibiotic or antimicrobial concentration which prevented visible growth (Takahata et al., 1999). After examining wells for turbidity, 40 µl of *p*-iodonitrotetrazolium violet (INT) (Sigma) dissolved in water (0.2 mg/ml) was added to each well and plates were incubated for one to 2 h at 37 ºC to detect viability (Langfield et al., 2004; Eloff, 1998). The MIC was determined as the lowest agent concentration at which no red color (loss of metabolic activity) appeared.

3.3.4 Synergy testing

The checkerboard method, which is commonly used for measurement of interactive inhibition (Rand et al., 1993; White et al., 1996; Vigil et al., 2005; Pillai et al., 2005), was used for the determination of synergy between the antibiotics and natural antimicrobials. The checkerboard method is often combined with calculation of a fractional inhibitory concentration (FIC) index to test the antimicrobial potencies of drugs in medical laboratories (Pei et al., 2009). Synergistic interactions involving the natural antimicrobials (e.g. drug A) plus the antibiotics (drug B) to which the bacteria were normally resistant were tested. The concentrations of the agents used were started from their MIC value and were serially diluted in two-fold steps. The effects of combinations were evaluated by calculating the FIC index for each combination using the following formula:

FIC of drug A= MIC of drug A in combination/MIC of drug A alone; FIC of drug B = MIC of drug B in combination/MIC of drug B alone; FIC index = FIC of drug A+ FIC of drug B (Vigil et al, 2005; Pillai et al., 2005). Synergy was defined as an FIC index ≤ 0.5. When the FIC index fell between 0.5 and 4.0 it indicated there was 'no interaction' between the agents. An FIC>4.0 would indicate there was antagonism between the two agents (Odds, 2003; Hemaiswarya et al., 2008; Zhao et al., 2002).

The logarithm of difference in viable population (Log DP) was calculated after plating broth from separate tests done in screw-capped tubes using agent combinations which were found to be synergistic from the observed FIC value. The tubes were incubated for 16 h at 37 ºC before plating. The populations at time zero and 16 h were recovered on TSA plates using an autoplate 4000 spiral plater (Spiral-Biotech, Exotech, Inc., Gaithersburg, MD, USA). The log DP of the combination was calculated using the equation;

Log DP = log (N/ N_0) = (log N – log N_0) according to Zhou et al. (2007a) and Pei et al. (2009), where N and N_0 represented the bacterial populations (cfu/ml) at 16h and zero time, respectively.

If a natural antimicrobial – antibiotic combination appeared synergistic (FIC $\leq$ 0.5), this observation was confirmed only after it was demonstrated that the combination also caused a significant decrease (>90%) in pathogen viability- that is, it yielded a DP < 0.1 (log DP < -1).

3.4 Results

3.4.1 Antibiotic resistance

The resistance patterns of the bacterial strains to the tested antibiotics are shown in Table 3.1. *Salmonella* Typhimurium and *E. coli* were resistant to all 6 antibiotics with a higher level of resistance being shown to ampicillin, penicillin, erythromycin and bacitracin. *S. aureus* was resistant to ampicillin, penicillin and bacitracin. *S. pyogenes* was sensitive to 5 antibiotics, but a high level of resistance was found toward erythromycin.

3.4.2 MIC's of natural antimicrobials

MIC results from tests of the 5 natural antimicrobials against the 4 bacterial strains are presented in Table 3.2. In general, the natural antimicrobials were effective against these drug resistant microbes. It was found that eugenol, thymol, cinnamaldehyde and carvacrol at 2.5 mM were inhibitory to *Salmonella* Typhimurium, *E. coli* and *S. aureus*. At 1.25 mM, carvacrol was effective against *S. aureus.* Concentrations of eugenol, thymol, AIT and

cinnamaldehyde at 0.63 mM and carvacrol at 0.31 mM were inhibitory to *S. pyogenes*. Moreover, the concentration of AIT required for inhibition was comparitively less than that required for the other antimicrobials tested. At 0.31 mM, AIT was found to be inhibitory toward *S.* Typhimurium plus *E. coli* and at 0.15 mM it was inhibitory toward *S. aureus*.

3.4.3 Effect of natural antimicrobial and antibiotic combinations

The potential reduction of antibiotic resistance by the natural antimicrobials was examined by calculation of FIC indices and log DP following bacterial challenge by pairs of agents and these are listed in Table 3.3. For *Salmonella* Typhimurium, a synergistic interaction was found for carvacrol and thymol with all antibiotics when assessed by FIC values. Thymol at 0.31 mM was found to be synergistic with all antibiotics except erythromycin. However, a synergistic effect was seen at 0.63 mM thymol. Similarly for carvacrol (except with ampicillin and erythromycin) 0.31 mM was synergistic with the antibiotics. With ampicillin and erythromycin at 0.63 mM carvacrol became synergistic. Eugenol at 0.63 mM was synergistic with tetracycline and novobiocin, but with the other antibiotics, synergism was seen at half of the MIC's (1.25 mM). Cinnamaldehyde interacted synergistically with the antibiotics (except for penicillin) at 0.31 to 1.25 mM. With AIT, no interaction was found with tetracycline, penicillin or novobiocin. Synergistic interactions were observed with ampicillin, erythromycin and bacitracin at 0.07 to 0.15 mM. However when log DP was calculated, none of their individual combinations with thymol, eugenol or cinnamaldehyde was synergistic. Three combinations with carvacrol and two combinations with AIT were found to be synergistic using these criteria.

Similarly for *E. coli*, eugenol was synergistic with tetracycline at 0.31 mM; however, 1.25 mM was needed to detect synergism with the other antibiotics. The results were the same with *Salmonella* Typhimurium when thymol and cinnamaldehyde were used. However, no interaction was noticed with bacitracin and carvacrol plus erythromycin or novobiocin. With AIT, synergism was observed only with bacitracin at 0.07 mM. By log DP assessment two combinations with thymol and carvacrol, one combination each with cinnamaldehyde or AIT showed synergistic interaction.

S. aureus was resistant to ampicillin, penicillin and bacitracin. No interaction was found between bacitracin and eugenol, but a synergistic interaction was found between penicillin and 0.31 mM of eugenol, and with ampicillin and 1.25 mM eugenol. A synergistic interaction was found for thymol (0.31 mM) and cinnamaldehyde (0.63 mM) with ampicillin and bacitracin; 0.15 mM and 0.31 mM with penicillin, respectively; and carvacrol (0.16 mM) with ampicillin or penicillin, and at 0.31 mM carvacrol with bacitracin. No interaction was found for AIT with ampicillin or penicillin, but AIT at 0.02 mM was synergistic with bacitracin. When log DP was calculated, all the combinations showed synergistic interaction except bacitracin/cinnamaldehyde.

S. pyogenes was resistant to erythromycin, but a synergistic interaction was seen with thymol, carvacrol or AIT at 0.31 mM, 0.08 mM and 0.07 mM, respectively. However, no interaction was found between erythromycin and eugenol or cinnamaldehyde. Thymol and carvacrol were synergistic with erythromycin according to log DP values.

In all these synergistic interactions, the inhibitory antibiotic concentrations were reduced by 1/4 to 1/8 of the MIC.

