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# Evaluation of the quality of chicken carcasses used as feed for Nile crocodiles on commercial farms

by

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Albert de Wet

2020/08/10

## ABSTRACT

The average mortality rate on chicken farms in South Africa is ± 4.0% per annum. Commercial crocodile farmers have access to some of the ±750 000 chicken carcasses available per week (nearly 3 million carcasses per month). The feeding of chicken carcasses to crocodiles was investigated to identify the potential hazards. The hazards investigated in more depth, focussed on bacteria present in chicken carcasses during different stages of decomposition and the prevalence of antibiotic residues. Chicken carcasses used as crocodile feed, differ in states of decomposition - ranging from fresh to severely decomposed. Carcass "quality" has become a concern to commercial crocodile farmers, with many farmers questioning the value of these severely decomposed carcasses, with some stating that feeding of severely decomposed chicken carcasses to crocodiles makes them sick. Fifty-four chicken carcasses were left in an environmentally controlled poultry house. Carcasses were removed in 6-hour intervals after death; with the last group exposed to typical in-house environmental conditions for 36-hours. Carcasses were frozen immediately after collection (each group after removal from the house). When these carcasses were processed again afterwards, they were thawed and eviscerated for sampling of the gastrointestinal tracts (GITs) and rest of the carcasses, separately. The bacterial contamination of the GITs and rest of the carcasses was determined using standard laboratory methods. *Salmonella spp., Listeria monocytogenes* and *Staphylococcus aureus* were detected in 88%, 61% and 36% of the tissue samples, respectively. Samples testing positive for *Salmonella spp.* were serotyped and identified as *Salmonella hamburg.* Further results showed that mean log CFU/g for *E.* coli were significantly higher ($P<0.05$) in GIT (gastrointestinal tract) (5.433 log CFU/g) compared to MBF (muscle, bone and feathers) (4.783 log CFU/g). A difference of 1.9 log CFU/g between the same group suggest that mean log CFU/g increased over 12-hours for *E. coli* on carcasses. Carcass pH and temperature was measured and recorded after each 6-hour exposure period in each carcass, just before freezing, by making a 1 cm incision in the pectoral muscle and inserting a portable meat pH meter and temperature probe (HANNA HI 99163). The measurements recorded showed an average decrease in carcass temperature of 3.6ºC between the 6-hour groups. The carcass pH decreased at a steadily rate of 0.15 units of pH/6-hours for the first three groups (12-hours exposed) and increased at a rate of 0.085 units of pH/6-hours for the last groups (36-hours exposed). Chicken carcasses used as crocodile feed vary in age and origin. Although withdrawal periods are adhered to in post-finisher poultry rations, chickens on grower and finisher rations take in pharmaceutical drugs that may pose a hazard to crocodiles and farmers exporting meat. Five commercial crocodile farms were used for collecting samples from minced chicken carcasses - prepared as crocodile feed on the farms. Three separate samples (± 20 gr each) were collected from chicken carcasses from each crocodile farm. Totalling 15 samples that were screened for 15 antibiotic compounds using the LCMS/MS technique (liquid chromatography / mass spectrometry). All samples submitted tested negative (< 50 µg/kg). This result concludes that poultry carcasses do not pose as a hazard for

antibiotic residues when used as feed for commercial crocodiles. Distinct carcass observations in the decomposition trial were used to determine if they could be used in developing a practical carcass evaluation method. Skin colour and carcass deterioration were chosen and used in the evaluation. Carcasses were easily identified by skin colour (red, green and blue). “Blue” carcasses confirmed deterioration and “red” did not when subjected to the pull-test, a technique developed in this project and used to determine carcass deterioration by pulling the legs apart, confirming deterioration, or resisting the pull test and remaining intact. The carcass evaluation method was performed on the commercial crocodile farms taking part in the questionnaire and on-farm hazard identification. A questionnaire was discussed with farmers and managers from 5 crocodile farms to gather information and compare the different methods of chicken carcass evaluation, preparation and feeding. The information gathered from the questionnaire and hazard identification confirmed that severely decomposed carcasses pose as a possible hazard if included as feed for hatchling and grower rations. These carcasses have the posibilty of increasing or introducing antibiotic resistant bacteria in feed, thus increasing the risk of disease outbreak. A *management plan for feeding chicken carcasses to crocodiles* was developed for commercial crocodile farmers addressing the source, transport, processing and storage of carcasses. In conclusion, results from the first two sub-projects (first 2 sub-projects as discussed above), together with our on-farm questionnaire and investigation, were used to evaluate the potential hazards associated with using chicken carcasses as feed for crocodiles. In our opinion, the risks to crocodiles, on commercial crocodile farms consuming chicken carcasses, are relatively low. Especially, if farm managers are willing to apply the proposed *management plan* discussed in this thesis, as well as general biosecurity guidelines.

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| | |
|---|---|
| ABR | Antibiotic Residue |
| BUGM | Biolog Universal Growth Medium |
| CAR1 | 6-hour exposed poultry carcasses |
| CAR2 | 12-hour exposed poultry carcasses |
| CAR3 | 18-hour exposed poultry carcasses |
| CAR4 | 24-hour exposed poultry carcasses |
| CFU | Colony Forming Unit |
| CP | Crude Protein |
| ESI | Electrospray-ionisation |
| FDA labs | Food & Drug Assurance Laboratories |
| FRESH | 0-hour exposed poultry carcasses |
| g | gram |
| GIT | Gastrointestinal Tract |
| h | hour(s) |
| LCMS/MS | Liquid Chromatography Mass-Spectronomy |
| LOC | Location |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| MBF | Muscle, Bone and Feathers |
| MRM | Multiple Reaction Monitoring |
| SEVROT | 36-hour exposed poultry carcasses |
| UP | University of Pretoria |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background information

Commercial crocodilian farming is a relatively new form of intensive animal production (Manolis and Webb, 2016). In southern Africa, only the Nile crocodile (*Crocodylus niloticus*) is used for commercial crocodile farming purposes. As such, it lacks the long-accumulated scientific knowledge if compared to more conventional animals (e.g. livestock, pigs and poultry) used in intensive commercial farming (Manolis and Webb, 2016).

The type of biological protein, used as crocodile feed, varies depending on the life stage of the growing crocodile (hatchling vs. growers) and the availability of biological protein sources (Isberg, 2007). The South African crocodile industry relies heavily on chicken mortalities from poultry farms as a source of biological protein. Chicken carcasses in different stages of decomposition (from fresh to severely rotten) are used. The state of decomposition of the chicken carcasses vary, depending on the effectiveness of the poultry farm labourers to regularly remove the dead chickens from the heated poultry houses. Dead chickens are usually removed (6, 12, 24-hour intervals) from the houses, stored frozen or transported immeditely to the crocodile farms. The distance of the crocodile farm from the poultry farm is also a determining factor as farms closer will receive fresher carcasses, depending on the availability of cold storage and capacity on the poultry farm.

Some South African crocodile farmers believe that crocodiles could consume any carcass (meat) or any parts thereof, regardless of the state of decay, without any negative effects. However, unpublished observations (J G Myburgh, S van der Woude & J C A Steyl) indicate that severely decomposed chicken carcasses may cause poor feed intake and even affect the health of the fast-growing crocodiles. Over the past few decades poultry farming has gone through tremendous growth; however, with the increase in production, the use of specific pharmaceutical drugs and feed additives has become essential to prevent disease outbreaks and to support growth promotion. However, one of the negative side-effects of excessive use of antimicrobial drugs is that they may accumulate in the tissues and organs of treated animals as residues,

and eventually become part of the feed chain (Mund, 2016). The prevalence of antimicrobial residues in crocodiles may be a risk for humans consuming crocodile meat.

### 1.2 Hypothesis

The hazards associated with chicken carcasses negatively affect their quality as crocodile feed.

### 1.3 Justification

The quality of chicken carcasses will be investigated by focusing on normal bacterial decomposition after death and pharmaceutical residues in carcasses of different ages. Chicken carcasses, at varying stages of decomposition, are fed to farmed crocodiles in South Africa. The pathogens of concern associated with chicken carcasses destined for human consumption are well known (e.g. *Salmonella spp., Listeria spp., Staphylococcus aureus*). However, the bacterial count of chicken carcasses, in various stages of decomposition, is unknown. Stress septicaemia may be caused whenever crocodiles are exposed to high levels of bacterial contaminants and when their immune systems are compromised (cold ambient temperature) (Benedict and Shilton, 2016). Crocodiles are sensitive to cold ambient temperatures (Huchzermeyer, 2003). The level of these pathogens in chicken carcasses, therefore, becomes important to the crocodile farmers utilising them as a feed source. Bacterial decomposition of chicken carcasses will be investigated using similar environmental conditions as used on commercial poultry farms.

Another factor posing as a risk to crocodile farmers, using chicken carcasses as feed, is the concentrations and type of antimicrobial residues remaining in the carcasses. Chicken mortalities collected during the growth phase of production, not yet subjected to a withdrawal period, have a good chance of containing pharmaceutical drugs due to the administration in the feed. These mortalities are not discarded separately and form part of the total mortalities provided as feed to crocodile farmers. Farmers specialising in crocodile meat for export must adhere to strict export regulations and need to be extremely careful not to feed any chicken carcasses containing pharmaceuticals to their crocodiles. Chicken carcasses, collected from 5 commercial

crocodile farms, will be used to get an indication of the prevalence of antimicrobial residues in these carcasses.

The chicken carcass decomposition knowledge will be used during the investigation on 5 crocodile farms to evaluate the quality of the chicken carcasses used. The information gained from observing carcasses exposed in the decomposition trial can be used in the investigation as a method to identify between fresh and severely rotten carcasses. In addition, chicken carcass management on the crocodile farms will be evaluated using an on-farm inspection and questionnaire. As far as we could ascertain this has never been done before and will be of great value to the crocodile industry. In the end a practical management plan will be developed for crocodile farmers feeding chicken carcasses.

### 1.4 Objectives of this study

1. Studying the **decomposition of chicken carcasses**, left in a standard environmentally controlled chicken house, by determining the microbial load, pH and temperature of the carcasses at specific intervals after death
2. Determining **antimicrobial residues present in chicken carcasses** (mortalities) processed as crocodile feed
3. Developing a practical **carcass evaluation method** that can be used on commercial crocodile farms to evaluate the state of decomposition of chicken carcasses
4. Crocodile **farm questionnaire, investigation** and **hazard identification**
5. Development of a practical **management plan for feeding chicken carcasses to crocodiles**

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Broiler industry in South Africa

A total of 927.1 million broilers were slaughtered in South Africa during the year 2017; a decrease of 8.4 million (-0.9%) compared to the previous year. The average mortality rate on the contract growers' farms was 4.1% (SAPA, 2018). Therefore, an average of ±750 000 discarded chicken carcasses became available per week, adding up to nearly 3 million carcasses per month.

On average, 19.0 million broilers were produced per week in January 2018. This was 1.6 million birds (+9.3%) more than the previous month and 1.8 million birds (+11.0) more than the same month of the previous year (SAPA, 2018). For December 2018 17.8 million broilers were produced per week. In total 79.0 million broilers were produced for slaughter in December 2018 (SAPA, 2018). At a mortality rate of 7% per month the number of carcasses available would be roughly 5.5 million. Day-old chicks were obtained from the principal company's hatcheries. The 27 respondents placed a total of 3.4 million chicks per cycle during July, August and September of 2018; an average of 127 600 per farmer. The average mortality rate on the contract growers' farms was 3.1% (SAPA, 2018).

In summary, with a mortality rate ranging between 3-7% annually on contract grower farms, the average amount of carcasses that will not be used or processed for human consumption estimate to be roughly between 3–5.5 million carcasses a month, thus 36-66 million carcasses a year. These carcasses are either destined for rendering plants, waste management, fertilizer production or crocodile feed.

## 2.2 Use of chicken mortalities by crocodile farmers

Internationally, minced or finely diced red meat, abattoir offal, discarded carcasses from intensive farming enterprise (mammals, birds or fish), supplemented with vitamins and mineral, are commonly used for crocodilians (Manolis and Webb, 2006).

In South Africa commercial crocodile farmers rely heavily on chicken mortalities as a protein source due to high abundance and availability of carcasses from poultry farms.

Most mortalities occur during handling or catching for transport, in transit to abattoirs and throughout the production cycle due to high stocking density and poor management (SAPA, 2018). Certain farmers only make use of abattoir rejects (breast blisters, burnt hocks and offcuts).

Beyeler (2011) reported that South African crocodile farmers, in general, do not feed their crocodiles a balanced diet, as most farmers feed any type of animal protein (e.g. whole chicken or carcass remnants from the abattoir) available, resulting in poor growth performance and poor skin quality. Beyeler (2011) reported that crocodiles that were fed a high crude protein (CP) diet, outperformed all the other crocodiles on a ,lower CP formulation. The formulation that contained a high quantity of raw minced chicken, elicited a more intensive feed response if compared to crocodiles on other feeds. The smell and taste of the meat most probably stimulated the high intake of the feed. In addition, wastage was low confirming the excellent palatability of the ration (Beyeler, 2011).

## 2.3 Handling of chicken carcasses on poultry farms and waste disposal

The correct disposal of chicken carcasses and waste has environmental, biological and financial concerns for the poultry industry. World-wide, there are several ways of disposing of poultry waste including: burial; rendering; incineration; composting; feed for livestock; fertilizer; or source of energy. Slaughterhouse wastes like feathers, blood, and innards are processed and utilised as high-protein animal feed sources or as fertilizer due to its high nitrogen content (Singh, 2018).

Due to the heated environment in a commercial chicken house, the microbial load of chicken carcasses (mortalities) may increase exponentially, depending on the ambient temperature in the house, length of time the carcasses were left in the house before removal (usually frozen after removal) and overall biosecurity on the farm. Housing units maintain a steady temperature of 24-25$^0$C, 55-60% Relative Humidity (RH) and 1.34 m$^3$/h/bird ventilation during production. An open chicken housing system, commonly used in poultry production, usually have lower temperatures and humidity, depending on the location and season of the year, but may have a higher biosecurity risk. This, together with the microbes found in the litter and stocking density of the house, will give rise to a high diversity and number of microbes found in and on chicken carcasses. The type and number of pathogens present in carcasses need to be known

as to determine whether they pose a threat to crocodiles when used as feed. Any savings achieved as a result of providing lower quality food will ultimately be offset by negative health and growth implications for the crocodiles.

Poultry housing units are routinely checked by workers to collect mortalities in the pens, this is either done on 6/12-hour shifts or daily. Carcasses are collected by hand and stored in walk-in freezers before transporting to crocodile farms or disposed of. Most mortalities occur during catching/handling pre-slaughter or during transport to abattoir, but some occur before this. Carcasses collected before the withdrawal period for specific pharmaceutical drugs, which are included in the chicken feed, may contain a higher concentration of residues than carcasses collected after the withdrawal period.

## 2.4 Classification of decomposed chicken carcasses

There is limited data available for poultry carcasses as far as the bacterial status of decomposing carcases is concerned. Reports stated that *Pseudomonas* counts on the neck skin were 1.8, 1.7, 3.1 $\log_{10}$ CFU/g, before scalding, after scalding and after defeathering, respectively. The concentration was 3.96 $\log_{10}$ CFU/$cm^2$ for whole carcasses (Panel and Hazards, 2016).

In general, red or white meat is considered to be spoiled when discolouration, off-odour and/or slime develop, this is usually caused by bacteria. *Pseudomonas, Lactobacillus* and *Enterococcus,* for example, produce slime on meat. *Enterococcus* may also produce hydrogen peroxide greening, similar to hydrogen sulphide greening caused by *Clostridium* spp. The growth of bacteria on meat is influenced by temperature, pH, nutrient availability, storage atmosphere and competition from other organisms. Small changes in these factors may greatly influence spoilage (Panel and Hazards, 2016).

The spoilage microflora of fresh poultry carcasses usually consists, almost exclusively, of Gram-negative rods (mainly pseudomonads) and micrococci (mainly *Micrococcus* spp. and *Staphylococcus* spp.). In addition, Gram-negative bacteria such as *Acinetobacter, Alcaligenes, Moraxella* and Enterobacteriaceae, and Gram-positive species including spore-forming bacteria, lactic acid-producing bacteria, as well as yeast and moulds, may be present in small numbers (Koutsoumanis and Sofos, 2004).

Although indigenous enzymes may also be involved, their contribution is considered to be negligible compared with bacterial action. The dominant spoilage bacteria genera are determined by the specific storage conditions. Under aerobic conditions, the spoilage consortium of bacteria is usually dominated by pseudomonads (Panel and Hazards, 2016).