3.5 Discussion

Antibiotic resistant bacteria were challenged with natural antimicrobials to determine whether the latter could interfere with phenotypic expression of genetically determined antibiotic resistance. The drug resistant bacteria were notably susceptible to these plant-derived natural antimicrobials. Pei at al. (2009), studied eugenol, cinnamaldehyde, thymol and carvacrol, and found that *E. coli* was inhibited at 1600 (9.7), 400 (2.6), 400 (3) and 400 (2.6) mg/L (mM), respectively. In the present study, the antibiotic resistant *E. coli* strain had MIC values in response to carvacrol, cinnamaldehyde and thymol challenge which were similar to those presented by Pei at al. (2009) except for eugenol. The present results are also consistent with those obtained by Helander et al. (1998). In that study it was shown that carvacrol, thymol and cinnamaldehyde inhibited *E. coli* and *S.* Typhimurium at 1 to 3 mM. Oussalah et al. (2007) evaluated 28 essential oils against 4 pathogens and found that most oils inhibited *Salmonella* Typhimurium, *E. coli*, *S. aureus* and *Listera monocytogenes*.

Olasupo et al. (2003) used carvacrol, eugenol and thymol against *Salmonella* Typhimurium and *E. coli* and observed MIC values of 1 and 1.5 mM carvacrol, 3 and 2.5 mM of eugenol and 1 and 1.2 mM of thymol, respectively. In the present work the MIC values of eugenol found were consistent with data from Olasupo et al. (2003). However, the MIC's for carvacrol and thymol were slightly higher for *S.* Typhimurium and *E. coli.* These differences may have been due to use of strains here which had high levels of resistance to the antibiotics tested. It has often been reported that Gram negative bacteria are more resistant to essential oils than Gram positive bacteria (Smith-Palmer et al., 1998; Blaszyk and Holley, 1998; Shelef, 1983). Results presented here are consistent with that observation. Among the bacteria tested, *S. pyogenes* was found to be most sensitive to the essential oils used.

Similarly, the concentrations of carvacrol and AIT needed to inhibit *S. aureus* were lower when compared to *S.* Typhimurium and *E. coli*.

Some plant-derived active compounds are known to reduce antibiotic resistance (Kim et al., 1995, 1998, 2000; Aburjai et al., 2001; Park et al., 1997). When the antibiotics and natural antimicrobials were tested in paired combinations for possible synergistic effects against the 4 antibiotic resistant bacteria, a considerable reduction in the MIC's of the antibiotics was noted. Synergy is generally recognized when the combined effect of two compounds is greater than the sum of the effects of each compound alone (Rand et al., 1993; Kobilinsky et al., 2007).

In the present study it was found using both the FIC index and log DP values that thymol, carvacrol, cinnamaldehyde or AIT individually acted synergistically with at least three antibiotics to which the bacteria were resistant. Eugenol was less effective in reducing resistance to the antibiotics, though it had the same effect as the other natural antimicrobials when used alone. AIT was highly effective when used alone; the concentration needed to inhibit growth was much less than with the other tested compounds, but in combination carvacrol was found to be more effective. The exact mechanism for the reduction in antibiotic resistance by the natural antimicrobials is unknown but is likely due to some structural change in the resistant bacteria. For example, the natural antimicrobials may have facilitated penetration of the drug through the outer layers of the bacterial cell wall or acted by blocking the inhibitory effects of protective enzymes, or interfered with single or multiple metabolic targets of the antibiotic (Darwish et al., 2002; Aburjai et al., 2001; Hemaiswarya et al., 2008; Blaszyk and Holley, 1998). The mechanism of antibacterial action might be different or the combined action might vary with different bacteria (Pei et al., 2009). Zhao et al. (2002) found that epigallocatechin gallate (EGCg) from green tea inhibited the activity

of penicillinase produced by *S. aureus* and it restored the activity of penicillin. Similar results were reported by Hu et al. (2002) where they showed that EGCg synergistically enhanced the activity of carbapenems against methicillin-resistant *S. aureus* (MRSA). Several studies have reported the synergistic inhibitory interaction of β-lactam antibiotics (BLA) with natural compounds toward resistant microbes (Shiota et al., 2000; Liu et al., 2000; Shimizu et al., 2001). Shiota et al. (2000) reported that tellimagrandin I from red rose (*Rosa canina .L*) petal extract greatly reduced the MIC of BLA's against MRSA. Similarly, corilagin, an active compound extracted from *Aretostaphylos Uva-ursi* was found to reduce the MIC's of two BLA (oxacillin and cefmetazole) against MRSA (Shimizu et al., 2001).

Studies on the use of these compounds in animal diets are very limited. Si et al. (2006a) in a study of the antimicrobial activity of carvacrol, thymol and cinnamaldehyde against *Salmonella* in pig diets reported that higher concentrations were needed to retain their antimicrobial activity when added to the diets. Alcicek et al. (2003) concluded that inclusion of 48 mg/kg of an essential oil combination (EOC) in a broiler diet significantly improved bodyweight, feed conversion ratio and carcass yield after a growth period of 42days. Similarly, a beneficial effect on broiler performance following use of an herbal feed additive was reported by Langeroudi et al. (2008). No negative effects on the growth performance of Japanese quail was noted when thyme extract products were added to their diets (Sengul et al., 2008).

It is difficult and expensive to screen for or develop new drugs, and so it becomes important to find new methods to reduce the development of resistance to antibiotics by pathogenic organisms. The present work showed that substituted phenolic (eugenol, thymol, carvacrol) and non-phenolic (cinnamaldehyde, AIT) compounds from plants have the potential for use

to control the development of antibiotic resistant bacteria in non-human hosts or even in contaminated food.

The natural antimicrobials tested in this study were either synergistic or showed no interaction (did not interfere) with antibiotic activity. Gram positive bacteria were more sensitive to the natural antimicrobials than the Gram negative organisms when tested individually and in paired combination with antibiotics. *S. pyogenes* was highly resistant to erythromycin but was more sensitive to the natural antimicrobials than the other microbes tested. The results showed that natural antimicrobials were able to substantially decrease the MIC of antibiotics in a diverse group of bacteria containing genetic elements responsible for drug resistance. Further studies are needed to evaluate the efficacy of these compounds in animal intestinal models and in animals.

Table 3.1- Resistance pattern of bacteria to different antibiotics

Bacteria	Minimum inhibitory concentration (μg/ml)					
	Amp	Pen	Tet	Ery	Bac	Nov
S, Typhimurium SGI 1	>512	>512	64	1024	>512	256
E. coli N00 666	>512	>512	128	512	>512	64
S. aureus blaZ	32	128	S	S	32	S
S. pyogenes ermB	S	S	S	>512	S	S

Amp-ampicillin, Pen-penicillin, Tet-tetracycline, Ery-erythromycin, Bac-bacitracin, Nov-novobiocin, S-sensitive

Table 3.2- MIC values of natural antimicrobials against antibiotic resistant bacteria

Antimicrobials	**Bacteria(MIC mM)**			
	S. Typhimurium	*E. coli*	*S. aureus*	*S. pyogenes*
Eugenol	2.5	2.5	2.5	0.63
Thymol	2.5	2.5	2.5	0.63
Cinnamaldehyde	2.5	2.5	2.5	0.63
Carvacrol	2.5	2.5	1.25	0.31
AIT	0.31	0.31	0.15	0.63

Table 3.3- Response of antibiotic resistant bacteria to the combined effects of an antibiotic and a natural antimicrobial expressed as the FIC index [a] and log DP [b]

***Salmonella* Typhimurium SGI 1**

	Natural antimicrobial FIC (log DP)				
Antibiotics	Eugenol	Thymol	Carvacrol	Cinnamaldehyde	AIT
Ampicillin	>0.5	0.12	0.25	0.25	0.35 (-3.3)
Penicillin	>0.5	0.13	0.37 (-3.3)	0.63	0.63
Tetracycline	0.22	0.1	0.18 (-3.5)	0.37	0.73
Erythromycin	0.63	0.25	0.25	0.24	0.48 (-2.0)
Bacitracin	>0.5	0.15	0.25	0.24	0.5
Novobiocin	0.40	0.37	0.37 (-1.9)	0.24	1.0

***Escherichia coli* N00 666**

	Eugenol	Thymol	Carvacrol	Cinnamaldehyde	AIT
Ampicillin	>0.5	0.12	0.25	0.37	0.63
Penicillin	>0.5	0.20	0.37 (-3.3)	0.24	1.0
Tetracycline	0.16	0.15	0.15 (-3.8)	0.37 (-1.7)	0.75
Erythromycin	1.1	0.25 (-1.1)	1.0	0.24	0.73
Bacitracin	>0.5	0.56	0.25	0.63	0.5 (-1.5)
Novobiocin	1.1	0.37 (-1.5)	0.63	0.24	1.0

Continued Table 3.3

Staphylococcus aureus blaZ

	Eugenol	Thymol	Carvacrol	Cinnamaldehyde	AIT
Ampicillin	1.0	0.12 (-2.1)	0.15 (-1.6)	0.25 (-1.7)	0.73
Penicillin	0.33 (-1.6)	0.18 (-1.5)	0.11 (-1.7)	0.37 (-2.2)	1.0
Bacitracin	>0.5	0.25 (-3.8)	0.25 (-1.1)	0.24	0.17 (-1.9)

Streptococcus pyogenes ermB

	Eugenol	Thymol	Carvacrol	Cinnamaldehyde	AIT
Erythromycin	1.0	0.40 (-1.6)	0.25 (-1.1)	1.0	<0.36

a. Synergy = an FIC index ≤ 0.5. An FIC index between 0.5 and 4.0 indicated 'no interaction'. An FIC>4.0 indicated antagonism between the two agents.

b. Log DP < -1 combinations had significant antibacterial activity. Log DP results are shown only for those tests where its value was < -1. The analysis was done for all combinations having an FIC index ≤ 0.5.

CHAPTER 4

In vitro evaluation of Termin 8 and thymol use in chicken caecal content and poultry feed against antibiotic resistant bacteria

4.1 Abstract

Bacterial contamination has been a persistent problem in the food industry, because bacteria can contaminate animal carcasses at slaughter or cross contaminate other food items which leads to human illness. The link between the *Salmonella* contamination of poultry carcasses and human salmonellosis has long been recognized. The beginning of the food safety chain starts with animal feed. Contamination of animal feed contributes to infection and colonization of food producing animals with the pathogens. Individual activity of a formaldehyde-based feed additive Termin 8 and its activity when used in paired combination with an antibiotic against four antibiotic resistant bacteria were studied using broth microdilution and checker board assays. The interaction between the paired compounds was assessed using fractional inhibitory concentration (FIC) and logarithmic difference in population count (Log DP). A notable susceptibility of antibiotic resistant bacteria towards Termin 8 and a considerable reduction in MICs of antibiotics were noted in paired combination. Even though when Termin 8 was used against *Salmonella* Typhimurium and *Escherichia coli* synergistic reactions were found with 5/6 and 3/6 antibiotics by the FIC index, respectively, only one combination with erythromycin was synergistic according to the Log DP value. When tested against *Staphylococcus aureus*, Termin 8 was synergistic with penicillin by both FIC and Log DP values. A study using Termin 8 and the natural antimicrobial thymol with chicken caecal content and chicken feed was conducted. Both antibacterial compounds required a higher concentration than the respective MIC to have antimicrobial activity in digesta/ feed. At concentrations of 0.7 μl/g Termin 8 in feed and

caecal content and 200 mM and 35 mM thymol in feed and caecal content, respectively, *S.* Typhimurium, *E.coli* and *S. aureus* were rapidly eliminated.

4.2 Introduction

Between 1998- 2002, a total of 6647 outbreaks of foodborne disease were reported and 128,370 persons became ill in the United States. The largest percentage of outbreaks was caused by bacterial pathogens (55%). Among these *Salmonella* Enteritidis accounted for largest number of outbreaks and *Listeria monocytogenes* accounted for the majority of deaths (Lynch et al., 2006). Food producing animals are considered to be the major reservoir of these organisms and they acquire these pathogens by ingestion (Mead et al., 1999). Animal feed may serve as a carrier for wide variety of microorganisms (Maciorowski et al., 2007) and microbial pathogens in feed are a major problem in the food animal industry. The emergence of prion disease in humans in the United Kingdom created concern about the contribution of contaminated animal feed to human foodborne illness (Crump et al., 2002). Feed is considered a key route for introduction of infections into poultry flocks, particularly in young birds because of a poorly developed competitive intestinal flora and an immature immune system which are exacerbated by the effects of stress (Arnold and Holt, 1995; Corrier et al., 1992). Pathogenic microorganisms may be consumed by pullets, the birds may become carriers and subsequently shed the organisms which can contaminate eggs during the production cycle (Anderson et al., 2001).

There are numerous ways contaminating microorganisms can enter into feed. Feeds may acquire diverse microflora from multiple environmental sources including dust, soil, water, insects and may be contaminated at any time during growing, harvesting, processing, storage

and distribution (Maciorowski et al., 2007). Feed mills combine ingredients from animals such as meat trimmings and other slaughter by-products plus plant products to produce feed mixes (Crump et al., 2002). Once contaminated, feed acts as a carrier for animal and human pathogens. When birds or animals consume the contaminated feed, the contaminant may colonize the gut and contaminate the carcass during slaughter or may pass to chicks by vertical transmission through the contaminated egg. The vertical transmission of *S.* Enteritidis and *S.* Typhimurium from parent flocks to day-old chicks when they leave the hatchery has often been reported (Heyndrickx et al., 2002). Humphrey (1994) reported that *S.* Enteritidis can be isolated from the contents of clean intact eggs as a result of infection of the reproductive tract of the bird. The poultry industry is under continuous pressure to produce pathogen-free meat, but the issues are complex and involve the rapidity with which the birds are killed and their close contact on the slaughter line (Humphrey and Lanning, 1988). Use of pathogen-free feed would be useful if other environmental sources of the major pathogens transmitted by food could be controlled.

To reduce this problem, antibiotics are used continuously in feed as growth promoters at low concentration. Continuous use of growth promoters has been associated with the development of antibiotic resistant bacterial strains and may enhance their persistence in the environment (Anonymous, 2001). So the animal industry is searching to find alternatives to address this problem. Herbs and natural feed additives are being investigated as natural sources of antimicrobials and it has been demonstrated that they have positive effects on broiler growth performance (Demir et al., 2003). One study demonstrated that young chickens can be protected from bacterial infection by formic acid treatment of contaminated feed (Humphrey and Lanning, 1988). In the Swedish *Salmonella* control programme, contaminated raw materials of plant origin are treated with 1-2% formic acid for 48 h before

pelleting (Haggblom, 2006). Chemical products such as formaldehyde-based feed additives can act as antibacterial substances which help to both decrease and prevent feed recontamination by bacteria (DeRouchey et al., 2004). Summers et al. (1980) found that treatment of culinary waste with formaldehyde kept the material stable for several weeks. Similarly, treatment of poultry waste with formaldehyde was reported to destroy microorganisms (Koenig et al., 1978). The FDA has approved formaldehyde for use up to a level of 3 mg/kg in complete pig diets (DeRouchey et al., 2004).

The objectives of this study were to first examine the effect of the formaldehyde-based commercial feed additive Termin 8 against four antibiotic resistant bacteria and determine whether there was any interaction between the antibiotics and Termin 8. A second objective was to do an *in vitro* study on chicken gut digesta and formulated chicken feed with Termin 8 and the natural antimicrobial thymol, which had previously been shown to be inhibitory to the test bacteria (Palaniappan and Holley, 2010) to determine the effectiveness of these agents for pathogen control in feed and chicken digesta.