There is limited data available for poultry carcasses as far as the bacterial status of decomposing carcases is concerned. Reports stated that *Pseudomonas* counts on the neck skin were 1.8, 1.7, 3.1 $\log_{10}$ CFU/g, before scalding, after scalding and after defeathering, respectively. The concentration was 3.96 $\log_{10}$ CFU/$cm^2$ for whole carcasses (Panel and Hazards, 2016).

Unfortunately, there is an (incorrect) perception that crocodiles can and will consume any animal protein, regardless of the state of decomposition, without it causing any negative effects. Despite unscientific reports of different crocodilian species in the wild feeding on carrion and “storing” food until decomposed, farmed crocodiles prefer fresh feed (Brien *et al.*, 2010).

In South Africa, some commercial crocodile farmers have developed practical methods of classifying the state of decomposition of chicken carcasses. Others make use of numerous photographs of carcasses in different stages of decomposition to help the workers responsible for processing. Farmers discard carcasses based on the conformation and/or colour at processing. On most farms, chicken carcasses that are in a severe state of decomposition are not used a crocodile feed. However, some farmers believe that rotten chicken carcasses would not affect the health of crocodiles, especially adult crocodiles. Deformed, flattened and rotten carcasses are usually discarded based on the workers’ perceptions and experience.

Severely rotten carcasses, or ‘’blue’” carcasses, are usually characterized by deep blue skin discoloration. The blueish colour may also be due to sub-dermal bleeding as a result of incorrect handling or during the transportation and loading, and not correlated with the stage of decay. Some discarded carcasses are used for the breeders on the farm, as it is perceived that breeders can consume any carcass, regardless of the stage of decay.

A standardised method to classify the state of decomposition of chicken carcasses has not been developed or published for commercial crocodile farms in South Africa.

## 2.5 Pharmaceuticals used in poultry industry

The projected increase in antibiotic use in food animals is directly related to the increase in the human population (from 7 billion to an expected 9 billion to 10 billion by 2050) and the increase in global prosperity. The demand for meat and other animal products is predicted to nearly double in the next 35 years. Most of the population growth, and an even more significant increase in food demand, will come from sub-Saharan Africa and Asia. Increase in income stimulates the increase in caloric intake and the demand for higher food quality (CDDEP, 2015). Unfortunately, the use of pharmaceuticals to keep animals healthy and maintain productivity, effectively stimulates an increase in antimicrobial use and thereby bacterial resistance (Van Boeckel *et al.*, 2015).

Antimicrobials have three roles in animal production to: (1) treat individual animals with bacterial infections; (2) prevent infections; and (3) promote growth (CDDEP, 2015). The global average annual consumption of antimicrobials per kilogram of animal body mass produced was: 45 $mg \cdot kg^{-1}$; 148 $mg \cdot kg^{-1}$; and 172 $mg \cdot kg^{-1}$ for cattle, chicken, and pigs, respectively. Starting from this baseline, between 2010 and 2030, the global consumption of antimicrobials will most likely increase by 67%, from ± 63 000 tons to 105 000 tons (Van Boeckel *et al.*, 2015).

Data on the quantity of antimicrobials used in production animals are scarce in South Africa and information is lacking about the patterns of antibiotic consumption in production animals. Moreover, considering the lack of information on the total quantity of antibiotics produced, it is not surprising that information on quantities used for specific purposes in agriculture and human medicine is also limited (Moyane *et al.,* 2013). Of all the available antibiotics used in livestock production in South Africa, ±29% are mixed into pre-mixes and represent a large percentage of all the registered antimicrobials used in the agricultural sector. Picard and Sinthumule (2002) together with Eager (2008) reported that the most frequent uses of antibiotics by weight (as measured by sales) were those for treating and preventing diseases in poultry, pigs and as growth promoters.

A study by Eager et al. (2012) found that the majority of consumed antimicrobials in production animals were from the macrolide and pleuromutilin groups, followed by the tetracyclines, sulphonamides and penicillins. Their survey results showed that 68.5% of the antimicrobials were administered as in-feed medications. About 17.5% of the total quantity of antibiotics utilized were parenterally administered, whereas antibiotics for water medication constituted 12% of the total and other dosage forms (topical and aural dosage) constituted 1.5% of the total. From the numbers above, it is not surprising that many poultry farms widely use antimicrobials as prophylactic drugs and as growth stimulants. However, this is particularly problematic because antimicrobials for growth promotion are used without veterinary prescriptions or administered for long periods of time at sub-therapeutic concentrations to entire groups or herds of animals (Moyane et al., 2013).

Despite prohibition, many broiler chickens are administered overdoses or inappropriate doses of antimicrobials for therapeutic, prophylactic, as well as non-therapeutic purposes throughout their entire lifespan (Mund *et al.,* 2017). If these drugs are not absorbed or if they are metabolized by the animal, residues would be insignificant. Unfortunately, that is not always what happens. Hence, unsafe drug residues tend to accumulate in various concentrations in edible parts of treated animals. These residues are primarily comprised of parent and derivative compounds including metabolites, conjugated and remnants bound with macromolecules (Mund *et al.*, 2017).

Various researchers have investigated the presence of drug residues in treated animals' edible tissues and/or offal's including heart, liver, kidney and gizzard through different techniques (Cohen *et al.,* 2013., Hakem *et al.,* 2013., Mund *et al.*, 2017.) Studies have confirmed the prevalence of high concentrations of levamisole residues in liver of broiler chickens as compared to other body tissues, including thigh muscles, due to the lipid-soluble nature of the drug. The deposition of oxytetracycline and chloramphenicol residues in substantially higher amounts was reported in the livers and kidneys of broilers. Similarly, higher concentrations of ciprofloxacin and enrofloxacin residues in livers and kidneys of broiler chickens, as well as lower concentrations of flumequine have also been detected (Mund *et al.*, 2017).

In poultry farming, several varieties of pharmaceuticals are used, including antibiotics. However, the irrational using and no respect for the withdrawal periods can lead to residues in meat (Hakem *et al.*, 2013). In addition, the misuse of antimicrobials may select multi-resistant pathogenic strains of bacteria, which may be transmitted to crocodilians consuming the chicken carcasses.

## 2.6 Risk of feeding poultry mortalities to crocodiles

A poor diet in crocodiles will lead to decreased growth, gastrointestinal tract disorders (diarrhoea and/or poor feed intake), bone and teeth pathology and reduced immunity to disease (J G Myburgh, S van der Woude & J C A Steyl, 2018, personal communication). It is especially important to strictly adhere to a high-quality protein and mineral-rich feed for hatchlings and juveniles less than one year old, as they are highly susceptible to diseases (Brien *et al.*, 2010).

Chicken carcasses used as crocodile feed, differ in states of decomposition - ranging from fresh to severely decomposed. Carcass “quality” has become a concern to commercial crocodile farmers, with many farmers questioning the value of these severely decomposed carcasses, with some stating that feeding of severely decomposed chicken carcasses to crocodiles makes them sick. The pathogens present in these carcasses, their numbers and the species involved are generally unknown to the crocodile industry and pose a potential risk.

Pathogens associated with poultry like *Salmonella spp.* are known to cause enteritis in reptiles and although *E. coli* are abundant in the gastrointestinal tract of crocodiles, they are also opportunist that will cause infection if ingested in large numbers. *Staphylococcus spp.* are known to cause abscesses in reptiles (Huchzermeyer, 1997).

Like many other animals, especially poultry, some pathogens carried by crocodilians may be potential zoonotic diseases for humans (e.g. *Salmonella, Trichinella*) (Manolis and Webb, 2006). Some crocodile farms mix chicken heads into the ration. However, anecdotal evidence suggested that hatchling mortality caused by *Salmonella spp.* may increase when chicken heads are used (Isberg, 2007).

For crocodile meat, there is a distinct possibility of contamination with *Salmonella spp.*, depending on housing management, feed storage and preparation, slaughter technique and hygiene practices on the crocodile farm. Under stressful conditions the

salmonellae can invade the visceral organs and, from there, other tissues. In young crocodiles, this may cause mortality. Similarly, and particularly under handling stress, the pathogens can invade the meat of crocodiles before slaughter and faecal contamination can occur post-slaughter. *Chlamydia* infections are common on some crocodile farms in southern Africa (Huchzermeyer, 1997).

Pinheiro and Lavorenti (2001) reported that caimans, especially the ones fed chickens, performed well, but the use of an antimicrobial agents such as the zinc bacitracin or olaquindox, added to the vitamin mixture of crocodilians (to get extra growth) is a concern. It is especially important to strictly adhere to a high protein and mineral-rich diet for hatchlings and juveniles less than one year, as they are highly susceptible to diseases (Brien *et al.*, 2010).

The use of chicken carcasses by crocodile farms may pose a high risk, regarding antimicrobial residues. Mortality of chickens before the end of the withdrawal period pose as a risk due to accumulation and prevalence of antimicrobials in the different internal organs and tissues, on the skin and in the feathers, and in the gastrointestinal tract of carcasses. A younger chicken carcass (mortality before slaughter age) may have a higher probability of containing antimicrobials. A normal withdrawal period for these pharmaceuticals is usually only instituted just before slaughter. Most chicken carcasses are usually fed to crocodiles (except for the severely decomposed carcasses), irrespective if the chickens received medicated feed, or not, at the time of death. Factors contributing to this risk are, firstly, the of the chicken and secondly, the type of pharmaceutical used. Drugs commonly used in the poultry industry include tetracycline, doxycycline and zinc bacitracin. Antimicrobial residues in chicken carcasses pose as a threat to the commercial crocodile industry, especially commercial farms who sell meat.

## 2.7 Hazard identification and risk assessment

A hazard is any natural or man-made substance, chemical, physical or biological agent, that is capable of causing an adverse health outcome in certain circumstances. Risk is an estimate of the effect of an adverse health outcome when exposed to a hazard (Chartres *et al*., 2019).

Risk Assessment is one of three components of Risk Analysis, the others being Risk Management and Risk Communication. *Risk Assessment* is the measurement of risk and the identification of factors that influence it. *Risk Management* is the development and implementation of strategies to control that risk, and *Risk Communication* is the exchange of information relevant to the risk among interested parties. The role which hazard identification plays in *Risk Assessment* can be seen in Figure 2.7.1 below.

In the terminology adopted by the OIE (World Organization for Animal Health), the first step in a *Risk Assessment* is called '*hazard identification*'. Because the *International Animal Health Code* is focused on trade, hazard identification is defined as '*the process of identifying any pathogenic agents which could potentially be introduced in the commodity considered for importation*'. However, the risk analysis is equally applicable to other areas of decision making, such as those affecting disease surveillance or control programmes, therefore hazard identification is merely the step of identifying what it is that might go wrong in whatever activity being considered (MacDiarmid and Pharo, 2003).

There are a number of challenges in conducting hazard identification and risk assessment of environmental hazards that are distinct from assessments of the effectiveness of clinical interventions. The causal chain linking of harmful substances with adverse outcomes is complex, with various interactions and often considerable time periods between exposure and effects. Hazardous substances may be comprised of many toxic components, with various interactions amongst them, making it difficult to identify the precise toxic component that causes an adverse health outcome. There is no one single measurement to assess the association of a harmful substance and an adverse outcome (Chartres ***et al***., 2019)

The OIE Working Group on Informatics and Epidemiology drafted advice on the way in which Veterinary Services should conduct risk analysis. As a range of different skills are required to perform the different components of a risk analysis adequately, the Group recommended that a team approach be adopted. An animal health import risk analysis requires the expertise of the epidemiologist, with his or her understanding of the patterns of disease. Depending on the commodity being considered, the analysis may also require the specialized skills of virologists, microbiologists , parasitologist and toxicologists. In some instances, it may be necessary to seek advice from experts

as diverse as climatologists, entomologists, wildlife experts, industry technologists, statisticians and economists. It is unlikely that all this expertise can be incorporated into a single risk analysis unit, even in the most developed countries. It follows then, that each major risk analysis should be treated as a project, and people with the necessary skills should be integrated into the project team as appropriate (MacDiarmid and Pharo, 2003).

Risk assessment may be qualitative, in which cases the likelihood of the outcome, or the magnitude of the consequences, is expressed in terms of such as '*high*', '*medium*', or '*low*', or it may be quantitative. In quantitative risk assessments the likelihood is expressed in terms such as '*one disease introduction in 100 years of trade*' or '*failure to correctly identify one diseased herd out of 100*'. Both qualitative and quantitative approaches to risk assessment are valid and, in fact, every risk assessment must first be conducted qualitatively. Only if further insight is required is it necessary to attempt to quantify the risk. Risk analysis is best used to develop insights, and not to develop numerical results which might mistakenly be considered to be highly precise. The discipline of numerical calculation can help to sharpen thinking about risks involving high levels of complexity and uncertainty, and thereby enable conclusions to be drawn which could not have been reached solely on the basis of qualitative reasoning (MacDiarmid and Pharo, 2003).

In this study, the possible hazards investigated for in poultry carcasses used as feed in commercial crocodile farming were; 1) bacterial quality of carcasses in various stages of decomposition and 2) antibiotic residues derived from the agricultural industry. The hazard identification done in this study can be used as the first step in a

risk assessment focusing on the hazards identified and the possible affect they might have on the crocodiles or commercial crocodile industry.

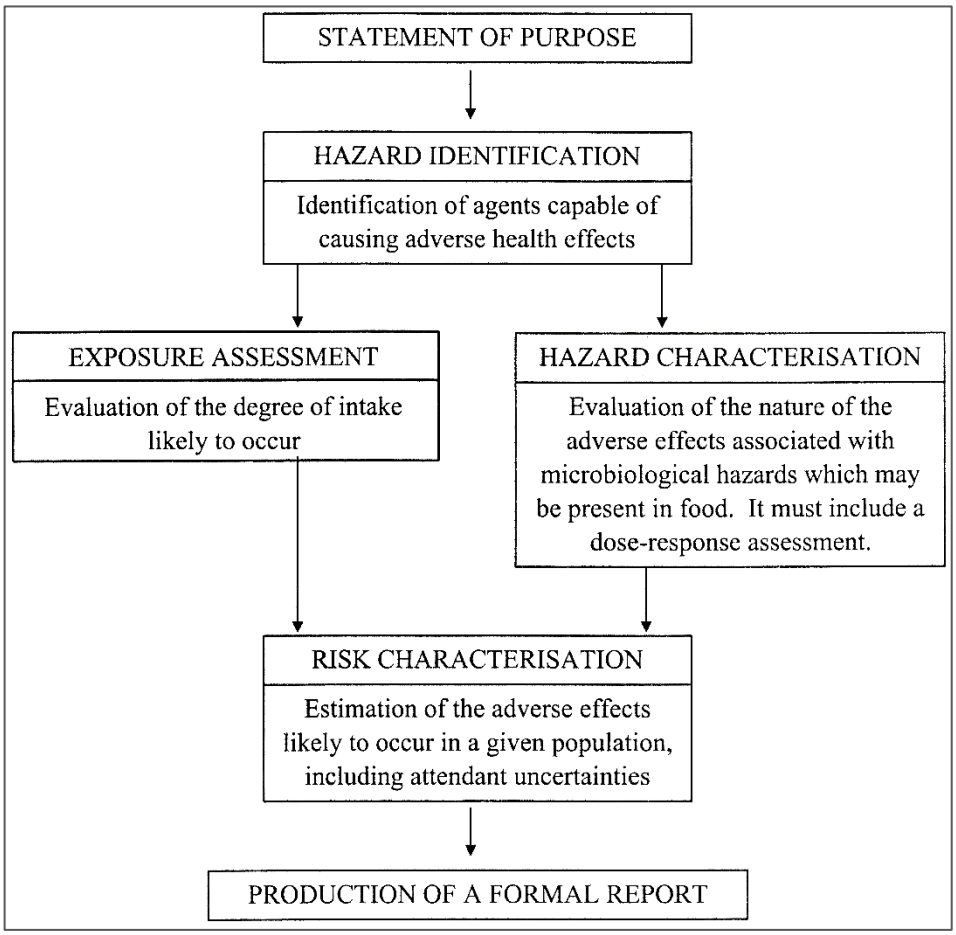

**Figure 2.7.1: Risk Assessment scheme for foodborne microbiological hazards (European Commission, 1997).**

## CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Experimental design

This project was designed to address the 5 Objectives listed below:

### 3.1.1 Decomposition trial (Objective 1)

Chicken carcasses were used to study the decomposition of poultry mortalities, left in a standard environmentally controlled chicken house, by determining the bacterial load at specific intervals after death, as well as muscle pH and temperature changes.