4.3 Materials and methods

4.3.1 Organisms

Bacterial strains used were selected from groups having known genetic determinants (genes) for resistance to some of the test antibiotics used in animal agriculture. Cultures were obtained from Dr. M. Mulvey, Public Health Agency of Canada, Winnipeg, MB. The tetracycline resistant Gram-negative bacteria used were *Salmonella* Typhimurium SGI1 (*tet A*) and *Escherichia coli* N00-666. The Gram-positive bacteria used were *Staphylococcus*

aureus blaZ, resistant to penicillin, and erythromycin resistant *Streptococcus pyogenes ermB*.

4.3.2 Antimicrobial agents

The antibiotics used were selected from those drugs that are permitted for use in animals in Canada as feed additives. The following antibiotics were included: tetracycline, ampicillin, penicillin G, erythromycin, bacitracin, novobiocin and virginiamycin (Sigma-Aldrich Canada Ltd., Oakville, ON). Thymol was purchased from Sigma-Aldrich. Termin 8 was obtained from Anitox Corporation, Lawrenceville, GA, USA. For *in vitro* tests Stafac-22 (a commercial feed additive containing 22g virginiamycin/kg) was obtained from Pfizer, Canada (Phibro Animal Health, Regina, SK, Canada) and it was added to the feed at a concentration of 500 g /1000kg of feed. The coccidiostat coban was obtained from Elanco Animal Health (Division of Eli Lilly and Company, Greenfield, IN, USA). Broiler feed samples and caecal contents were provided by Dr. Bogdan Slominski, Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada. The birds had been fed a standard commercial broiler starter diet which contained the antibiotic chlortetracycline hydrochloride (220 mg/kg) (FeedRite: a division of Ridley Inc., Winnipeg, Manitoba, Canada). Seven birds were sacrificed at 4 weeks of age for caecal sample collection. The samples were stored at -80˚C immediately after collection and thawed to room temperature before use.

4.3.3 Individual and combined effect of Termin 8 with antibiotics

The MIC of Termin 8 and its interaction with antibiotics against the 4 bacteria was studied. For MIC and interaction studies, the method described earlier (broth microdilution and checker board assay, Chapter 3) was used. The broth microdilution method was used to determine the antibiotic sensitivity of bacteria toward virginiamycin and Stafac-22.

4.3.4 Chicken caecal content and feed analysis

The thin agar layer (TAL) method described by Wu et al. (2001) was used to study the effect of Termin 8 and thymol on chicken gut digesta and chicken feed. Two types of poultry feed were used in the *in vitro* trials and these were formulated at the University of Manitoba. The first contained virginiamycin (11 mg/kg diet) and the coccidiostat coban (99 mg/kg diet), while the second type was identical but did not contain an antibiotic or a coccidiostat.

Tryptic Soy Agar (TSA; Oxoid, Fisher Scientific, Edmonton, AB, Canada) was used as a non-selective medium. The selective medium Xylose Lysine Deoxycholate Agar (XLD; Difco, Fisher Scientific, Edmonton, AB, Canada) + 0.1% Brilliant Green (BG; Sigma-Aldrich Canada Ltd., Oakville, ON) was used for *S.* Typhimurium (black colonies), MacConkey Agar (Difco) was used for *E. coli* (pink colonies), Mannitol Salt Agar (MSA; Oxoid) was used for *S. aureus* (yellow colonies) and Brain Heart Infusion Agar (BHI; Oxoid) + 300 μg/ml erythromycin was used for *S. pyogenes* (this organism was resistant to > 512 μg/ml erythromycin).

Cultures were prepared by growing *S.* Typhimurium, *E. coli*, and *S. aureus* in Tryptic Soy Broth (TSB; Difco) and *S. pyogenes* in Brain Heart Infusion Broth (BHI; Oxoid) at 37˚C for 16 h. Then 100 μl of those grown in TSB were recultured in 5 ml of fresh TSB and

incubated for 1 to 2 h at 37˚C to reach a bacterial density of 1×10^8 cfu/ml. The *S. pyogenes* culture was used directly after 16 h growth. Cultures were adjusted to 1×10^7 cfu/ml by adding 1ml culture to 9 ml saline. From this, 0.5 ml was added to one g of chicken caecal content or feed in 3.5 ml saline. The Termin 8 and thymol were added over a range of concentrations (from the MIC and serially increased to achieve maximum bacterial reductions. The final concentrations ranged from 5 mM to 250 mM of thymol and from 0.084 to 0.672 μl/g of Termin 8). The final bacterial concentration in the digesta or feed was 1×10^6 cfu/gm. For controls, the cultures were inoculated into digesta and separately into both types of feed without additional treatment. All samples were incubated for 3 h at 37˚C. After incubation, the inoculated digesta or feed samples were serially diluted and plated on the agars noted previously to get countable numbers of bacteria.

4.3.5 One step TAL method

To enumerate both injured and non-injured pathogens, TSA was used as a non-selective medium, and XLD + BG, MacConkey Agar and MSA were used as selective media for the different pathogens. After solidification of the sterilized selective agar in a Petri dish, 7 ml of melted TSA (48˚C) was overlaid. After this first layer solidified for one min, another 7 ml of TSA was overlaid and allowed to solidify. Then the diluted cultures from digesta or feed were plated using an Autoplate 4000 spiral plater (Spiral-Biotech, Exotech Inc., Gaithersburg, MD, USA). Samples inoculated with cultures without treatment were also plated as controls. The plates were incubated at 37˚C for 16 h and colonies were counted.

4.4 Results

The MIC of Termin 8 and the natural antimicrobial thymol are shown in Table 4.1 and the results of the interaction tests are presented in Table 4.2.

Termin 8 was found to be effective against the tested antibiotic resistant bacteria. The MIC ranged from 0.042 to 0.084 µl/ml for all 4 organisms. In the interaction study, 0.021 µl/ml of Termin 8 was found to be synergistic with 5 antibiotics (ampicillin, tetracycline, penicillin, erythromycin, bacitracin) against *S.* Typhimurium, with three antibiotics (erythromycin, bacitracin, novobiocin) against *E. coli* of 6 tested and with one antibiotic (penicillin) against *S. aureus* of three tested. Termin 8 showed a synergistic reaction with erythromycin against *S. pyogenes* at a concentration of 0.042 µl/ml. Not all the previously noted combinations were synergistic when the decrease in viable population (Log DP) was considered. Two combinations with several single organisms; that is, Termin 8 × erythromycin against *S.* Typhimurium as well as *E. coli*, and Termin 8 × penicillin against *S. aureus* were found to be synergistic. No synergism was found between Termin 8 and erythromycin against *S. pyogenes*.

4.4.1 *In vitro* effects of thymol and Termin 8 in chicken caecal content and chicken feed

The effects of Termin 8 and thymol were tested in chicken caecal content and feed in the presence of the antibiotic resistant bacteria and the results are shown in Figs 4.1-4.6.

In the chicken caeca, a concentration of thymol higher than the MIC was necessary for bacterial reduction, especially against *S.* Typhimurium and *E. coli*. A concentration of 35 mM thymol was needed to eliminate the organisms from the caecal digesta in 3 h (Fig.4.1), which was > 20 times greater than its MIC of 2.5 mM against *S.* Typhimurium and *E. coli*.