### 3.1.2 Multi-residue screening for antibiotic residues (Objective 2)

Samples were collected from processed chicken carcasses (minced) on 5 crocodile farms. These tissue samples were tested for pharmaceutical drug residues.

### 3.1.3 Carcass evaluation method (Objective 3)

A practical chicken carcass evaluation method was developed to evaluate the state of decomposition of chicken carcasses on crocodile farms. The results and on-farm observations from the: decomposition trial (Objective 1); on-farm investigations (Objective 2); and completing the crocodile farm questionnaires (Objective 4) were used to develop a practical chicken carcass evaluation method for crocodile farmers.

### 3.1.4 Questionnaire and on-farm hazard identification (Objective 4)

Farmers or managers of commercial crocodile farms were interviewed and a questionnaire completed. In addition, an on-farm investigation was done to identify specific hazards, specifically related to the storage, preparation and feeding of chicken carcasses.

### 3.1.5 Management plan for feeding of chicken carcasses to crocodiles (Objective 5)

The results of Objectives 1 and 2, together with the on-farm feeding evaluation and hazard identification (Objective 4), and the chicken carcass evaluation method (Objective 3) were used to develop a specific *management plan for feeding chicken carcasses to crocodiles*.

## 3.2 Location of study sites

The following section provides the locations of the farms or facilities used for: the decomposition trial, the storage, processing and sampling of carcasses for the microbiological assay (Objective 1); collecting of the multi-residue tissue samples (objective 2); and who participated in the carcass evaluation investigation (Objective 3), on-farm hazard identification and completing the questionnaire (Objective 4). The crocodile farms are located in the Gauteng, North-West and Limpopo Provinces of South Africa.

### 3.2.1 Carcass decomposition study (Objective 1)

The decomposition trial was performed at the Experimental Farm of the University of Pretoria. The chickens used for this study came from another poultry research project (AEC EC040-18) and were housed in the UP-Poultry Unit.

The University of Pretoria abattoir was used for the storage, processing and sampling of the chicken carcasses.

**Figure 3.2.1.1: UP Poultry Unit, University of Pretoria Experimental Farm.** The location where the decomposition trail was conducted.

3.2.2 Locations of crocodile farms used for: chicken tissue sample collection and carcass evaluation; as well as crocodile farm questionnaire, on-farm investigation and hazard identification

The crocodile farms used for the collection of chicken carcass tissue samples for multi-residue screening (Objective 2), carcass evaluation (Objective 3), hazard identification and taking part in the questionnaire surveys (Objective 4) were located in the North-West and Limpopo Provinces. Only the locations of the crocodile farms (district or nearest town) were reported to protect the identity of the specific crocodile farms.


**Figure 3.2.2.1: Locations of crocodile farms visited.** At the farms chicken tissue samples were collected, chicken carcasses evaluated, on-farm hazard identification done and questionnaire completed.

| Locations of crocodile farms | Questionnaire completed | Carcass evaluation | Number of residue samples collected |
|---|---|---|---|
| 1. Naboomspruit, Limpopo Province | Yes | Yes | 3 |
| 2. Brits, North West Province | Yes | Yes | 3 |
| 3. Hartbeespoort, North West Province | Yes | Yes | 3 |
| 4. Bela-Bela, Limpopo Province | Yes | Yes | 3 |

| | | | |
|---|---|---|---|
| 5. Letsitele, Limpopo Province | Yes | Yes | 3 |
| | | **Total** | **15** |

**Table 3.2.2.1 Locations of crocodile farms visited.** At the farms chicken tissue samples were collected, chicken carcasses evaluated, on-farm hazard identification done and questionnaire completed.

## 3.3 Study animals, procedures and sample collection

### 3.3.1 Decomposition trial (Objective 1)

#### a. Chicken carcasses used for decomposition trial

Fifty-four chicken carcasses were obtained from an UP-research project AEC EC040-18. Only birds from the control group were used for this study to prevent any of the pharmaceuticals having an influence on the decomposition of the chickens. The control birds (EC040-18) did not receive any medicated feed. Carcasses were placed randomly in different pens in the Poultry House after humane killing.

#### b. Decomposition trial and chicken carcass management

The objective of this decomposition trial was to simulate the conditions broiler carcasses (n=54) would be exposed to after death in an environmentally controlled poultry housing unit. Commercial poultry housing units are routinely checked by workers to collect mortalities; this is either done at 6, 12- or 24-hour intervals. Carcasses are usually stored in freezer rooms after collection or transported immediately to nearby crocodile farms.

For this investigation, the Poultry Unit was not sterilized before the decomposition trial started with each bird placed on the same type of litter. The housing unit maintained a steady temperature of 24 - 25$^0$C, 55-60% RH and 1.34m$^3$/h/bird ventilation. Each group (I - VI) represented a certain stage of decomposition (in-house exposure) (Table 3.3.1.1), while each sub-group (A and B) represented the composition of the tissue samples (Table 3.3.1.2).

Birds were weighed the day before (n = 54) to select birds of equal weight and size (1.7 - 1.9 kg) and were kept in specific pens for less than 24 hours before the start of the decomposition trial. After euthanasia, by cervical dislocation, the carcasses were placed, randomly, in pens in the middle of the housing unit. Carcasses collected directly after euthanasia (exposed to poultry house conditions for 0 hours), were marked as the FRESH group. The other carcasses were kept in the poultry unit for 6, 12, 18, 24 and 36 hours (Table 3.3.1.1), with carcasses from the last group (SEVROT) exposed to 25°C and 60% RH for 36 hours. Nine carcasses were randomly collected once the different exposure times (Table 3.3.1.1*9-) were reached. The time periods (exposure time) that the carcasses were left in the poultry house are given in Table 3.3.1.1.

| Group | Group identification | Number of carcasses collected per group | Exposure time (hours) in house for each group |
|---|---|---|---|
| I | FRESH | 9 | 0 |
| II | CAR1 | 9 | 6 |
| III | CAR2 | 9 | 12 |
| IV | CAR3 | 9 | 18 |
| V | CAR4 | 9 | 24 |
| VI | SEVROT | 9 | 36 |
| | **TOTAL** | 54 | |

**Table 3.3.1.1: Time period of carcasses collected after exposure time in poultry unit.** Groups: I=0h, II=6h, III=12h, IV=18h, V=24h, VI=36h

The pH and temperature of each carcass were recorded by making an incision in the pectoral muscle (± 3 cm deep) of the carcass and inserting the probe of the portable meat pH and temperature meter (HANNA portable meat pH/temperature meter: HI 99163 with PVDF body FC232D amplified pH electrode and FC099 removable

stainless steel blade) into the muscles (Fig 3.3.1.1). A reading was recorded once the probe displayed the "*stable*" sign. The probe was cleaned with alcohol wipes after each carcass was evaluated. Scalpel blades and gloves used for each carcass were disposed after use. Whilst recording carcass pH and temperature, each carcass was examined (skin colour, onset of rigor mortis and any distinct pathophysiological changes). After the pH and temperature recordings were completed, the carcasses were placed in sterile bags, per group, and transported to the abattoir freezer room for storage at -12$^{o}$C. The HANNA portable meat pH/temperature meter was calibrated before each group of carcasses (Table 3.3.1.1) was tested.

**Figure 3.3.1.1: Carcass pH and temperature recordings during decomposition trial.** Each carcass collected during the decomposition trial was recorded for carcass pH and temperature after the designated time intervals (FRESH=0h, CAR1=6h, CAR2=12h, CAR3=18h, CAR4=24h, SEVROT=36h).

Materials used for carcass collection, pH and temperature recording, storage and marking of groups:

- HANNA meat pH meter HI 99163 with PVDF body FC232D amplified pH electrode and FC099 removable stainless-steel blade
- Two scalpel handles
- Fifty-four scalpel blades for incision of pectoral muscles
- Sterile bags for storing carcasses in freezer

- F10 Aerosol Disinfectant spray for sterilizing working area
- 100 latex gloves and 2 facemasks
- Stopwatch for timekeeping
- Insolation tape for marking groups and carcasses
- Permanent marker for labelling
- Scale

**c. Collecting samples for bacteriology testing in decomposition trial**

Carcasses were stored in the abattoir freezer room at -12$^{o}$C for at least 24-hours before processing for sampling. In most cases, the carcasses were kept for several weeks in the freezer room. Carcasses were only processed once the Food Microbiology Laboratory of the Department of Consumer and Food Science was ready to receive the tissue samples. Only one group was processed at a time, one group every week to prevent cross-contamination between groups and due to the laborious task to prepare each group for laboratory testing. Due to the budget available for bacteriology testing, the carcasses were grouped as shown in Table 3.3.1.2.

| **Group** | **Carcasses grouped before evisceration** | **Subgroup A** Sample 50g | **Subgroup B** Sample 50g | **Date sampled** |
|---|---|---|---|---|
| FRESH n=9 | 3 | GIT 1 | MBF 1 | 05/10/2018 |
| | 3 | GIT 2 | MBF 2 | |
| | 3 | GIT 3 | MBF 3 | |
| CAR1 n=9 | 3 | GIT 1 | MBF 1 | 29/10/2018 |
| | 3 | GIT 2 | MBF 2 | |
| | 3 | GIT 3 | MBF 3 | |
| CAR2 n=9 | 3 | GIT 1 | MBF 1 | 03/11/2018 |
| | 3 | GIT 2 | MBF 2 | |
| | 3 | GIT 3 | MBF 3 | |
| CAR3 n=9 | 3 | GIT 1 | MBF 1 | 09/11/2018 |
| | 3 | GIT 2 | MBF 2 | |
| | 3 | GIT 3 | MBF 3 | |
| CAR4 n=9 | 3 | GIT 1 | MBF 1 | 16/11/2018 |
| | 3 | GIT 2 | MBF 2 | |
| | 3 | GIT 3 | MBF 3 | |
| SEVROT n=9 | 3 | GIT 1 | MBF 1 | 23/11/2018 |
| | 3 | GIT 2 | MBF 2 | |
| | 3 | GIT 3 | MBF 3 | |
| **TOTAL** | 54 carcasses | **18 samples** | **18 samples** | **36 samples** |

**Table 3.3.1.2: Grouping of carcasses for representative samples.** Nine carcasses in each group (n=54) were divided into 3 groups for evisceration, mincing of GIT (gastrointestinal tract) and MBF (eviscerated carcass) for sample collection.

Before further processing and mincing, the carcasses were grouped into three carcasses per sample. Eviscerated carcasses were minced (sub-group B) together, representing Muscle+Bone+Feather (MBF). The 3 gastrointestinal tracts (GITs) (sub-group A) were minced separately. Tissue samples were collected from sub-groups A and B and properly marked.

Processing of the carcasses for sampling was done in the following order:

i. Carcasses were removed from the abattoir freezer at 06:00 to thaw for at least 6-hours in sterile plastic bag before eviscerating. Carcass temperature was recorded hourly with infrared thermometer (Smart Sensor AR360A+ Infrared Thermometer Digital Non-Contact Lazer). Processing started when the average carcass skin temperature was >15$^{o}$C.
ii. The carcass mincer was sterilized before processing and a separate small mincer for the gastrointestinal tracts. This was done by soaking the mincer instruments overnight in a bleach solution and washing with disinfectant soap and coarse salt scrub on the day of sampling. Instruments were left to dry while recording carcass temperature during thawing.
iii. The workspace area for dissecting carcasses was cleaned with F10 disinfectant spray.
iv. Zip-lock bags were laid out and labelled in correct sampling order for sample collection.
v. 12:00. Three carcasses were eviscerated by opening the abdominal and pleural cavity via cross-sectional incisions with scalpel. The gastrointestinal tract was collected from crop to rectum without puncturing the surrounding organs. Each carcass was eviscerated with a new and sterile scalpel blade and latex gloves.
vi. The 3 gastrointestinal tracts were minced together and collected by a sterile plastic spoon until the samples weighed 50g. Zip-lock bags sealed, and samples placed in polystyrene cooler in fridge.

vii. The three eviscerated carcasses were minced together in the carcass mincer and samples were collected with sterile spoons until it weighed 50g.
viii. Samples were placed in cool-box and in abattoir fridge until rest the of samples in the group were processed and collected.
ix. Mincers were washed and sterilized before processing the next carcasses in the group that were kept at 10ºC in abattoir fridge.
x. Once all the samples were collected, they were stored in the abattoir fridge while cleaning and sterilizing the workspace.
xi. Samples were transported to the Department of Food and Consumer Sciences' Food Microbiology Lab for analysis at +- 16h00.

A total of 36 samples were submitted for bacteriology testing, 18 GIT and 18 MBF samples (Table 3.3.1.2). Eviscerating the carcasses and testing the GIT and MBF samples separately were done to see whether the bacterial load differed between the GITs and rest of the carcasses (MBF).

The materials used for processing and collecting samples:

- Fifty scalpel blades for evisceration
- Two scalpel handles
- One hundred latex gloves
- Two facemasks
- Thirty-six Zip-lock bags for samples
- Thirty-six plastic disposable spoons for sample collection
- One hundred alcohol swabs
- Weighing scale
- F10 disinfectant spray to clean working area
- Coarse salt and steel wool brush for cleaning mincers
- 3.5% m/v Sodium Hypochlorite solution (NaClO) for overnight soaking of mincer parts
- Wolfking® Frozen Meat Grinder C 300 FBG (Carcass mincer)
- OKTO meat mincer (Eviscerated carcasses GIT)
- Infrared thermometer Smart Sensor AR360A+ Infrared Thermometer Digital Non-Contact Lazer

- Polystyrene cool-box for transporting and storing samples below 5ºC

3.3.2. Screening for antibiotic residues (Objective 2)

**a. Chicken carcasses used for residue screening**

Three tissue samples for laboratory analysis were collected from 5 different farms totalling 15 samples to be screened for antibiotic residues. Three representative samples were collected per farm, in 50 ml test tubes, from minced chicken carcasses prepared on that specific day as crocodile feed by the farm. The tissue samples were collected before any pre-mixes or other nutrients were added.

It was decided to test for residues using minced whole carcasses, because most farmers prefer to prepare the chicken carcasses in this way. We were concerned that pharmaceuticals may be concentrated in-or-on the feathers of these birds. By only testing livers samples, this additional source of pharmaceuticals for crocodiles might have been missed.

**b. Chicken carcass tissue sample collection and testing**

Chicken carcass samples were collected from 5 commercial crocodile farms that use chickens as their main protein source. Samples were collected, at random times during feed preparation process, before any additives were added. Three samples were collected, per farm, by removing at least 20 gr (per sample) of the processed material from the mincer bin (mixture of different chicken carcasses). Each sample was collected with a new latex glove at random times during feed processing. Each sample was sealed in a 50 ml laboratory test tube and transported, on ice, in a polystyrene box to the Faculty of Veterinary Science, Onderstepoort. On arrival, the 50 ml tubes were stored in a chest freezer until testing.

The source of the chicken carcasses and age, and the specific feeding schedules of the various crocodile farms were observed and noted during the on-farm investigation.

The 15 frozen tissue samples (in 50 ml test tubes) were transported to the Food & Drug Assurance Laboratories (Food & Drug Assurance Laboratories (Pty) Ltd, Reg No.:2007/010792/07, Cnr Justice Mohammed and Alexander Street, Brooklyn,

Pretoria, South Africa) for testing, as soon as all the samples were collected from the 5 crocodile farms.

Materials used for collecting poultry samples on farms:

- Thirty latex gloves and facemasks
- Permanent marker for labeling
- 15 x 50 ml collection tubes
- Polystyrene cooler and gel packs

### 3.3.3 Chicken carcass evaluation method (Objective 3)

**Developing the carcass evaluation method**

During the decomposition trial (Objective 1), carcasses were observed for distinct physical changes (subjective) that could be used for the development of a practical carcass evaluation method. Muscle pH were evaluated as a potential parameter to monitor the changes in the carcasses after death. The recorded pH values were compared with the physical changes of the carcasses. Each carcass was studied to find possible post mortal changes to be used in the classification of chicken carcases on crocodile farms in different decomposing groups.

The most prevalent and distinguishable changes noted in the clinical trial (Objective 1) were used for the on-farm carcass evaluation (to determine whether carcasses were fit for use (fresh) or not (severely rotten)). Practical parameters were identified to be used for the carcass evaluation method (Objective 3), as workers on crocodile farms need to be able to use this system during processing. For the practical carcass evaluation on the crocodile farm we focused on skin colour changes and a Pull test.

The Pull test (deterioration test) idea was first evaluated on 50 carcasses, in several stages of decomposition, to see if it can be used as a rapid and effective way of testing carcass deterioration. Carcasses used to develop the Pull test were tested on a nearby crocodile farm (Brits), as the carcasses in the Decomposition trial (Objective 1) needed to stay intact for sampling.