When used against *S. aureus*, 20 mM thymol was needed which was 8 times greater than the MIC. Termin 8 was also needed at higher concentrations than the MIC. When used against *S.* Typhimurium and *E. coli*, the concentration needed was 0.336 µl/g. A higher concentration (> 0.672 µl/g) was required against *S. aureus* in the caecal content tests (Fig.4.2).

Even greater concentrations of thymol and Termin 8 were needed to show satisfactory antibacterial activity in feed. Similar results were obtained in feed with or without antibiotic (Fig.4.3-Fig.4.6). A relatively high concentration of thymol (200 mM) was used in feed to obtain suitable antimicrobial activity, which was 80 times more than the MIC against *S.* Typhimurium and *E. coli*. In the case of *S. aureus*, a slightly lower concentration (175 mM) was necessary. When the "pure" form of virginiamycin was tested against these bacteria for its activity, *S.* Typhimurium and *E. coli* were highly resistant to the antibiotic levels used, whereas *S. aureus* and *S. pyogenes* showed sensitivity. Concentrations of virginiamycin in the "pure" form of the drug were found to be equally effective when the feed additive Stafac-22 was used as the antibiotic source. The results for virginiamycin and Stafac-22 are shown in Table 4.3. At 0.672 µl/g Termin 8 was found to be effective against all three bacteria (*S.* Typhimurium, *E. coli* and *S. aureus*) in feed (Fig 4.5 and 4.6).

In the untreated digesta sample, a one log increase in growth was seen for *S.* Typhimurium and two log increases in growth were noted for *E. coli* after 3 h incubation at 37˚C. Even though the feed used for broiler growth unexpectedly contained chlortetracycline hydrochloride (220 mg/kg), residual inhibitory effects of this antibiotic were not observed in inoculated control caecal digesta, with the possible exception of *S. pyogenes* (Table 4.4). Numbers of *S. aureus* in digesta were unchanged after incubation. In feed with or without antibiotic and coccidiostat, a two log increase in growth was observed for both *S.*

Typhimurium and *E. coli* after 3 h incubation at 37˚C (Table 4.4). However no growth of *S. aureus* occurred; bacterial numbers remained essentially the same in both digesta and feed samples (Table 4.4). In contrast, *S. pyogenes* numbers were reduced in digesta and feed samples to negligible levels (< 2 log cfu/g, Table 4.4).

4.5 Discussion

Microbes colonize the intestinal tract of poultry and can cause contamination of carcasses during processing. Poultry carcass contamination with *Salmonella* varies from 5 to 100 % (Carraminana et al., 1997) and in many countries poultry is a major food vehicle for transmission of illness to humans. Epidemiological investigations have found that the ultimate source of *Salmonella* and toxigenic *E. coli* in food animals are feed and feed ingredients (Maciorowski et al., 2007; Davies et al., 1997; Jones et al., 1991). There is considerable opportunity for contaminated animal feed ingredients to move among and within countries. Furthermore this could cause rapid and widespread dissemination of a pathogen to animals/foods over large geographic areas (Crump et al., 2002). The feed industry is under continuous pressure to supply feed which is free of microbial contaminants to the poultry industry.

Nayak et al. (2003) observed a dramatic reduction in the levels of *Salmonella* in feed by the addition of Termin 8 at the rate of 0.2 to 0.3% (w/w). Treatment of poultry feed with Termin 8 at the manufacturer's recommended rate of 5.9 lb/tonne (2.72 kg/tonne) resulted in a 2 log reduction in feed contamination and a significant reduction of microbiological loads on the egg shell surface (Anderson and Richardson, 2000). A reduced mortality rate in brown egg pullets was observed by Anderson et al. (2001) when Termin 8 treated-feed (2.72

kg/tonne) was included in their diets. Improved growth performance during the initial period after weaning in nursery pigs was noted when they were fed with Termin 8 treated spray-dried animal plasma. The Termin 8 was included at a level of 5.9 lb/ tonne (2.72 kg/tonne) of total product (plasma or complete diet). The lower mortality rate and improved growth performance could have been due to reduced microbial load. While Termin 8 treatment of whole diets did not generate any difference in growth performance from the control, no negative effects were reported (DeRouchey et al., 2001).

In the present study, Termin 8 concentrations lower than the manufacturer's recommended levels were sufficient to reduce the numbers of antibiotic resistant bacteria in laboratory media within 3 h to almost undetectable numbers. The manufacturer's recommended level of Termin 8 in feed equates to 2.7 µl/ml in laboratory media, which is 32 times greater than the highest bacterial MIC observed. Although a concentration about 16 times higher than the MIC was needed to control antibiotic resistant bacteria in feed and digesta, this is still one-half its recommended use rate in feed and should eliminate these pathogens from feed under moist conditions. In addition, Termin 8 was effective in reducing the MIC's of antibiotics when used in combination with them.

Termin 8 is a liquid preservative which contains 33% formaldehyde. A study by Anitox Corporation showed that the minimum effective dose of formaldehyde needed to retard the growth of *Clostridium perfringens* in the poultry feed up to 14 d was 0.91 kg/tonne (Anonymous, 2009). An European commission report noted that inclusion of formaldehyde up to 660 mg/ kg in complete poultry and pig feeds had no adverse effects on performance of the target animals, detectable tissue levels or contamination of the environment (Anonymous, 2002). No adverse effects were noted in birds fed with formalin (37% formaldehyde) - treated feed for 8 weeks (Khan et al., 2003). This study further reported that

since formalin is volatile, it almost completely evaporates when exposed to the atmosphere. So its concentration at consumption is much less than when mixed with the feed.

Similarly, no negative effect on feed efficiency in steers fed formaldehyde-treated poultry waste was observed by Koenig et al. (1978). Waldo et al. (1972) and Madsen (1982) reported that feeding formaldehyde-treated silage to ruminants had a protective effect on protein degradation in the rumen. Rumen undegraded protein (RUP) increased feed efficiency and milk fat content in dairy cows (Broderick et al., 2009).

Even though many studies report application of essential oils in food to improve shelf life and control pathogens, published studies on their use in feed and digesta are still limited. Blends of essential oil compounds have been shown to have stimulatory effects on the digestive system of birds which significantly improved their performance (Jang et al., 2007; Sengul et al., 2008; Alcicek et al., 2003). Si et al. (2006a) developed an *in vitro* assay with pig diets to assess whether the use of essential oil components could control *Salmonella* contaminants in the feed. It was concluded that a concentration higher than the MIC of the essential oil cinnamaldehyde was required in the diets to exhibit antimicrobial activity. The present results are in agreement with those obtained by Si et al. (2006a), since it was necessary to use a high concentration of thymol and Termin 8 in both the poultry feed and digesta to demonstrate a satisfactory antimicrobial effect.

When used individually, 2.5 mM of thymol effectively inhibited the growth of *S.* Typhimurium, *E. coli*, *S. aureus* and 0.63 mM was found to be inhibitory against *S. pyogenes.* These findings were consistent with the results reported by Pei et al. (2009) where 2.6 mM of thymol inhibited the growth of *E. coli*. Similarly, Helander et al. (1998) showed 1 to 3 mM thymol was effective against *S.* Typhimurium and *E. coli*. In combination with

antibiotics, it was more effective against Gram-positive bacteria (*S. aureus* and *S. pyogenes*) than the Gram-negative bacteria tested (Chapter 3; Table 3.3).