The Pull test is a simple test of deterioration that follows after skin colour observation and is only tested on carcasses observing a green/blue colouration. By gripping the carcass legs and pulling it away from each other, deterioration was confirmed when the carcass was pulled apart (not-resist) and not confirmed when the carcass remained intact (resist). The aim of the Pull test is not to split the carcass open, but to test whether the carcass will deteriorate when subjected to the test. The Pull test was confirmed to be effective in distinguishing carcass deterioration of fresh and severely rotten carcasses and could be used in the carcass evaluation, together with carcass skin colour.

Chicken carcass observations and evaluation were made whilst visiting the 5 crocodile farms for sample collections, focusing on the storage, handling, processing and the general state of decomposition of the carcasses.

Skin colour and pull test were classified as:

- Colour: red (fresh); yellow (partially fresh); green (rotten)
- Pull test: resist (fresh); no-resist (rotten)

### 3.3.4 Crocodile farm: questionnaire, investigation and hazard identification (Objective 4)

**a. Crocodile farm questionnaire**

The most likely answers are in brackets.

**Chicken carcass information**

1. How many chicken farms supply carcasses to you and from where? (number)
2. Do the carcasses consist mainly of broiler or layer birds? (broilers of layers)
3. Are carcasses from different sources mixed or separated? (mixed or separated)
4. Do you receive chickens weighing? (yes or no)
    a. <0.7 kg
    b. 0.7-1.5kg
    c. >1.5kg

5. How regularly are the carcasses delivered or collected? (period: days or weeks))

**Sources, transport and storage of carcasses**

6. Are the carcasses delivered/collected frozen or unfrozen? (frozen or fresh)
7. How are the carcasses stored and for how long before being fed? (how stored and period)

**Chicken carcass management on the crocodile farm**

8. Are the carcasses classified, based on the stage of decay, before being processed? (yes or no)
9. Are the carcasses minced before feeding? (yes or no)
10. How much chicken, in kg's, is given per croc/pen a week or day? (feeding schedule) (kg)
11. How are the carcasses prepared (whole, disembowelled, skinned, feathers on, etc.) before mincing or feeding? (whole, disembowelled, skinned, feathers on, etc.)
12. If the carcasses are prepared before feeding, how is it done and the reason why? (how and why)
13. If another protein feed source is used, please state the reason why? (yes or no; why)
14. List the most common feed related diseases observed in the crocodiles on the farm. (list the diseases)
15. How regularly are the pens cleaned? (days)
16. Have any of the workers ever fallen ill due to the handling the chicken carcasses? (yes or no)
17. If any pre-mix is added and how much per kilogram of feed? (premix management)

The aim of the questionnaire was to gather information regarding the sources of chicken carcasses, transport, storage, preparation, processing and feeding to growers. This information, together with the *on-farm investigation* and *carcass*

*evaluation* was used to identify possible hazards. Every farm (n=5) visited for the multi-residue tissue sample collection and on-farm evaluation, completed the questionnaire.

**b. On-farm investigation and hazard identification**

The crocodile farms visited for chicken carcass evaluation and tissue sample collection were also used for the on-farm hazard identification. Although the two most important hazards that we identified before commencing with this study (see Literature Review), were carcass quality (bacterial decomposition) and antimicrobial residues, and on-farm investigation was done to try and identify other potential hazards.

The experience gained from Objective 3 was used to evaluate the quality of the chicken carcasses used on commercial crocodile farms. In addition, the questionnaire completed on the farm (probability of antibiotic residue in chicken carcasses) was used to identify specific hazards associated with sources, methods of transport, storage and processing of chicken carcasses. After gathering the information from the questionnaire, the investigation observed and noted the vehicles used for transport, the facilities used for storing carcasses, followed by the method of processing carcasses and finally carcass evaluation.

The information gathered from the questionnaire, together with the observations recorded and carcass evaluation, were used to compare the overall carcass quality between farms, i.e. identifying possible hazards associated with a specific system used (e.g. no refrigerated transport from a source >100km), thus higher amount of blue (rotten) carcasses observed in carcass evaluation.

**c. On-farm carcass evaluation**

After the questionnaires were completed, the feeding routine and general practices on the 5 commercial crocodile farms were observed, as well as the storage facilities. Our practical evaluation method, which was developed, was used to inspect 30 chicken carcasses, available that day (before processing) on each of the 5 farms. Skin colour changes and the Pull test were used to estimate the stages of decomposition.

Skin colour observations were conducted first, selecting red, yellow/green and green/blue skin colour carcasses. The colour of the skin covering the abdominal cavity

was most often used. After 5 carcasses were confirmed for each skin colour group a pull test was performed on each carcass to determine the deterioration.

The Pull test was executed by pulling the two carcass legs away from each other, by hand. Deterioration was confirmed once the carcass was pulled apart and not confirmed when resisting. The Pull test was done by only one person the investigation. Carcasses with no resistance were classified as rotten. After the evaluation all the carcasses were processed by the farm workers as routinely done on the farm.

## 3.4 Design of a practical management plan for feeding chicken carcasses to crocodiles (Objective 5)

The management plan for crocodile farmers feeding chicken carcasses was based on the observations made during the on-farm investigations (Objective 4), information coming from the crocodile farm questionnaires, the results of the decomposition trial (Objective 1) and residue tests (Objective 2), as well as the experience gained during the development of the chicken carcass evaluation method (Objective 3).

## 3.5 Laboratory testing of collected samples

### 3.5.1 Microbiological analysis

The tissue samples were collected, prepared and numbered as discussed before and summarised in Table 3.3.1.2. A total of 36 tissue samples were submitted to the Food Microbiological Laboratory for microbiological analysis.

To determine the most prevalent bacterial species within the samples, three different colonies (visual discrimination) were randomly picked from the highest dilution standard plate count agar petri-dish. Gram stain and catalase tests were done. Isolates were identified to species level with the Omnilog® Data Collection Software Identification System version 2.1 (Biolog Inc., Hayward, Calafornia) following prior isolation on plate count agar and subsequently on Biolog Universal Growth medium (BUG) (Biolog). Bacterial counts log CFU/g were determined by the most probable number method.

Bacterial pathogens (e.g. *E. coli, S. aureus, Listeria monocytogenes* and *Salmonella spp.)* were identified and enumerated. Direct plating (0.1 mL) of serially diluted samples onto respective enumeration and identification agar was done for *S. aureus*. To identify presumptive *E. coli*, positive tubes containing EC broth were streaked onto Eosin Methylene Blue Agar. Enrichment of *L. monocytogenes* was done using half Fraser and Full Fraser media with contents from each medium subsequently streaked onto Oxford and Palcam selective agars after incubation. Enrichment of *Salmonella spp*. was done using buffered peptone water, *Salmonella* enrichment broth and Selenite Cysteine broth.

The *Salmonella spp.* positive samples were serotyped at the Microbiology Laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, University of Pretoria.

3.5.2 Multi-residue screening

Samples were stored at -5ºC before being taken to Food & Drug Assurance Laboratories for multi-residue screening following the liquid chromatography – mass spectrometry technique (LCMS/MS). LC-MS/MS is a chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (Food & Drug Assurance Laboratories website). LC-MS/MS is a powerful technique used for many applications that has a very high sensitivity and selectivity. Generally, its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture).

LCMS/MS instrumentation

The LC–MS/MS system consisted of a Nanospace SI-2 (Shiseido, Tokyo, Japan) and API 4000 Q trap tandem mass spectrometer (Applied Biosystems, Foster City, CA). Analyst 1.4.1 software was used to control the instruments and process data. The analytical column was a Capcell Pak $C_{18}$ (5 µm, 2.0 mm inner diameter × 150 mm; Shiseido, Japan) which was operated at 40 °C. The mobile phase consisted of (A) 10 mM ammonium formate and 1 vol.% formic acid in water and (B) methanol. A linear gradient programme was applied as follows: a mixture of 95% (A)/5% (B) for 2 min; increased to 100% (B) from 2 to 5 min; then the system was held for 4.5 min, returned

to 95% (A)/5% (B) over a period of 1 min and equilibrated to 5 min. The mobile phase was pumped at a flow rate of 0.3 ml/min. The electrospray-ionisation (ESI) source was operated in the positive mode. Data acquisition for identification was performed by working in the multiple reaction monitoring (MRM) mode. The operating MS parameters were optimised to be as follows: curtain gas, 20 psi; ion spray voltage, +5500 V; collision gas, nitrogen; and source temperature, 450 °C.

Mixed poultry matrix preparation

Acetonitrile (6 mL) was added to each 15 mL polypropylene tube containing 2 g mixed poultry muscles. The tubes were tightly sealed and shaken sideways vigorously and centrifuged at approximately 1650*g* at 4 °C for 5 min. The samples were placed for 30 min at −20 °C and the supernatant solution (6 mL) was transferred to glass tubes. Acetonitrile was evaporated under a nitrogen stream in a water bath at 50 °C and then a 0.2 M phosphate buffer solution (3 mL) was added and the contents were mixed.

Validation procedure

The analytical method in this study was validated according to Codex guidelines for the establishment of a regulatory programme for control of veterinary drug residues in foods CAC/GL 16-1993 concerning the performance of analytical methods of veterinary drug residues (Codex Alimentarius volume 3,1993). The limit of detection (LOD) and limit of quantification (LOQ) were based on the standard deviation of the response and the slope of the calibration curve of the analytes. A specific calibration curve was obtained using samples, containing an analyte in the range of the lowest calibration concentration. The standard deviation of the *y*-intercepts of the regression lines may be used as the standard deviation. All samples were prepared in triplicate.

The samples were screened for the following 15 antibacterial compounds used in the agricultural industry: Chlortetracycline, Doxycycline, Oxytetracycline, Tetracycline, Ciprofloxacin, Norfloxacin, Enrofloxacin, Lincomycin, Olaquindox metabolite, Sulfadiazine, Sulfadimidine, Sulfamethoxazole, Tiamulin, Trimethoprim and Tylosin.

## 3.6 Data analysis

### 3.6.1 Statistical analysis of bacterial concentrations

A total of (n=36) samples were submitted for microbiological analysis comprising out of 2 sub-groups, namely (GIT) and (MBF), containing 18 samples per group.

Mean bacterial counts for *E. coli* were expressed as $\log_{10}$ colony-forming units per gram ($\log_{10}$ CFU/g). Means were calculated by group average. One-way analysis of variance (ANOVA) was performed between group means plated for *E. coli* at a confidence interval of 5% following the GLM procedure of SAS. A Fisher's protected least significance difference test was done comparing mean values in groups and sub-groups. *Staphylococcus aureus* was not detected at lowest dilution set log at $\log10^{-1}$ CFU/g in 4/6 groups, thus the data was transformed to a binary form where values equal to 0 were not-detected and values greater than 0 were presented as detected. *Salmonella spp.* and *Listeria monocytogenes* were analyzed by a summary of detection in groups and sub-groups as they were qualitatively analyzed as either positive or negative in the samples.

3.6.2 Multi-residue screening

Multi-residue screening was performed on a total of 15 samples collected from 5 different crocodile farms. A summary of detection was used from the laboratory results, indicating if a pharmaceutical was detected or not, as well as the highest concentration of the specific compound detected.

## 3.7 Biological material disposal

Any biological material that was not used or on which experimentation is completed, such as the minced carcasses and microbiological culture plates, were discarded by placing them in acceptable biomedical waste containers and disposed of by the appointed waste disposal company used by the University of Pretoria. We adhered to the following prerequisites and rules received from the waste disposal company:

1. *Waste must be packed in a proper waste container with a red plastic liner inside.*
2. *Medical or pharmaceutical waste must be kept in a safe storage area until the service provider/contractor collect such waste.*
3. *Care must be taken that waste boxes/containers do not leak. No wet boxes will be removed.*

4. *Medical or pharmaceutical waste boxes must be sealed with adhesive tape (bio-hazardous tape).*
5. *Boxes must not weigh more than 15 kg.*
6. *Special care must be taken to ensure the bottom of boxes does not separate.*
7. *No medical waste removal request may be sent directly to the service provider/contractor.*
8. *Only requests sent to the contractor/service provider by the UP Environmental Management Department will be collected.*

# CHAPTER 4

# RESULTS

## 4.1 Bacteriology, pH and temperature of carcasses in decomposition trial

A total of 36 samples were submitted for bacteriology testing, 18 samples comprised of minced gastrointestinal tracts (GIT) and 18 samples comprised of minced muscle, bone and feathers (MBF). All samples were screened for the following bacteria: *Staphylococcus aureus; Salmonella spp.; Listeria monocytogenes;* and *Escherichia coli.* The objective was to identify a group (e.g. CAR4) and/or sub-group (e.g. GIT) with an increase in bacterial load that could be linked to the period the carcasses (different groups) were left in the poultry house.

### 4.1.1 Bacteriology of chicken carcasses

The *S. aureus*, *Salmonella spp.* and *L. monocytogenes* bacterial counts were described using a *summary of detection method*. *E. coli* laboratory results were provided as mean bacterial loads and could be used in a one-way ANOVA in a *general linear model* (GLM). See Table 4.1.1 for a summary of the bacteriology results of the tissue samples collected during the decomposition trial.

| **GROUPS** | **HOURS exposed** | **SUB-GROUPS** | ***E. coli*** logCFU/g | ***L. monocytogenes*** POS/NEG | ***Salmonella hamburg*** POS/NEG | ***Staph. aureus*** logCFU/g | **Avg. pH before freezing** | **Avg. pH before process** | **Avg. Carcass temp before freezing** ⁰ Celsius |
|---|---|---|---|---|---|---|---|---|---|
| FRESH | 0 | GIT | 5 | Pos | Pos | 3.5 | 6.017 | 6.034 | 35 |
| | | MBF | 6.9 | Neg | Pos | 5.4 | | | |
| CAR1 | 6 | GIT | 4.6 | Pos | Pos | 0 | 5.63 | 6.17 | 32 |
| | | MBF | 5.3 | Neg | Pos | 3.5 | | | |
| CAR2 | 12 | GIT | 3.9 | Pos | Pos | 0 | 5.604 | 6.24 | 27 |
| | | MBF | 4.5 | Neg | Pos | 0 | | | |
| CAR3 | 18 | GIT | 4.2 | Pos | Pos | 0 | 5.56 | 6.32 | 24 |
| | | MBF | 5.3 | Pos | Pos | 0 | | | |
| CAR4 | 24 | GIT | 5.4 | Pos | Pos | 0 | 5.65 | 5.50 | 22 |
| | | MBF | 5.8 | Neg | Pos | 0 | | | |
| SEVROT | 36 | GIT | 5.3 | Pos | Pos | 0 | 5.72 | 6.33 | 17 |
| | | MBF | 5.3 | Neg | Pos | 2.9 | | | |

**Table 4.1.1: Summary of the bacteriology results of samples collected during the decomposition trial.** Note 0 representing "not detected at lowest dilution set at $log10^{-1}$ CFU/g.

### 4.1.2 *Staphylococcus aureus*

*Staphylococcus aureus* was not detected at lowest dilution set log at $log10^{-1}$ CFU/g in 4/6 groups, thus the data was transformed to a binary form where values equal to 0 were not-detected and values greater than 0 were presented as detected in Table 4.1.2

| *S. aureus* | | | | |
|---|---|---|---|---|
| **Sub-group** | **Not detected** | **Detected** | **No. tested** | **% detected in sub-group** |
| GIT | 10 | 8 | 18 | 44,44% |
| MBF | 13 | 5 | 18 | 27,78% |
| **Total** | 23 | 13 | 36 | |
| **% = detected/no. tested** | 63,89% | 36,11% | | |

**Table 4.1.2: Summary of the *S. aureus* results.**

### 4.1.3 *Salmonella hamburg*

The *Salmonella* spp. results are summarized in Table 4.1.3. A summary of detection in groups and sub-groups was used.

*Salmonella spp.* was detected in 32/36 samples submitted with sub-group MBF showing the highest level of detection, 94.44%.