From the results of the present study, either Termin 8 or thymol could be used as alternative feed additives to control antibiotic resistant bacteria in feed. A reduction in microbial pathogens in feeds will benefit the bird by reducing competition in the digestive tract between indigenous microorganisms and feed borne pathogens attempting to colonize the small intestine, lowering microbial populations in the environment and enhancing the birds growth performance (Anderson and Richardson, 2000; Anderson et al., 2001). There is also benefit through the potential decrease in egg shell contamination by the pathogens. Reduction of pathogenic bacteria in birds will reduce public health risks associated with poultry products. Termin 8 contains a mixture of formaldehyde, propionic acid, d-limonene, as well as mono and di-glycerides of edible oils (DeRouchey et al., 2001). Although 1-2 % formic acid is used in Europe (Haggblom, 2006), there is no indication in any study of a minimum concentration of formaldehyde which might be used alone as a feed additive. Thus further studies are needed to determine an appropriate concentration of formaldehyde that will achieve complete bacterial pathogen control in feed. It is also important to understand the factors controlling the action of Termin 8 or thymol as feed additives and their effects on beneficial gut microbes in animals.

Table 4.1- MIC values of thymol and Termin 8 against antibiotic resistant bacteria

Bacteria	MIC	
	Thymol (mM)	Termin 8 (μl/ml)
*S.*Typhimurium	2.5	0.042-0.084
E.coli	2.5	0.084
S.aureus	2.5	0.042-0.084
S.pyogenes	0.625	0.084

Table 4.2- Response of antibiotic resistant bacteria to the combined effects of an antibiotic and Termin 8 expressed as the FIC index [a] and log DP [b]

Antibiotic	FIC (log DP)			
	S. Typhimurium	*E. coli*	*S. aureus*	*S. pyogenes*
Ampicillin	0.5 (0.9)	0.75	1.25	
Tetracycline	0.38 (1.0)	0.75		
Penicillin	0.5 (0.6)	1.25	0.27 (-1.5)	
Erythromycin	0.5 (-1.2)	0.45 (-3.0)		0.5 (0.8)
Bacitracin	0.38 (-0.2)	0.5 (-0.4)	0.75	
Novobiocin	0.75	0.31 (0.2)		

a. Synergy = an FIC index ≤ 0.5. An FIC index between 0.5 and 4.0 indicated 'no interaction'. An FIC>4.0 indicated antagonism between the two agents.

b. Log DP < -1 combinations had significant antibacterial activity. The analysis was done for all combinations having an FIC index ≤ 0.5 and the results are shown.

Table 4.3- MIC values of virginiamycin and Stafac 22 against antibiotic resistant bacteria

Bacteria	MIC	
	Virginiamycin μg/ml	Stafac-22 (mg/ml)
S. Typhimurium	500	23
E. coli	250	23
S. aureus	3.12 (S)	0.07
S. pyogenes	0.039 (S)	0.0018

S- Sensitive

23 mg of Stafac contained ~ 500 μg virginiamycin

Similarly 0.07 mg of Stafac contained ~ 1.56 μg virginiamycin which is half of the MIC and 0.0018 mg of Stafac contained ~ 0.039 μg virginiamycin

These values are calculated based on the manufacturer's (Pfizer Canada; Phibro Animal Health, Regina, SK, Canada) information given below:

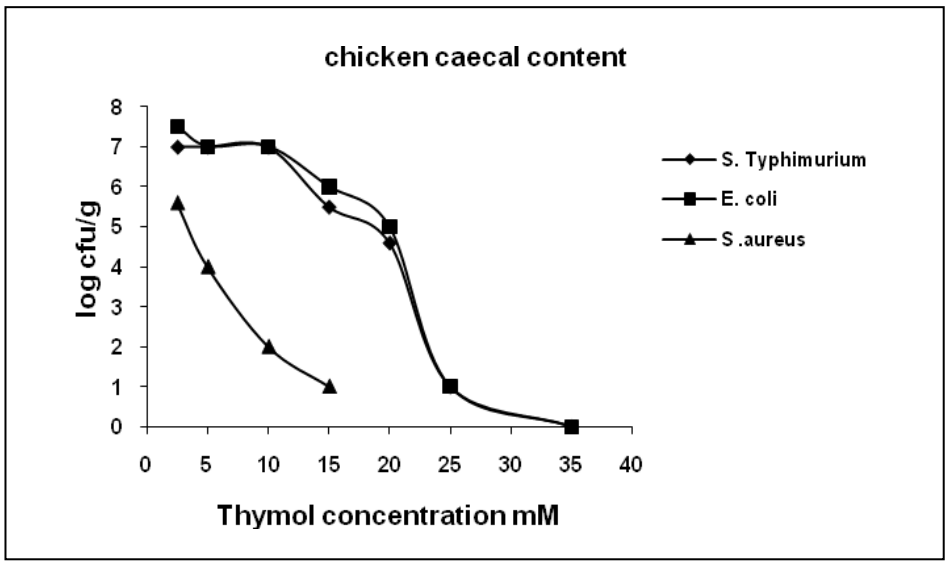
22 g of virginiamycin in one kg of Stafac-22

Table 4.4- Bacterial numbers in chicken caecal content and feeds after 3 h at 37˚C

Bacteria	Log CFU/g			
	Number of bacteria inoculated	Bacteria recovered from inoculated chicken caecal content	Bacteria recovered from inoculated feed with antibiotic and coccidiostat	Bacteria recovered from inoculated feed without antibiotic and coccidiostat
S. Typhimurium	6	7.0	8.1	8.1
E. coli	6	8.0	8.2	8.0
S. aureus	6	6.0	6.1	6.5
S. pyogenes	6	1.0	ND	ND

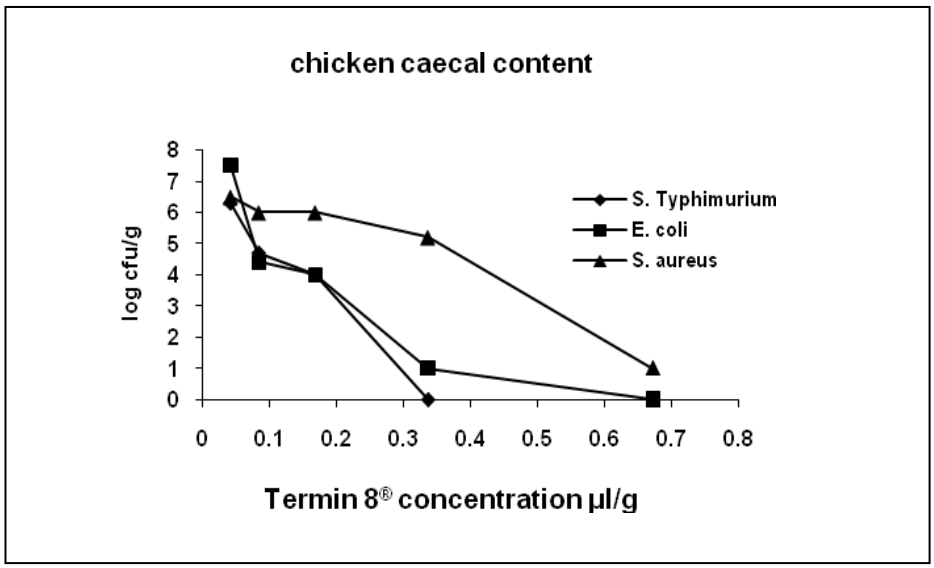
ND-Not detected at 10^1 dilution

Fig. 4.1- Viable antibiotic resistant bacterial numbers (log cfu/g) in chicken caecal digesta at different thymol concentrations after 3 h at 37˚C