Sixteen *Salmonella* samples were serotyped. Eleven of the samples were identified as *Salmonella hamburg*. Two out of the five samples identified as *Pseudomonas aeruginosa*, two samples as *Stenothrophomonas maltophilia* and one as *Proteus mirabilis.*

| *Salmonella spp.* | | | | |
|---|---|---|---|---|
| **Sub-group** | **Not detected** | **Detected** | **No. tested** | **% detected in sub-group** |
| GIT | 3 | 15 | 18 | 83,33% |
| MBF | 1 | 17 | 18 | 94,44% |
| **Total** | 4 | 32 | 36 | |

| | | | | |
|---|---|---|---|---|
| **% = detected/no. tested** | 11,11% | 88,89% | | |

**Table 4.1.3: Summary of the *Salmonella spp.* results.**

### 4.1.4 *Listeria monocytogenes*

*Listeria monocytogenes* was detected in 22/36 samples submitted with MBF (Muscle, bone and feathers) samples showing the highest level of detection, 94.44%. Results for *L. monocytogenes* are summarized in Table 4.1.4.

| ***L. monocytogenes*** | | | | |
|---|---|---|---|---|
| **Sub-group** | **Not detected** | **Detected** | **No. tested** | **% detected in sub-group** |
| GIT | 13 | 5 | 18 | 27,78% |
| MBF | 1 | 17 | 18 | 94,44% |
| **Total** | 14 | 22 | 36 | |
| **% = detected/no. tested** | 38,89% | 61,11% | | |

**Table 4.1.4: Summary of the *Listeria monocytogenes* results.**

### 4.1.5 *Escherichia coli*

Fig. 4.1.5.1 illustrates the mean values of bacterial counts for *E. coli* of GIT and MBF groups over the 36-hour exposure time.

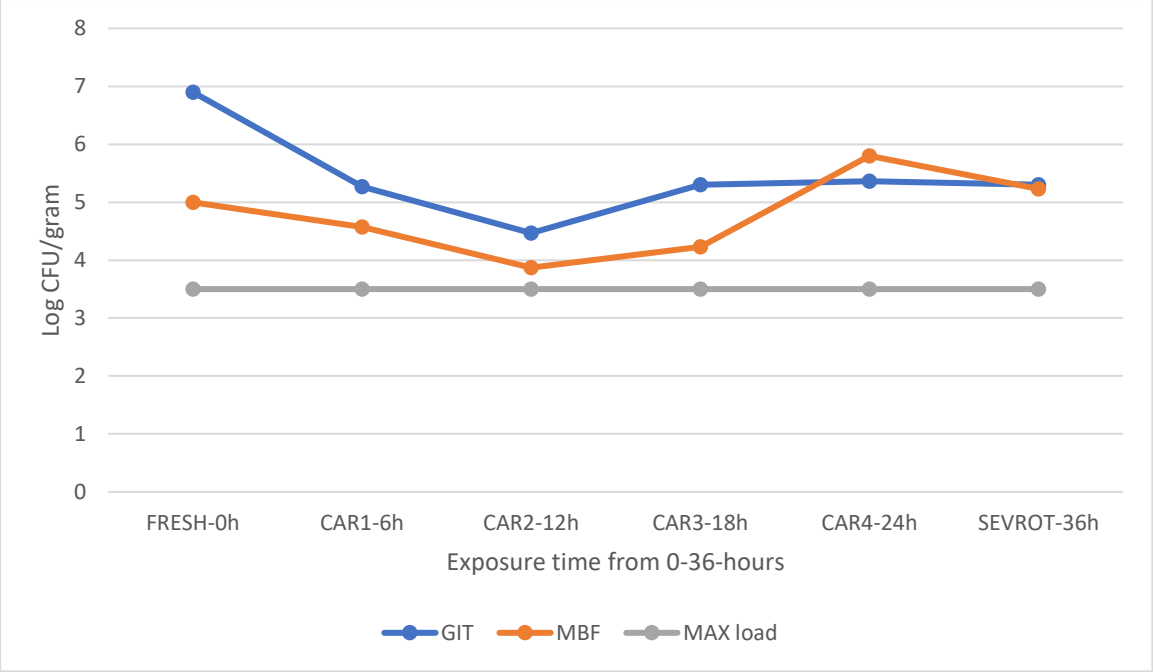


**Figure 4.1.5.1: Group means of bacterial counts for *E. coli* over the 36-hour exposure time.** MAX load represents the maximum log(3.5) cfu/gram of *E. coli* allowed for human consumption.

One-way ANOVA was used to analise whether there is a significant difference between bacterial counts of *E. coli* in groups and sub-groups. A general linear model (GLM) procedure using a factorial design was followed on SAS. One-way ANOVA table illustrated in Table 4.1.5.1.

Analysis of variance

Variate: *E. coli*

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Group | 5 | 11.9958 | 2.3992 | 16.61 | <.001 |
| Sub-group | 1 | 3.8025 | 3.8025 | 26.32 | <.001 |
| Group.Sub-group | 5 | 4.8825 | 0.9765 | 6.76 | <.001 |
| Residual | 24 | 3.4667 | 0.1444 | | |
| Total | 35 | 24.1475 | | | |

**Table 4.1.5.1: One-way ANOVA performed on groups and sub-groups plated for *E. coli* following a GLM (general linear model) procedure factorial design on SAS.**

Mean bacterial counts and standard deviation of *E. coli* in sub-groups GIT and MBF showed in Table 4.1.5.2

| Sub-group **GIT** | | | |
|---|---|---|---|
| | No observed | Mean | s.d. |
| Group | | | |
| FRESH | 3 | 6.900 | 0.4359 |
| CAR1 | 3 | 5.267 | 0.6028 |
| CAR2 | 3 | 4.467 | 0.0577 |
| CAR3 | 3 | 5.300 | 0.1732 |
| CAR4 | 3 | 5.367 | 0.2517 |
| SEVROT | 3 | 5.300 | 0.1000 |
| Margin | 18 | 5.433 | 0.7963 |

| Sub-group **MBF** | | | |
|---|---|---|---|
| | No observed | Mean | s.d. |
| Group | | | |
| FRESH | 3 | 5.000 | 0.6083 |
| CAR1 | 3 | 4.567 | 0.0577 |
| CAR2 | 3 | 3.867 | 0.5508 |
| CAR3 | 3 | 4.233 | 0.5686 |
| CAR4 | 3 | 5.800 | 0.1732 |
| SEVROT | 3 | 5.233 | 0.2082 |
| Margin | 18 | 4.783 | 0.7501 |

**Table 4.1.5.2: *E. coli* counts in sub-groups (18 GIT and 18 MBF).** Displaying the means and standard deviation (s.d.) of bacterial counts plated for *E. coli* in GIT and MBF sub-groups of the six carcass groups (FRESH-0h, CAR1-6h, CAR2-12h, CAR3-18h, CAR4-24, SEVROT-36h) exposed.

Fisher's least significant difference test was used to compare the mean bacterial counts of *E. coli* between groups and sub-groups. Fisher's l.s.d. test illustrated in Table 4.1.5.3.

| **Group.Sub-group** | Mean bacterial count | Single letter significant difference |
|---|---|---|
| CAR2/MBF | 3.867 | a |
| CAR3/MBF | 4.233 | ab |
| CAR2/GIT | 4.467 | abc |
| CAR1/MBF | 4.567 | bc |
| FRESH/MBF | 5.000 | cd |
| SEVROT/MBF | 5.223 | de |

| | | |
|---|---|---|
| CAR1/GIT | 5.267 | de |
| CAR3/GIT | 5.300 | de |
| SEVROT/GIT | 5.300 | de |
| CAR4/GIT | 5.367 | de |
| CAR4/GIT | 5.800 | e |
| FRESH/GIT | 3.900 | f |

**Table 4.1.5.3: Fisher's l.s.d. test comparing mean values of groups and sub-groups (type).** Sub-groups assigned with a single letter (a, e and f) representing significant difference, the letters (ab, abc, bc, cd, de) represent non-significant difference. CAR2/MBF showed the lowest bacterial load with CAR4/MBF and FRESH/GIT showing the highest bacterial load for *E. coli*.

The mean log CFU/g for *E.* coli was significantly higher ($P$<0.05) in GIT (5.433 log CFU/g) samples than in MBF (4.783 log CFU/g) samples with FRESH/GIT the highest (6.9 log CFU/g) and CAR2/MBF (3.86 log CFU/g) the lowest (Table 4.1.1). Due to the low sample size (n=36) and high level of significance between groups, Fisher's protected test of least significant difference (l.s.d.) was applied on the groups and sub-group means (Table 4.1.5.3). A significant difference between mean values of FRESH/GIT, CAR2/MBF, CAR/4MBF were identified by a single letter for values of 6.9, 3.87 and 5.8 log CFU/g, respectively. A difference of 1.93 log CFU/g between CAR4/MBF and CAR2/MBF mean values indicates an increase in mean log CFU/g for *E. coli* in MBF sub-group over 12-hour exposure time. A 1.9 log CFU/g difference between FRESH/GIT and FRESH/MBF sample means indicates a higher prevalence of *E. coli* in the gastrointestinal tract than in the rest of the carcass (MBF).

### 4.1.6 Carcass pH and temperature in decomposition trial

Carcass pH and temperature were recorded from each carcass evaluated during the decomposition trial (Objective 1). The aim in recording these values were to observe whether there would be significant differences in carcass pH and temperature between the different groups exposed in-house. If a certain group (e.g. SEVROT) resulted in excessively high bacterial loads for a certain pathogen, the carcass pH recordings were evaluated for possible use to identify carcass quality on farms .

Average group carcass pH and temperatures are represented in Table 4.1.1. Average carcass pH of groups over time exposed in poultry housing unit is illustrated in Figure 4.6.1.1. The average carcass temperature of groups is illustrated in Figure 4.1.6.2

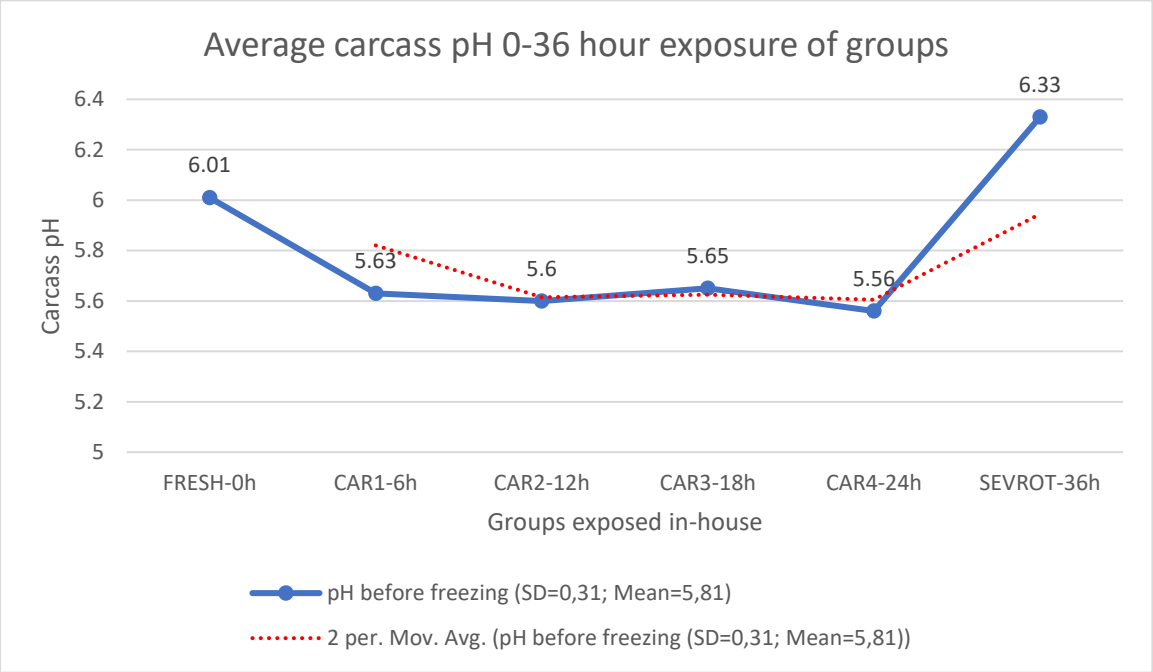


**Figure 4.1.6.1: Average carcass pH of groups exposed in decomposition trial.** The average carcass pH was recorded at the designated time intervals (FRESH-0h, CAR1-6h, CAR2-12h, CAR3-18h, CAR4-24, SEVROT-36h) before freezing.

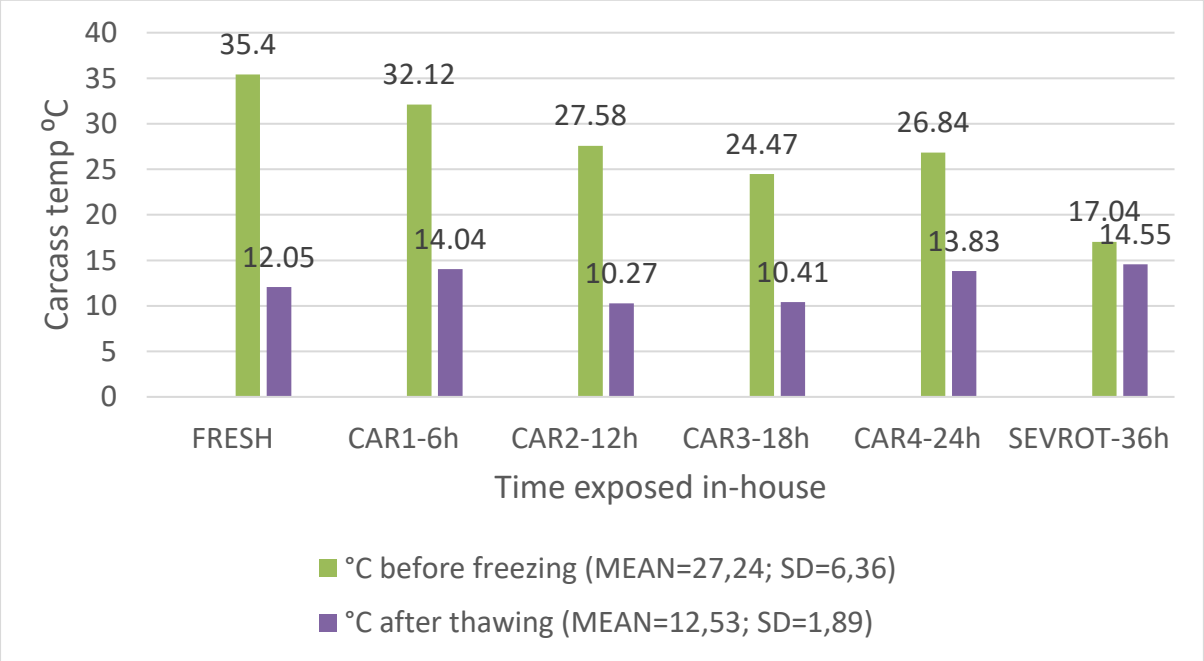


**Figure 4.1.6.2: Average carcass temperature (degrees Celsius) versus time exposed (hours).** Average carcass temperature was recorded at the time intervals

(FRESH-0h, CAR1-6h, CAR2-12h, CAR3-18h, CAR4-24h, SEVROT-36h) before freezing (after collected) and after thawing (before processing for sampling) carcasses.

The carcass pH measured before freezing decreased at a steadily rate of 0.15 units of pH/6-hours for FRESH, CAR1, CAR2 and increased at a rate of 0.085 units of pH/6-hours from CAR3, CAR4 and SEVROT (Fig 4.1.6.1). Carcass temperature decreased at a rate of 3.67ºC/6-hours between groups, as expected (Fig 4.1.6.2). Linear regression analysis was performed on carcass pH and temperature before freezing. The high percentage of variation accounted for $R^2$=16.6, displaying outliers on the regression model and a high degree of heterogeneous variation.

### 4.2 Multi residue screening results of poultry samples

The aim of this objective was to determine whether antimicrobial compounds are present in poultry carcasses being used as feed.

All the chicken tissue samples (n =15) tested negative for the 15 pharmaceuticals screened for (Table 4.2.1). The lowest detection limit of the laboratory was 50 µg/kg for these compounds.

| | | |
|---|---|---|
| Chlortetracycline | Doxycycline | Oxytetracycline |
| Tetracycline | Ciprofloxacin | Norfloxacin |
| Enrofloxacin | Lincomycin | Olaquindox metabolite |
| Sulfadiazine | Sulfadimidine | Sulfamethoxazole |
| Tiamulin | Trimethoprim | Tylosin |

**Table 4.2.1: 15 Pharmaceuticals screened for in feed samples collected on commercial crocodile farms.** All of the 15 samples collected on the 5 farms visited tested negative for the pharmaceutical compounds.

This indicates zero detection of antibiotic residues in poultry carcasses with the diagnostic method used by the laboratory. All farms used in sampling rely on different sources for carcasses, ranging from broiler farm mortalities to abattoir rejects and transport/handling mortalities. Our findings strongly suggest that there is no difference between sources in the prevalence of pharmaceutical residues present in chicken carcasses used as crocodile feed.

### 4.3 Carcass evaluation method

Practical carcass evaluation method for crocodile farmers feeding chicken carcasses. The aim of this objective was to develop a rapid and practical carcass evaluation method that can be used on commercial crocodile farms to discard severely rotten carcasses whilst processing carcasses for feed. Resulting in a higher amount of quality carcasses used as feed. Carcasses used in the decomposition trial were observed and used in the development of this evaluation method.

### 4.3.1 Physical changes observed in decomposition trial used for developing carcass evaluation method

Over the 36-hour exposure period the groups were observed for physical changes (colour and deterioration of carcass) at different intervals. The most obvious and distinguishable changes between the different groups were skin discolouration and carcass deterioration. The pull test was tested on carcasses in different stages of decomposition (decomposition trial).

| Groups | Time exposed (hours) | Skin colour | Pull test | pH average | Temp average |
|---|---|---|---|---|---|
| FRESH | 0 | Red | resist | 6.01 | 35.4 |
| CAR1 | 6 | Red/yellow | resist | 5.63 | 32.12 |
| CAR2 | 12 | Yellow/red | resist | 5.60 | 27.58 |
| CAR3 | 18 | Yellow/green | resist | 5.56 | 24.47 |
| CAR4 | 24 | Green/Yellow | resist | 5.65 | 22.37 |
| SEVROT | 36 | Green | resist | 5.73 | 17.04 |

**Table 4.3.1: Physical changes of carcasses exposed in decomposition trial.** Carcasses were exposed over 36-hours (0,6,12,18,24,36 hours) and divided into six groups (FRESH, CAR1, CAR2, CAR3, CAR4, SEVROT). Skin colour and the pull-test was recorded in each group as well as the carcass pH and temperature.

**Figure 4.3.1:Chicken carcass skin colour observed in clinical trial.** The yellow skin colour was observed in the 12-hour exposed group, CAR2 (left) and the green skin discolouration observed in CAR3, CAR4 and SEVROT after 18-hour exposure (right).

## 4.4 Crocodile farm: questionnaire, investigation and hazard identification

### 4.4.1 Crocodile farm questionnaire

| Questions | LOC 1 | LOC 2 | LOC 3 | LOC 4 | LOC 5 |
|---|---|---|---|---|---|
| **Chicken carcass information** | | | | | |
| How many chicken farms supply carcasses to you and from where? | 1 Naboomspruit | 6 in Gauteng | 2 Abattoirs in Brits and Hartbeespoort dam | 1 Farm and 2 abattoirs Bela-Bela | 4 Farms in Letsitele |
| Do the carcasses consist mainly of broiler or layer birds? | Broiler | Both | Broiler | Broiler | Broiler |
| Are carcasses from different sources mixed or separated? | Mixed | Mixed | Mixed | Mixed | Mixed |
| Do you receive chickens weighing <0.7kg , 0.7-1.5kg and/or >1.5 kg? | All | >1.5kg | All | <0.7 and >1.5kg | All |
| How regularly are the carcasses delivered or collected? | Weekly | Daily | Weekly | Daily | Weekly |
| **Storage of carcasses** | | | | | |
| Are the carcasses delivered/collected frozen or unfrozen? | Unfrozen | Frozen | Frozen | Unfrozen | Unfrozen |
| How are the carcasses stored and for how long before being fed? | -12°C 12 hours | -10°C used daily | Used on arrival | Used on arrival | -12°C for 24 hours |
| **Chicken carcass management on the crocodile farm** | | | | | |
| Are the carcasses classified, based on the stage of decay, before being processed? | Yes | Yes | No | Yes, based on colour | Yes |
| Are the carcasses minced before feeding? | Yes, not breeders | Yes, not breeders | Yes | Yes | Yes, not breeders |
| How much chicken, in kg's, is given per croc/pen a week or day? (feeding schedule) | 1.5kg/croc/2xweek | 1.5kg/croc/2xweek | 2kg/croc/2xweek | 1.5kg/croc/2xweek | 1.5kg/croc/2xweek |
| How are the carcasses prepared (whole, disembowelled, skinned, feathers on, etc.) before mincing or feeding? | Meat for hatchlings, whole for breeders | Defeathered, legs off and eviscerated | Defeathered and eviscerated by source | Meat only for hatchling, whole for breeders | Defeathered and eviscerated by hand |
| How are carcasses processed before feeding? | Discarded on decay by workers perception (colour, deformity) | Discarded on decay by visual pictures, chlorine bath solution | Minced, no need due to off cuts and rejects from abattoir | Hatchling ration meat only | Discarded on colour |
| If another protein feed source is used, please state the reason why? | Full fat soya, carcass meal, fish meal for amino acid profile | Full fat soya, carcass meal, fish meal, blood meal for amino acid profile | No, 100% poultry | No | Blood meal, carcass meal, fish meal for amino acid profile |
| List the most common feed related diseases observed in the crocodiles on the farm. | Coccidiosis rarely | Impurities in feed | None | None | None |
| How regularly are the pens cleaned? | 2x week | 2x week | 2x week | 2x week | 2x week |
| Have any of the workers ever fallen ill due to the handling the chicken carcasses? | No | No | No | No | No |
| Is any pre-mix added and how much per kilogram of feed? | Yes 250g/50kg feed | Yes 125g/25kg feed | No | Yes 125g/25kg feed | No |

**Table 4.4.1: Crocodile farm questionnaire**.

This questionnaire (Table 4.4.1) was provided to commercial crocodile farmers from five different locations (LOC 1, LOC 2, LOC 3, LOC 4, LOC 5). The questions were grouped into three categories (Chicken carcass information, storage of carcasses, chicken carcass management on the crocodile farm).

### 4.4.2 On-farm hazard identification

An on-farm hazard identification was done to evaluate the quality of chicken carcasses used on commercial crocodile farms in essence to identify possible hazardous carcasses associated with decay and antimicrobial residues. Carcass 'quality' was evaluated by following the chicken carcass evaluation method (see 4.3) before feed processing. Minced carcass samples were collected after feed processing and tested for pharmaceutical compounds (see 4.2).

#### 4.4.2.1 Quality of chicken carcasses used as feed on commercial crocodile farms.

Chicken carcass evaluation was done on the 5 crocodile farms visited for multi-residue screening sample collection. The carcass evaluation method was used to evaluate the quality of chicken carcasses used as feed.

#### 4.4.2.2 On-farm chicken carcass evaluation

With the knowledge gained from the carcass deterioration trial, chicken carcasses on commercial crocodile farms were inspected to confirm if the determined parameters could be applied as a practical method on crocodile farms. Skin discolouration and carcass deterioration (pull-test) were used as a practical method for classifying stages of decomposition or carcass quality.

We made an effort to evaluate 100 chicken carcasses on each crocodile farm (Fig 4.4.2.2.1). The following observations were made during the on-farm evaluation of the different crocodile farms: skin colour was a very practical parameter to evaluate chicken carcass quality; red/yellow was most often observed, while green/blue was seldom seen on the crocodile farms evaluated.

During the decomposition trial the most severe decomposition of the chicken carcasses (SEVROT) was classified as green. However, on the commercial crocodile farms some chicken carcasses appeared to be in a more advanced stage of decomposition and displayed a blue skin colour (Figure 4.4.2.2.1).

**Figure 4.4.2.2.1: Chicken carcass skin discolouration observed on farms visited.** This displays the two main colours observed on the majority farms visited. Distinct skin colours of red (left) and blue (right) are shown.

| Farm number | Skin colour (%) | Pull test |
|---|---|---|
| 1 | 90 Red | Resist |
| | 0 Green | - |
| | 10 Blue | No resist |
| 2 | 80 Red | Resist |
| | 10 Green | Resist |
| | 10 Blue | No resist |
| 3 | 90 Red | Resist |
| | 0 Green | - |
| | 10 Blue | No resist |
| 4 | 90 Red | Resist |
| | 0 Green | - |
| | 10 Blue | No resist |
| 5 | 75 Red | Resist |
| | 15 Green | Resist |
| | 5 Blue | No resist |

**Table 4.4.2.2.1: Results observed during the carcass evaluation done on the different crocodile farms**. The carcass evaluation was done by using skin colour (Red, Green, Blue %) and carcass deterioration (carcass resistance pull-test) as parameters to distinguish between fresh and severely rotten carcasses.

Very few green carcasses were identified on the crocodile farms as the majority carcasses consisted out of red/yellow carcasses with the remaining few observed as blue (Table 4.4.2.2.1). Green carcasses that were observed on some farms were evaluated for carcass quality. All the red/yellow carcasses used in the pull test resisted, whilst all the blue carcasses deteriorated with ease when pulled. Pull test was not performed on frozen or semi-frozen chicken carcasses.

We observed that the majority of carcasses used on crocodile farms resemble a red/yellow skin colour, with all resisting when subjected to the pull test. These carcasses are in a “fresher” stage of decomposition compared to blue skin colour carcasses (Table 4.4.2.2.1).

Information that we gathered during the farm questionnaire confirmed that most farmers use some or other classification system to discard the very decomposed

chicken carcasses, resembling the blue chicken carcasses observed in our investigation.

During one farm visit umbilical stump abscesses were observed in younger chickens during processing for feed. The infected umbilical stumps were collected and sent in for microbiological analysis at the Microbiology Laboratory of the Department of Veterinary Tropical Diseases, Onderstepoort. The results from the analysis indicated an abundance of microorganisms including: *Salmonella spp.; Staphylococcus saprophyticus; Staphylococcus gallinarum;* and *E. coli.* Antibiograms were also done for the microorganisms identified (Tables 4.4.2.2.2 and 4.4.2.2.3). The antibiogram indicated that *E. coli* was resistant to 5/11 (45%), *Salmonella* spp. resistant to 3/11 (27%), *S. saprophyticus* resistant to 4/11 (36%) and *S. gallinarium* resistant to 4/11 (36%) of the antibiotics tested.

| | *E. coli*, non-haemolytic rough – 4 | *Salmonella spp.* - 2 |
|---|---|---|
| Ampicillin | R | S |
| Doxycycline | S | S |
| Enrofloxacin | S | S |
| Erythromycin | R | R |
| Fosbac | S | * |
| Fosfomycin | S | S |
| Kanamycin | R | R |
| Neomycin | S | R |
| Sulphisoxazole | R | I |
| Tetracycline | S | S |
| Trimeth/Sulpha | R | S |

| | *Staphylococcus saphrophyticus* - 1 | *Staphylococcus spp.* – 3 |
|---|---|---|
| Ampicillin | R | R |
| Doxycycline | R | S |
| Enrofloxacin | S | S |
| Erythromycin | S | R |
| Fosbac | * | * |
| Fosfomycin | R | R |
| Kanamycin | S | S |
| Neomycin | S | S |
| Sulphisoxazole | S | R |
| Tetracycline | R | S |
| Trimeth/Sulpha | S | S |

**Tables 4.4.2.2.2 and 4.4.2.2.3: Antibiotic susceptibility pattern.** Samples collected from infected yolk sacs observed in chicken carcasses during the hazard identification. R=resistant, S=sensitive

### 4.4.2.3 Antimicrobial residue levels in carcasses

Farmers use chicken carcasses at different ages that suggest a younger chicken mortality has not been subjected to a withdrawal period in contrast to an older chicken mortality. This increases the risk of antimicrobial residues accumulating in the gastrointestinal tract and muscle tissue of the carcass, thus being processed as feed for crocodiles, and posing as a possible hazard. However, the zero detection of antibiotic residues in the tissue samples tested suggest that chicken carcasses from various origins do not pose as an antibiotic residue hazard regarding to crocodiles. See: 4.2 *Multi residue screening results of poultry samples*.

## 4.5 Practical chicken carcass management and feeding plan for crocodile farmers

The development of a practical chicken carcass management and feeding plan for a crocodile farm was aimed at providing farmers with information ranging from sourcing and transport of carcasses to processing, formulating feed and discarding of chicken carcasses not fit for use as feed. The information gathered from the questionnaire (Table 4.4.1) and results from this project served as valid sources in compiling this plan.

### Chicken carcass management plan

**Aim:** Providing crocodile farmers with a standard operating plan that can be used to maintain a constant supply of good quality chicken carcasses fit for use as feed. The management plan outline will be as follows:

a) *What has been observed during this project*

*b) What is recommended*

1. Sourcing

a) Commercial poultry farms that follow a strict biosecurity protocol and use freeze/cold rooms for storing carcasses can provide a continuous supply of carcasses throughout the year. Abattoirs are another source of chicken carcasses or off-cuts that can be utilized as crocodile feed. A method of sourcing that ensures high carcass quality fed to crocodiles is the “all in all out” system. This involves the use of carcasses on arrival or in less than 24 hours. No carcasses are stored in a cold room over an extended period of time. This method requires a readily proximal source of chicken carcasses.

b) The nearest accredited commercial poultry farm is recommended to be used as a source. The closest source of chicken carcasses, in proximity to the crocodile farm, is preferred as it is economically beneficial, regarding transport costs, and can provide higher quality feed to the crocodile farm on a constant basis. Local poultry farmers that do not adhere to strict biosecurity protocols and have the facilities to store carcasses below 0ºC, should not be considered as viable sources.

2. Transport

a) The method of transport is dependent on the economic viability of the farmer, the available transport and distance of the source from the farm. Farmers located near their source (<10km) do not make use of refrigerated transport as carcasses are delivered daily and fresh. These farmers make use of the “all in all out” system due to constant supply of fresh carcasses.

b) Refrigerated transport is recommended if the source is located > 50km away. Storing carcasses below -5ºC during transport and upon arrival on the crocodile farm for at least 12-24 hours before processing is advised.

3. Processing

a) The processing of carcasses on commercial crocodile farms is dependent on the production stage or age of crocodiles it will be fed to. It is recommended that hatchling and growers be fed a high-quality diet i.e. processing carcasses by removing the gastrointestinal tract (evisceration), the feathers, heads and feet of the carcass before mincing. This is to decrease the likelihood of including pathogens in the feed and increase the overall quality of carcasses used for hatchling and grower diets. Most farms that do not include rotten or severely rotten

carcasses in hatchling and grower diets, make use of them in breeder diets. These carcasses are not eviscerated or treated and are fed whole to breeders.

b) The treatment of carcasses in a chlorine bath solution is a recommended pre-processing method. This method disinfects and thaws the carcass as well as reduces the risk of human-animal contamination or transmission of pathogens. The chicken carcass evaluation method, mentioned in Chapter 4.3, is a simple and effective method to increase overall carcass quality fed to crocodiles by discarding severely rotten carcasses. Eviscerating carcasses during processing is recommended as the gastrointestinal tract has the possibility of serving as a reservoir for unwanted bacteria and antibiotic residues derived from feed. Mechanical evisceration is preferred as it is a faster method compared to manual evisceration. The final step in processing is mincing of the carcasses to be able to add the pre-mixes (optional). Pre-mixes usually consist of essential amino acids, vitamins and minerals for a balanced feed depending on the production stage. Using fresh or high-quality chicken carcasses in hatchling and grower diets are recommended. In any instance of a S*almonella* spp. outbreak derived from carcasses, all feed must be heat-treated to more than 80º C after processing and carcasses thawed in a chlorine bath solution for at least 12 hours before processing (Huchzermeyer, 1991). Although farms that feed breeder's whole, rotten and discarded carcasses have not experienced any metabolic or pathogenic induced illness, it is recommended that severely rotten carcasses be discarded following a biohazardous waste disposal program. The possibility of promoting a pathogenic outbreak among a breeder population receiving severely rotten and untreated carcasses is high.

4. <u>Discarding carcasses</u>

a) Farms adhere to strict biosecurity protocols by cleaning/removing remaining feed from pens/dams every second day or before feeding. Rinsing and cleaning water in pens every 6-8 weeks. Some farmers discard severely rotten carcasses by feeding them whole to breeder crocodiles. Farms that receive a high amount of good quality carcasses do not include severely rotten carcasses in any ration and discard carcasses by using a biosecurity protocol or waste disposal program.

b) To safely discard carcasses, workers should be supplied with adequate biohazardous working materials and maintain a strict biosecurity protocol when working with severely rotten carcasses. A hazardous waste disposal program must be set up and maintained. It is not recommended that discarded carcasses be used as feed for breeders. If a nearby source of good quality chicken carcasses can maintain a constant supply, the amount of severely rotten carcasses will decrease and increase the overall quality of carcasses used in all diets. Thus, decreasing the amount of severely rotten carcasses that need to be discarded.

# CHAPTER 5
# DISCUSSION

The findings of this research project are discussed under different sub-headings representing the objectives of this research project.