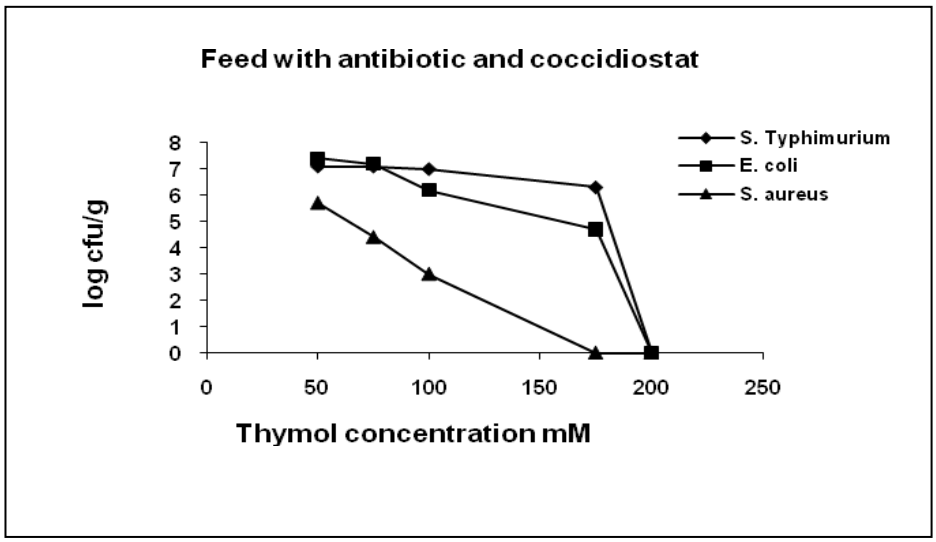
Chicken caecal material was inoculated with 6 log cfu / g of each bacterium

Fig. 4. 2- Viable antibiotic resistant bacterial numbers (log cfu/g) in chicken caecal digesta at different Termin 8 concentrations after 3 h at 37˚C



Chicken caecal material was inoculated with 6 log cfu / g of each bacterium

Fig. 4.3- Viable antibiotic resistant bacterial numbers (log cfu/g) in feed with antibiotic and a coccidiostat at different concentrations of thymol after 3 h at 37˚C



When added, the feed contained: virginiamycin 11 mg/ kg

coccidiostat coban: 99mg /kg diet

Fig. 4.4- Viable antibiotic resistant bacterial numbers (log cfu/g) in feed without antibiotic and a coccidiostat at different concentrations of thymol after 3 h at 37˚C

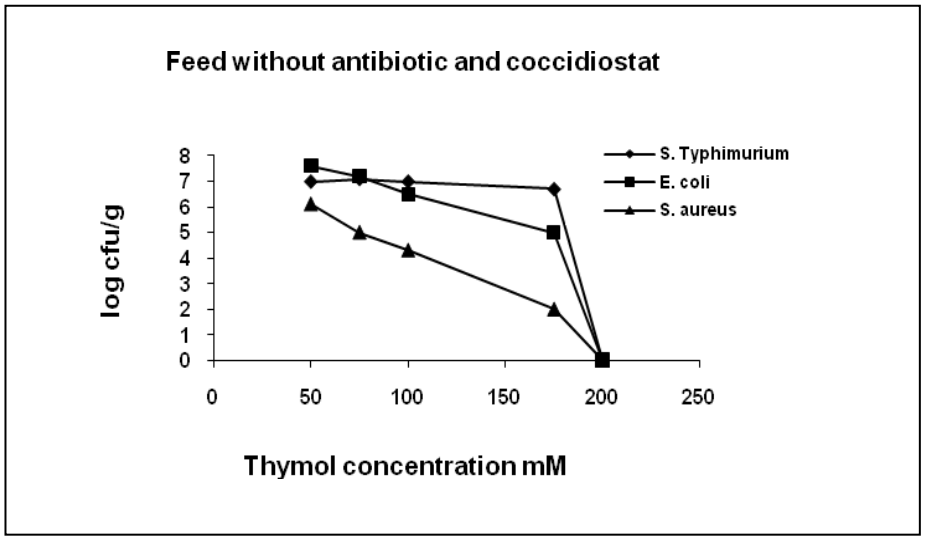
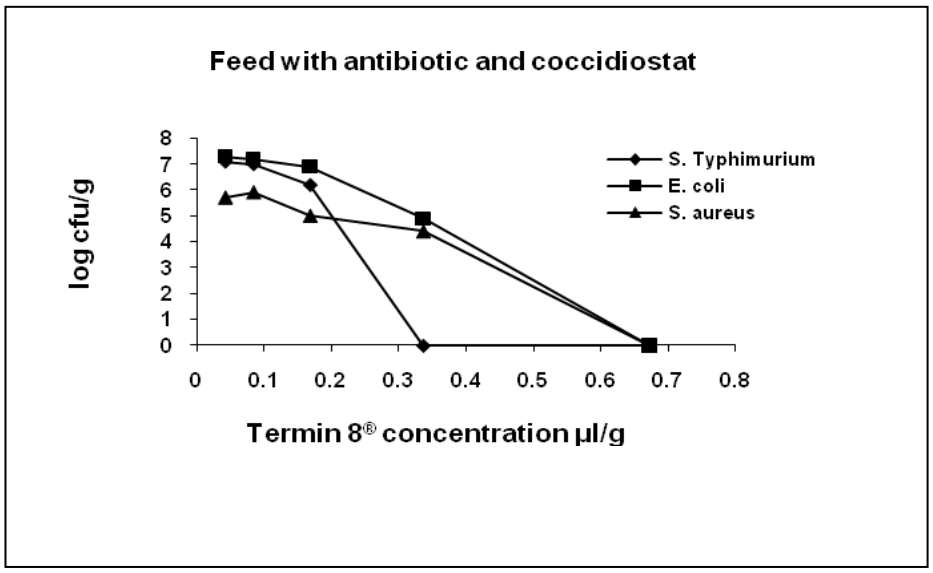


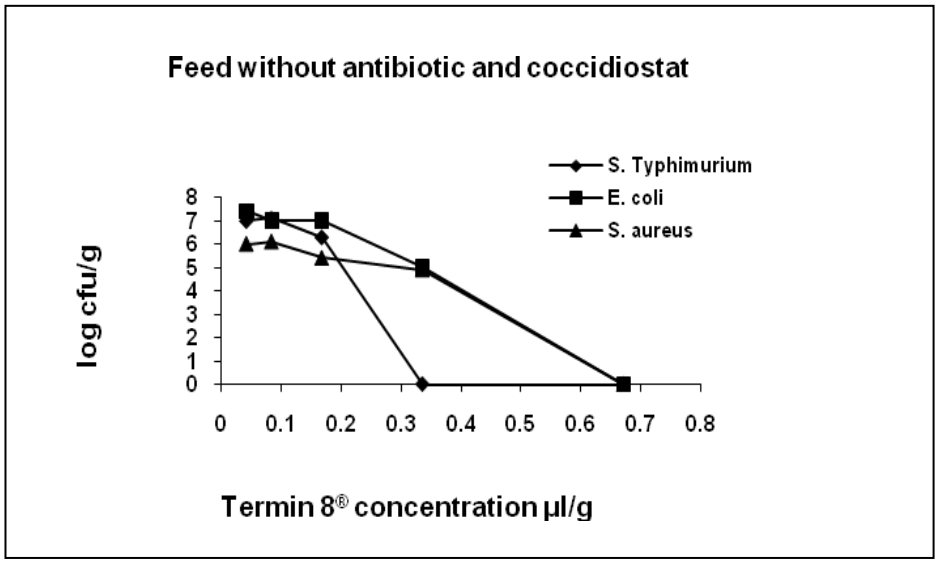
Fig. 4.5- Viable antibiotic resistant bacterial numbers (log cfu/g) in feed with antibiotic and a coccidiostat at different concentrations of Termin 8 after 3 h at 37˚C



When added, the feed contained: virginiamycin 11 mg/ kg

coccidiostat coban: 99mg /kg diet

Fig. 4.6- Viable antibiotic resistant bacterial numbers (log cfu/g) in feed without antibiotic and a coccidiostat at different concentrations of Termin 8 after 3 h at 37˚C



CHAPTER 5

Conclusions

From the results obtained during these studies, several conclusions can be reached. The main outcomes are:

- Consistent with observations where natural products have been shown to be a rich source of anti-infective and antimicrobial agents, the essential oils tested here were shown to have inhibitory activity against a group of 4 bacteria highly resistant to antibiotics.