This research project is unique in regard to the results observed in similar studies done on chicken carcasses. In comparison to other studies focussed on chicken carcasses intended for human consumption, the demand for information on carcasses intended for crocodile feed is significantly less. Thus, it is difficult to compare and discuss the results observed in this project, to others focussed on carcasses intended for human consumption. There are no other researchers that have focussed on the bacterial quality and antibiotic residue level of mortality carcasses intended for crocodile feed. Chicken mortalities, carcasses before point of slaughter, can not be intended for human consumption and need to be discarded or used. The crocodile industry can be seen as a sustainable resource utilizing chicken carcasses as protein in their feed.

## 5.1 Bacteriology, pH and temperature of carcasses in decomposition trial

Unprocessed meat from animal carcasses and meat products are easily contaminated if not properly handled, processed and preserved. Animal products may support the multiplication of microorganisms and serve as sources of various spoilage and pathogenic microorganisms (Koutsoumanis and Sofos, 2004).

It is widely believed that crocodiles may consume any meat or carcass in various stages of decay, without any health repercussions to them. However, this misperception may lead to higher crocodile mortality rates, poor growth in animals, lower quality skins and potential health implications for farm workers (personal communication from crocodile farmers in this study). Huchzermeyer (1997) reported that whenever crocodiles are severely stressed (e.g. before slaughter), microorganisms from the gastrointestinal tract may cross the intestinal barrier (gastrointestinal mucosa) and, via systemic circulation, may invade the muscles of affected animals. This may influence the quality of crocodile meat (Huchzermeyer, 1997).

In this study the focus was on microorganisms coming from chicken carcasses, namely the gastrointestinal tracts and the rest of the carcass. Although the focus was orientated towards the contribution of the poultry carcasses (feathers, meat and bones) and gastrointestinal tracts, several other potential sources of microorganisms also need to be taken into consideration. Live chickens are often very dirty – with dust, dirt and litter accumulating on their skins and feathers. High numbers of bacteria are usually present on the animals' skins, including the normal flora of the skin (staphylococci, micrococci, pseudomonads, yeasts and moulds) as well as organisms from soil, water, feed and faeces (Koutsoumanis and Sofos, 2004). All these different sources of microorganisms are a concern, because chicken carcasses are very often processed *as is* for crocodiles. Some farmers remove the internal organs to reduce the bacterial load before processing. However, it does not seem to make a big difference, if microorganism numbers are taken into consideration, with the skin and feathers containing large numbers of these organisms.

Chickens may also be asymptomatic carriers of pathogenic microorganisms with antibiotic resistance. We have observed small umbilical stump abscess in some chicken carcasses that we eviscerated during processing (unpublished data Myburgh, de Wet and Bredenkamp). This problem was especially prevalent in chickens growing slower (runts) than the other birds in their group. These birds were routinely removed and given to the crocodile farms (unpublished data Paul Bredenkamp).

Bacteria in processed crocodile feed may negatively affect the quality of crocodile feed if it is given enough time (not fed immediately) and ideal environmental conditions to support multiplication. It is therefore recommended that leftover feed must be stored in freezers, immediately after processing. It is not advised to process carcasses and store the feed for the next day. Good feed management and formulation will decrease the amount of feed wasted and lower the likelihood of bacterial multiplication in feed stored. Excess feed not eaten by crocodiles in pens after feeding must be discarded as waste and pens cleaned after each feeding. The high humidity and temperature in environmentally controlled housing units make ideal incubators for bacteria (Huchzermeyer, 1997).

### 5.1.1 *Staphylococcus aureus*

In this study, *S. aureus* was detected in 36% of the samples with 8/18 and 5/18 detected from the GIT and MBF sub-groups, respectively. The highest mean log CFU/g was from the FRESH/MBF samples at 5.4 log CFU/g, above the acceptable upper limit for raw poultry products of 3.7 log CFU/g. The low prevalence of detection could be due to other pathogens dominating the microenvironment, as *S. aureus* does not compete well with other microorganisms (Food Control Guideline, 2017).

A study done in Morocco, Cohen et al. (2007) concluded that the primary factor contributing to staphylococcal food poisoning outbreaks is an inadequate control of refrigeration temperatures. In our investigation (Objective 1), the chicken carcasses were placed in the abattoir freezer (-12ºC) immediately after collection and this could have played a role in the bacterial counts and levels of detection observed in the results. This is an important suggestion for the treatment of carcasses that may reduce the microbial load and thus the *S. aureus* prevalence. Most farmers store chicken carcasses in freezer rooms on the poultry farms.

A double enrichment method during bacteriology testing could have been used for enumeration to detect all *S. aureus* colonies. A larger sample size would have resulted in a more accurate estimate of the bacterial contamination associated with the carcasses, unfortunately this was not possible as limited resources were available for microbiological analysis.

### 5.1.2 *Salmonella spp.*

*Salmonella spp.* was detected in 88.89% of the samples submitted with 15/18 and 17/18 detected in GIT and MBF sub-groups, respectively. The samples that tested positive for *Salmonella* were serotyped and identified as *Salmonella hamburg.* The high prevalence of detection of this microorganism was expected in a favourable environment such as a poultry unit.

A study done by Gelaw et al., (2018) detecting *Salmonella* from animal sources in South Africa between 2007-2014, detected around 108 different serotypes from nine different food and non-food animal host species. The three most common serotypes were *Salmonella enterica* subspecies *enterica* serotype Heidelberg (*n* = 200), *Salmonella enterica* subspecies *enterica* serotype Enteritidis (*n* = 170) and *Salmonella enterica* subspecies *enterica* serotype Typhimurium (*n* = 146). Of the

total number of isolations recorded during the period under review, 871 (70.8%) were from poultry and other birds, 116 (9.4%) from cattle, 26 (2.1%) from sheep and goats, 16 (1.3%) from pigs and 8 (0.6%) from crocodiles. A similar study done by Van der Walt et al., (1997) over a 10-year period (1985-94), 173 isolates of *Salmonella* were obtained during routine isolation from reptiles. Of the 173 isolates, 92 different *Salmonella* serovars were identified. The majority of isolates were from farmed Nile crocodiles (145).

In a study done by Huchzermeyer (1997), it is mentioned that numerous *Salmonella* have been isolated from crocodiles. Most of these are believed to be harmless gut inhabitants. Under stressful conditions, however, the *Salmonella* organisms can invade visceral organs and, from there, other tissues. In some crocodiles, this may cause mortality. Most *Salmonella* infections are acquired from feed, thus a significant reduction in the infection rate is expected from feeding compounded pellets (Huchzermeyer, 1997).

In an earlier study by Huchzermeyer (1991) on the treatment and control of an outbreak of salmonellosis in hatchling Nile crocodiles, it was found that crocodiles appear to be particularly susceptible to salmonellosis during the early post hatchling period and it is important to minimize exposure to *Salmonella spp*. during this period. The pathology caused by salmonellosis in crocodiles is severe and may result in acute mortality. However, in many cases the course of the disease may be protracted, affected animals dying from chronic manifestations, such as intestinal occlusion, up to 3 weeks after being identified as ill. This was particularly evident amongst the force-fed crocodiles. The disease may therefore be associated with a protracted period during which the diseased crocodiles also refuse to eat. High humidity and temperatures make controlled housing units ideal incubators for bacteria. It appeared that the probable source of infection were beef feedlot carcasses, infected with *Salmonella* spp. (Huchzermeyer, 1991). The high detection of *Salmonella* spp. in this study proves the importance of treating carcasses before being used as feed to eliminate the chance of *Salmonella* outbreaks on crocodile farms. This can be done by heat-treating hatchling feed after processing to over 80º Celsius to prevent the introduction of *Salmonella* spp..

Using abattoir equipment and storage facilities in this investigation could have impacted the prevalence of detection, although thorough sterilization methods were used before, in-between and after sampling.

### 5.1.3 *Listeria monocytogenes*

*Listeria monocytogenes* was detected in 61.11% of the samples submitted with 5/18 and 17/18 detected in GIT and MBF sub-groups, respectively. The high prevalence of samples detected in the MBF, compared to the GIT sub-group, is no surprise due to *L. monocytogenes* being ubiquitous in the environment.

This microorganism is capable of tolerating cold temperatures (storage at -12ºC) and surviving in aerobic and anaerobic conditions. A study by Van Nierop et al, (2005) who focused on the contamination of chicken carcasses in the Gauteng Province, collected samples from street vendors (fresh), butcheries (fresh/frozen) and supermarkets (frozen). *L. monocytogenes* was isolated from 19 out of 99 carcasses and the extent of contamination was similar for fresh and frozen carcasses from all three outlets. This is again a confirmation how tolerant this organism is to extreme temperatures.

Microbial contamination of chickens is influenced by climate, geographic location, method and distance of transportation and holding conditions (Koutsoumanis and Sofos, 2004). The source of contamination could derive from the staff managing the chickens, moving daily in and out the housing unit without disinfecting boots, equipment etc. each time.

Listeriosis is an important human infection and is particularly harmful for pregnant women, neonates and older adults. Cases of listeriosis have not been confirmed in crocodiles.

The above-mentioned results and reports confirm the importance of treating chicken carcasses with chlorine before processing. The high detection of *L. monocytogenes* in samples could suggest cross-contamination from the abattoir equipment used, although sampling processes were done aseptically.

### 5.1.4 *Escherichia coli*

*E. coli* was detected in al samples submitted for microbacteriology analysis with GIT averaging a mean log CFU/g of 5.4 and MBF averaging a mean log CFU/g of 4.7. A

higher mean log CFU count was expected in the GIT compared to the outside of the carcass.

A potential source of *E. coli* contamination in the MBF samples could be the poultry litter and dust that accumulated between the feathers and on the skin of the carcasses. Staff handling birds during management, moving birds to pens, checking for bruising etc., could also be a source (SAPA, 2018). In a study done by Cohen et al. (2007), comparing bacterial quality of poultry samples in Morocco from supermarkets and traditional shops, showed that 22.4% of the total tested samples were above the safety limit in terms of faecal coliforms. The majority of the samples were recorded in the hot season, with 26 (13.5%) form traditional shops and 11 (5.7%) from supermarket samples. In the cold season, only 6 (3.1%) samples were beyond the safety limit and were found in traditional shops; none was recorded in supermarkets. Of all the samples analysed, 93 (48.4%) tested positive for *E. coli.* With the technique of hand evisceration predominantly practiced in the traditional shops under study and with infrequent hand washing, a high prevalence of bacteria related to human contact was expected in these samples.

Processing of chicken carcasses (e.g. evisceration) on farms is also done by hand. Although protective gloves are most often worn, the transmission from humans to carcasses or *vica versa* could become important if ingested in large numbers by crocodiles.

### 5.1.5 Carcass pH and temperature

Due to the inconclusive pH results in the decomposition trial (Objective 1), we were not able to use the chicken carcass pH as an indicator for the state of decomposition during the on-farm hazard identification (Objective 4). Low variation in fresh and severely rotten carcass pH values and effect of temperature on carcass pH data recorded made it difficult to use it as a practical and accurate indicator of the state of decomposition. In addition, to be able to monitor carcass pH, a portable meat pH meter is needed, which is expensive.

Post-mortem carcass temperatures decreased in our study at a steadily rate of 3.67ºC/6-hours between groups, as expected. Linear regression analysis was performed on carcass pH and temperature before freezing. The high percentage of

variation accounted for $R^2$=16.6, displaying outliers on the regression model and a high degree of heterogeneous variation. Carcasses prepared for sampling were thawed until an average carcass temperature ranging between 10 –15ºC was recorded before sampling started.

Information gathered from the questionnaire confirmed that most of the farmers either received the carcasses in a frozen state or unfrozen carcasses were placed in freezers immediately after arrival. Some farms processed fresh carcasses immediately on arrival, depending on the day of feeding or the availability of chicken carcasses. This makes it even more difficult to use carcass temperature as an indicator in carcass decomposition estimation. Mild to severely decomposed carcasses that arrive on the farm frozen have a low carcass temperature, but still pose as a possible hazard. Carcasses transported warm and unfrozen, depending on the proximity of the source to the crocodile farm, can still pose a threat as a high proportion of carcasses could be mild to severely decomposed. Although carcass temperature does play a major role in the rate of decomposition, it is not a practical indicator that can be used to identify low quality carcasses.

In conclusion, we were not able to establish that muscle pH can contribute as an objective indicator to distinguish between fresh and severely rotten carcasses. In general, most chicken carcasses processed on farms are subjected to temperature changes, potentially altering the muscle pH values, resulting in inconsistent values recorded with a high degree of variation. It is also a time consuming and expensive method of measuring for decomposition if compared to skin discolouration and deterioration.

### 5.2 Antibiotic residues in poultry carcasses used on farms

Although the majority of samples submitted for multi-residue screening tested below the lowest detection limit (50µg/kg) for the 15 compounds, a sufficient sample size from different farms is necessary to conduct an accurate screening.

Our negative results were not expected as antibiotic usage in the poultry industry is well-known. A study by Hakem et al. (2013), revealed that 124 out of 145 poultry meat samples (85.51%) were positive for antibiotic residues. However, most of them

(75.81%) contained β-lactams and/or tetracyclines against 44.35% for macrolides and/or β-Lactams and 36.29% for sulphonamides. Conversely, 13.71% of samples were positive to aminoglycosides. A study on global antimicrobial trends done by Van Boekel et al. (2015), showed that antimicrobial consumption for animals in the BRICS countries is expected to grow by 99% by 2030, whereas their human populations are only expected to grow by 13% over the same period. The study of Van Boekel et al. (2015), confirmed the general misuses and noncompliance's to withdrawal periods (period between the termination of antibiotic administration and slaughter). Globally, intensive livestock farming has increased food production and at a lower production cost per unit, however this is achieved by an increased use of antimicrobials and resistance developing (Van Boekel et al., 2014).

Our antibiotic residue results suggest that the poultry farms mostly adhere to the necessary withdrawal periods and regulations regarding pharmaceutical administrations. This is a positive finding for crocodile farmers who sell crocodile meat for human consumption. The current low income received by crocodile farmers from selling crocodile skins is forcing most crocodile farmers to consider selling the meat for human consumption for an additional income.

The samples tested in this study were all negative for the specific detection limit of the laboratory equipment used. In general, the use of antibiotics, to any level, will increase the resistance of microorganisms to the pharmaceuticals included in animal feed (Van Boekel et al., 2015). Especially if the exposure is on a continuous basis. In a study done by Benedict and Shilton (2016) a total of 139 *Providencia rettgeri* isolates were tested between 2010 and 2015, of which 44% were sensitive to all antibiotic treatments (sulphafurazole, tetracycline and sulphamethoxazole), 21% were resistant to all three antibiotic treatments, 21% were resistant only to tetracycline and 14% were resistant only to sulphonamides. The isolates derived from samples collected from juvenile farmed saltwater crocodiles that died due to bacterial septicaemia. Antimicrobial resistance in crocodilian bacterial isolates may be due to development either of resistance in response to antibiotics used on the farm when treating animals directly, or indirectly by antibiotics derived from chicken carcasses in the feed.

### 5.3 Chicken carcass evaluation

The chicken carcass evaluation method developed where the skin colour was used as an indicator of carcasses deterioration proved to be effective. This rapid and effective evaluation method of carcasses can be used on any crocodile farm feeding chicken carcasses. The evaluation can be done on carcasses in thawed, semi-thawed and unfrozen states, but not in fully frozen carcasses as they are too frozen to be pull-tested. This is beneficial as frozen carcasses cannot be processed until semi-thawed, to prevent damage to equipment. Skin colour observations can be made by any basically skilled farm worker and is simple to learn and understand.

The pull test is a method that can be easily shown and taught to workers on crocodile farms. The aim of this method is not to pull the carcass apart, but to observe the quality of the carcass as severely decomposed carcasses separate with ease when pulled. This technique might be of benefit to the crocodile farmer, as any worker can apply the pull test. By conducting the skin colour observations first, numerous carcasses are not selected for the pull test and it therefore does not slow down the processing. The disadvantage of the pull-test is that it is not very sensitive. The chicken carcass that is tested must be severely rotten before it becomes “positive”.

The muscle pH method that we investigated (Objective 1), unfortunately, did not work as a diagnostic tool to objectively determine the decomposition state of chicken carcasses. In conclusion, we found that the only practical method for chicken carcass decomposition evaluation is the skin colour changes. Although it is a subjective method, this is the only technique that we found to be simple, practical and repeatable for farm use.