- When used in paired combination with antibiotics, the essential oils were able to reduce the MIC's of antibiotics against drug resistant bacteria. Though the exact mechanism behind the synergism is not clear, the essential oil components (particularly carvacrol and thymol) enhanced the antimicrobial efficacy of the antibiotics.

- Gram-positive bacteria were more sensitive to natural antimicrobials than the Gram-negative bacteria, both in individual and combination tests with antibiotics. *S. aureus* was more sensitive and many combinations were synergistic against this organism, more so than with the other three bacteria (*S.* Typhimurium, *E. coli*, *S. pyogenes*) tested.

- Carvacrol was more effective in combination with antibiotics; it was synergistic with at least three antibiotics of 6 tested against *S.* Typhimurium and *E. coli*, two antibiotics with *S. aureus* and one antibiotic with *S. pyogenes*. When

measured by the FIC index alone, thymol was as frequently synergistic as carvacrol, however, when combined with log DP, carvacrol showed greater synergism. AIT was more effective than the other agents when tested individually.

- The formaldehyde-based feed additive Termin 8 also showed potential to inhibit the antibiotic resistant bacteria. In combination with antibiotics, it was synergistic with two of them (with erythromycin against *S*. Typhimurium and *E. coli*, and with penicillin against *S. aureus*).

- In feed and chicken caecal digesta, a concentration higher than the MIC of thymol and Termin 8 was necessary to essentially eliminate viable *S.* Typhmurium, *E. coli* and *S. aureus* cells. The level of Termin 8 required was one-half the recommended use rate in feed and the level of thymol necessary was not detectable by smell. *S. pyogenes* did not survive (≥6 log reduction) in feed (± virginiamycin) or in caecal digesta held at 37ºC for 3 h.

- Plant extracts tested showed great potential for use as antimicrobial agents against antibiotic resistant bacteria *in vitro*. They can be considered as a potential alternative to replace antibiotic growth promoters which are in part responsible for the current issue of antibiotic resistance. The combined effect of a natural antimicrobial and an antibiotic enhanced the inhibitory action of several of the antibiotics to which the bacteria were normally resistant.

CHAPTER 6

Future study

- The present study used only 5 essential oils against 4 antibiotic resistant bacteria. Further study should be expanded to include more plant extracts from different geographical areas. Whole extracts and as well as their major active components should be studied to evaluate these materials for antimicrobial activity.

- It is important to better understand the mode of inhibitory action of natural antimicrobials against bacterial cells. Research should also be focused on the toxicity of these compounds to human and animal cells and their effects *in vivo.*

- Further work is necessary to explain natural antimicrobial efficacy, stability in the atmosphere, palatability in animal diets and suitable concentrations for antimicrobial action in feed. More complete evaluations are required to establish their effects on animal performance.

- Understanding the mechanism of synergistic interaction is fundamental to the development of new pharmacological drugs to replace growth promoting antibiotics, which may be withdrawn from use in response to the antibiotic resistance issue. Studies should be undertaken to better define the full potential of natural antimicrobial and antibiotic synergy in feed and animal models.

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APPENDICES

Appendix A. Broiler starter diet without antibiotic and coccidiostat

Ingredient	Starter %
Wheat	27.00
Corn	27.00
SBM	27.50
Canola meal	8.00
Canola oil	5.93
Calcium carbonate	1.42
Di calcium phosphate	1.56
DL-met	0.09
Mineral premix	0.50
Vitamin premix without antibiotic and coccidistat	1.00
Total	100.00
Nutritional value	
Crude protein %	22
ME (kcal/kg)	3100
Ca %	1.1
P available %	0.45
Methionine %	0.5
Lysine %	1.15

Minerals provided: Manganese oxide, 70 mg; zinc oxide, 80 mg; ferrous sulfate, 80 mg; copper sulfate, 10 mg; sodium selenite, 0.3 mg; calcium iodide premix, 0.5 mg per kilogram of the diet.

Vitamins provided: Vitamin A, 8255.0 IU ; vitamin D_3, 3000.0 IU ; vitamin E, 30.0 IU ; vitamin B_{12}, 0.013 mg ; niacin, 23.1 mg; choline chloride, 1081 mg; folic acid 4.0 mg; biotin, 25 mg; pyridoxine (B_6) (HCL), 0.25 mg; thiamine (B_1) (mononitrate), 4 mg; endox (anti-ox), 125.0 mg per kilogram diet

SBM- Soya bean meal, Met - Methionine, ME- Metabolizable energy, Ca- Calcium, P- Phosphorus

Appendix B. Broiler starter diet with antibiotic and coccidiostat

Ingredient	Starter %
Wheat	27.00
Corn	27.00
SBM	27.50
Canola meal	8.00
Canola oil	5.93
Calcium carbonate	1.42
Di calcium phosphate	1.56
DL-met	0.09
Mineral premix	0.50
Vitamin premix without antibiotic and coccidistat	1.00
Total	100.00
Nutritional value	
Crude protein %	22
ME (kcal/kg)	3100
Ca %	1.1
P available %	0.45
Methionine %	0.5
Lysine %	1.15

Virginiamycin - 11 mg/ kg diet; Coccidiostat: Coban - 99 mg per 1kg diet.

Minerals provided: Manganese oxide, 70 mg; zinc oxide, 80 mg; ferrous sulfate, 80 mg; copper sulfate, 10 mg; sodium selenite, 0.3 mg; calcium iodide premix, 0.5 mg per kilogram of the diet.

Vitamins provided: Vitamin A, 8255.0 IU ; vitamin D_3, 3000.0 IU ; vitamin E, 30.0 IU ; vitamin B_{12}, 0.013 mg ; niacin, 23.1 mg; choline chloride, 1081 mg; folic acid 4.0 mg; biotin, 25 mg; pyridoxine (B_6) (HCL), 0.25 mg; thiamine (B_1) (mononitrate), 4 mg; endox (anti-ox), 125.0 mg per kilogram diet

SBM- Soya bean meal, Met - Methionine, ME- Metabolizable energy, Ca- Calcium, P- Phosphorus

Appendix C. Composition of diets fed to chickens from which digesta was taken for analysis

Standard commercial broiler diet in crumble form (FeedRite; a division of Ridley Inc., Winnipeg, Manitoba, Canada)

Ingredient	Starter
AME kcal/kg	2,713
Crude Protein (min)	21.0 %
Crude Fat (min)	1.9 %
Total Lys	0.96 %
Total Met	0.40 %
TSSA	0.80 %
Available P	0.7 %
Ca	1.0 %
Vitamin A (min)	9,000 IU/kg
Vitamin D3 (min)	3,000 IU/kg
Vitamin E (min)	30 IU/kg
Selenium	0.3 mg/kg
Chlortetracycline hydrochloride	220 mg/kg

AME- Apparent metabolizable energy, Lys-Lysine, Met- Methionine, TSSA- Total sulfur amino acid (methionine + cystine), P- Phosporous, Ca- Calcium