The removal of poor-quality carcasses not passing the evaluation from the processing line has benefits for the farmer. The farmer or workers may use the pull test in case they are not certain about the state of decomposition of the carcass e.g. the carcass has a dominant blue skin colour, but it resists when subjected to the pull test.

By using the chicken carcass evaluation method before processing, a higher proportion of good quality carcasses is included in feed rations intended for hatchlings and growers. Decreasing the number of severely rotten carcasses in crocodile feed, may potentially reduce the health hazard to crocodiles. The severely rotten chicken

carcasses must preferably be destroyed, but if crocodile farmers don't have any feed available, the poor-quality chicken carcasses could be used for the breeders (although not recommended).

## 5.4 Crocodile farm: questionnaire, on-farm investigation and hazard identification

### 5.4.1 Questionnaire

The following discussion is divided and discussed in separate forms as followed in Table 4.4.1 summarizing the results from the questionnaire conducted on crocodile farms.

a. Chicken carcass information

Carcass evisceration is practiced on most of the farms, especially when processing feed intended for grower and hatchling rations. This is important as juvenile crocodiles lack the level of immunity compared to breeders, adult crocodiles. Optimal growth and health of grower and hatchlings are crucial in the production of high-quality skins and maintenance of healthy animals.

Farms follow a similar schedule ranging between 1.5-2.0 kg minced poultry/crocodile fed twice a week or every second day, cleaning pens after feeding and rinsing dams every 8 weeks. 4/5 farms rely on 100% minced poultry ration.

Farms that rely on a 100% minced poultry ration differ between each other in the preparation of hatchling diets. Farm LOC4 utilizes a 'all-in all-out system' only using the breast meat from carcasses for hatchling diets, using the remaining carcass in grower rations. Farm LOC3 uses rejected carcasses and offcuts from abattoirs that have been processed, defeathered and eviscerated. These cuts are minced and fed to hatchlings in a pellet form. Farm LOC1 located near the source of carcasses receives a high amount of fresh carcasses daily, these carcasses are processed so only the meat is included in hatchling and grower diets. Farm LOC5 defeather and eviscerate carcasses intended to be minced for hatchling diets. Farm LOC2 follow a 50/30/0% inclusion rate for minced poultry depending on the age or stage of production ration. Breeders are fed whole carcasses discarded during the processing of carcasses for hatchling and grower diets on the majority of farms.

b. Sources, transport and storage of carcasses

The different source of carcasses that the crocodile farms use had an effect on the processing and storage methods. Farms located near broiler farms (<10km) or abattoirs followed a similar 'all-in all-out system', delivering "fresh" carcasses daily with minimum storage or freezing necessary. Carcasses were processed on arrival. Farms located further from the source of carcasses utilize refrigerated transport and freezer rooms ( -12ºC) storing carcasses on arrival for processing after freezing by thawing in a chlorine solution pre-processing. This is beneficial in terms of lowering bacterial contamination of carcasses subjected to freezing temperatures and a disinfectant solution. Depending on the distance of the source, carcasses are delivered weekly or daily. Farms with the necessary equipment for transporting frozen carcasses deliver carcasses frozen, with farms located close to a source receiving unfrozen carcasses. Majority of farms store carcasses in freezer rooms running between minus 10-15ºC.

One of the farms uses a first-in, first-out system based on time of carcasses arrival. First carcasses to be placed in freezer will be the first to be used, in the next processing. Another farm uses carcasses strictly form transport mortalities and rejected by abattoirs; this farm observed the highest quality of carcasses. Most of the other farms rely on intensive poultry production farm mortalities that are stored on farm. One of the farms do not accept carcasses weighing less that 800g, while the majority accept all sizes. The level of effect these techniques have on bacterial contamination must be studied in further investigations.

c. Chicken carcass management on crocodile farms

Farms utilize "fresh" carcasses or off-cuts during processing for grower/hatchlings diets while the more decomposed carcasses are included in the breeder ration. This could be seen as a possible hazard as some carcasses observed during evaluation were in a severe state of decomposition. However, no crocodile mortalities were reported in the last 6 months by any of the farms utilizing whole decomposed chicken carcasses as breeder feed. The level of immunity developed by adult crocodiles for these carcasses could be beneficial in regard to optimal utilization of all carcasses bought in for feed.

Farms discarding severely decomposed carcasses during processing pre-storage, reported no mortalities in the last 6 months. Farm LOC2, 4, 5 follow a similar holistic approach during carcass classification, discarding “blue” and deformed carcasses on the perception of workers processing them. Farm LOC2 follow a protocol of thawing frozen carcasses in a chlorine bath solution before classifying based on level of decomposition, deformity and visual signs of sepsis (pictures provided on wall in processing room). The remaining farms, LOC1 and LOC3, are in such close proximity to their source, in return they receive a constant supply of fresh carcasses that is less labour-intensive during processing. LOC3 has the least labour-intensive classification system due to the farm receiving processed carcasses and offcuts from abattoirs.

d. General conclusions

The information gathered from the questionnaire was crucial in conducting the hazard identification, as crocodile farmers use different sources, processing methods and storage of carcasses.

None of the farms recorded crocodile mortalities directly related to the feed (necrotic enteritis, salmonellosis or stress induced septicaemia) over the last 6 months.

### 5.4.2 On-farm investigation and hazard identification

The on-farm hazard identification (Objective 4) was conducted by taking into consideration the following: chicken carcass evaluation method that was developed (Objective 3); and the results from the multi-residue screening of chicken tissue samples (Objective 2) tests.

a. Quality of carcasses used as feed

The farms identified with no green skin coloured chicken carcasses differed in factors contributing to these results. LOC 1 is located <10 km from the poultry farm used as a source of the carcasses, thus being able to collect ‘fresher’ carcasses, exclude high transportation costs and maintain a high degree of selection when processing carcasses. LOC 3 uses a first-in first-out system, using this as a control measure to decrease the number of ‘rotten’ carcasses in storage. Although this system is an effective way of controlling the number of discarded carcasses, blue carcasses were still identified on this farm. This suggests that the carcasses were initially in this stage

of decay when sourced. LOC 4 strictly sources carcasses from transportation mortalities and processing plant “rejects”, carcasses rejected due to breast lesions or blistering, subdermal bleeding, etc. This farm observed the least number of blue carcasses due to the source. From our investigations, it is clear that the source of chicken carcasses plays a major role in the quality of the chicken carcasses.

Due to the heated environment in a commercial chicken houses, the microbial load of chicken carcasses (mortalities) may increase exponentially, depending on the ambient temperature in the house, length of time the carcasses were left in the house before removal (usually frozen after removal) and overall hygiene biosecurity on the farm. An enormous number of microorganisms exist in terrestrial, atmospheric, and aquatic environments and live together on and within other creatures.

Shortly after death, microbes (bacteria, fungi and protozoa) contribute to the decomposition of the carcass. These microorganisms metabolize the body’s macromolecules (carbohydrates, proteins, lipids and nucleic acids) into gas, liquids and simple molecules and are the main force in the putrefaction or bloat stage, which alters the appearance of the carcass significantly. Typically, a green discolouration appears while various gases, including hydrogen sulphide, carbon dioxide, methane, ammonia, sulphur dioxide and hydrogen, are released into the surrounding tissues (Ralebitsosenior T, 2018).

The information gathered from our on-farm chicken carcass investigation, suggests that the carcasses classified as severely decomposed, may pose as a hazard if included in processing rations for hatchlings and growers. These carcasses can be used as feed for breeder rations but is not advised and should be discarded. The level at which pathogen loads are present in feed will determine their effect on crocodiles and needs to be taken into consideration for all carcasses. Pathogen levels of carcasses, subjected to treatment before feeding, need to be investigated in order to determine whether the level of contamination still poses as a threat. The level at which these pathogens may cause an effect on crocodile health is currently not 100% clear. Although some rotten carcasses were observed on specific crocodile farms (mostly discarded), no crocodile morbidities or mortalities were recorded – see questionnaire results (Table ).

Umbilical abscesses were observed in young chicken carcasses used as feed on one farm during the chicken carcass investigation. The infected umbilical stalks or stumps were collected and the associated microorganisms identified. The condition was identified as chronic omphalitis, characterized by infected and unhealed navels in young birds. It is infectious, but non-contagious, and is associated with poor regulation of incubation temperature or humidity (Randall, C.J, 1985). The results from our laboratory analysis indicated an abundance of microorganisms including: *Salmonella spp.; Staphylococcus saprophyticus; Staphylococcus gallinarum;* and *E. coli.* The antibiogram indicated that *E. coli* was resistant to 5/11 (45%), *Salmonella* spp. resistant to 3/11 (27%), *S. saprophyticus* resistant to 4/11 (36%) and *S. gallinarium* resistant to 4/11 (36%) of the antibiotics tested. See also: *b. Antibiotic residues*, below.

These affected chicken carcasses are possible hazards as they serve as a source of bacteria that could influence the quality of the processed crocodile feed. The inclusion of these carcasses during processing may increase the number of microorganisms in the feed and potentially support antibiotic resistance developing on the crocodile farm, increasing the probability of a disease outbreak.

Minced chicken carcasses not fed immediately and stored will be exposed to bacterial multiplication in the minced crocodile feed. Adding to a higher probability of an outbreak and mortalities. Especially if you have sources of pathogenic microorganisms in the chicken carcasses, e.g. umbilical abscesses, or normal GIT microorganisms minced and mixed with the rest of the carcass during processing. Precision feed formulating and processing will decrease the amount of unnecessary carcasses processed and stored.

Any savings achieved as a result of providing lower quality food will ultimately be offset by negative health and growth implications in the crocodiles. A poor diet will lead to decreased growth, gastrointestinal tract disorders, bone and teeth pathology and reduced immunity to disease (J G Myburgh, S van der Woude & J C A Steyl, 2018, personal communication). It is especially important to strictly adhere to a high quality, protein and mineral-rich feed for hatchlings and juveniles less than one year, as they are highly susceptible to disease and mortality (Brien et al., 2010).

Proving the techniques used in storage, processing and attaining optimal biosecurity measures reduces the prevalence of pathogens. The log CFU/g value for each

pathogen present in a carcass will determine whether it could pose as a possible hazard in feed for crocodiles, these values can be affected via processing, storage and additives. The bacterial multiplication of processed feed exposed in crocodile housing environments before ingestion has to be taken into consideration. The bacteriology from the decomposition trial represented carcasses that were frozen before sampling, but not subjected to a chlorine bath solution or additives used in the commercial crocodile industry. The degree of intake and affect for each pathogen could not be determined.

Majority of the carcass samples tested observed to exceed the maximum acceptable upper limit for poultry products intended for human consumption, posing as a possible biohazard to workers processing and handling carcasses. Necessary sanitary measures need to be taken in to account when handling and processing carcasses *Salmonella* spp. levels of 5 log CFU/g is highly suggestive of the possibility of food poisoning occurring in humans. Levels ranging between 7 - 9 log CFU/g are adequate levels to cause salmonellosis (Food Control Guideline, 2017).

b. Antibiotic residues

In poultry farming, large numbers of pharmaceuticals are used, including antimicrobials. They are used either as growth promoters, to increase production yields, or as therapeutic remedies to treat and prevent specific infectious diseases. However, the irrational using and no respect for the withdrawal periods can lead to residues in meat (Hakem et al., 2013). Adding to this, the non-prudent use of antimicrobials may increase and select multi-resistant bacteria, which could be transmitted to the commercial crocodile farms.

A younger chicken carcass (mortality before slaughter age) may have a higher risk of containing antimicrobials. A normal withdrawal period for these pharmaceuticals is usually only instituted just before slaughter age. Most chicken carcasses are usually fed to crocodiles (except for the severely decomposed carcasses) irrespective if the chickens received medicated feed or not, at the time of death. The results obtained from this study suggest that the poultry farms that we monitored applied the necessary withdrawal periods.

Younger chicken carcasses eviscerated on a farm during the hazard identification observed to have infected yolk sacs. The bacteria prevalent in the umbilical stalks or stumps were identified to be resistant to the following antibiotics: ampicillin, doxycycline, erythromycin, kanamycin, sulphisoxazole, fosfomycin and tertracycline. See also: *a. Quality of chicken carcasses used as feed*, above.

These compounds are well known to be used in the agricultural industry. Younger chicken carcasses deriving from commercial poultry production pose a problem of increasing the risk of introducing microorganism resistant to some antibiotics. The resistant microorganisms may be introduced to crocodiles via the carcasses in the feed if no precautionary measures were taken to disinfect carcasses before processing. By eviscerating carcasses during processing and removing the infected yolk sacs and stalks from the carcass would decrease the risk of introducing these microorganisms

Farms using abattoir rejects and offcuts were expected not to have a problem with antimicrobial residues (due to drug withdrawal periods). However, most of the other crocodile farms participating in the questionnaire stated that they utilize carcasses of varying ages. This was confirmed in the observations made on the farms visited while evaluating carcasses. All the feed samples tested lower than the detection limit of the laboratory.

Antimicrobial residue concentrations should be tested on a regular basis, with a sufficient number of tissue samples collected to monitor this potential hazard. Farms specializing in exporting of crocodile meat should consider using sources of chicken carcasses where withdrawal periods are known to be properly applied, e.g. abattoir rejects, offcuts and transport mortalities.

### 5.5 Management plan for feeding chicken carcasses to crocodiles

The need for a standard practical carcass management and feeding plan is crucial for the commercial crocodile industry. By adhering to a standard protocol, the risks associated with feeding chicken carcasses may be reduced, e.g. prevalence of pathogenic disease outbreaks can be decreased, and mortalities prevented. Each

commercial crocodile farm should develop their own SOP based on the basic principles provided.

Regardless of the feeding system used, all carcasses need to be viewed as possible hazards due to the bacterial contamination and potential antimicrobial resistance developing on the farm. Bacterial contamination of carcasses is not only associated with decomposition, but also with the environment the chickens were raised in, means of storage, transport and handling. It is advised that carcasses frozen before processing be thawed by using a diluted disinfectant solution, e.g. 5% chlorine solution. Farms using an all-in all-out system are also advised to treat carcasses with a disinfectant solution.

High mean counts of *E. coli* and *Salmonella spp.* reported in the GIT of carcass observed in the decomposition trial suggest that evisceration of all carcasses before feeding could be beneficial. It is advised to discard all chicken carcasses not passing the chicken carcass evaluation test. Breeders play an important role in the production of viable eggs. A relationship between severely decomposed carcasses and increased morbidity and mortality rates in crocodiles has not been proved, but the risk of contaminating a breeder population with pathogenic organisms (e.g. *Salmonella*) is a possibility.

Accurate feed formulation and good management will decrease the likelihood of processed feed being stored and wasted. Processed feed must be consumed as soon as possible. Chicken carcasses can be thawed and treated overnight and processed the next morning for the correct amount of feed needed. The storage of minced carcasses or feed is not advised as it holds the potential to multiply in bacterial number and species. In no circumstance can feed not eaten (scraps) in pens be used as feed again, the controlled environment crocodiles are housed in make ideal incubators for pathogen multiplication and spread and increase the risk of outbreaks. Excess processed feed may be heat-treated and/or stored frozen to minimize the risk of bacterial multiplication.

Hazards and potential risk to crocodiles:

- Severely decomposed carcasses with high numbers of microorganisms especially if used as feed for hatchlings and growers.
- Chicken carcasses not subjected to an antimicrobial withdrawal period prior to slaughter.
- Chicken carcasses not selected, disinfected or processed before feeding.
- Chicken carcasses sourced from unregistered abattoirs and/or poultry farms.
- Stress septicaemia in crocodiles that may be influenced by high bacterial loads in feed.
- Immuno-suppression in young crocodiles that may influence their ability to cope with high bacterial loads.
- Microorganism contamination in breeder pens due to utilization of severely decomposed chicken carcasses as feed.
- Possible introduction of antibiotic resistant microorganisms to crocodile farms, if no precautionary measures are taken to minimize the bacterial level of carcasses used as feed.

# CHAPTER 6
# CONCLUSION

1. Chicken carcasses exposed in a commercial poultry rearing unit environment for 0-36 hours before collection are subjected to decomposition. Unfortunately, carcass pH, time of exposure and microbial load cannot be used as objective indicators to distinguish between fresh, mild and severely decomposed carcasses.

2. In our investigation it seems as if antibiotic residue levels in chicken carcasses processed as crocodile feed on commercial farms are not significant risks (<50µg/kg). The use of antibiotics in the agricultural industry does increase antibiotic resistance in microorganisms that could be exposed to crocodiles by including contaminated carcasses in feed.

3. The carcass evaluation method developed in this study can be used on all commercial crocodile farms to increase feed quality by evaluating, identifying and discarding rotten and unwanted chicken carcasses.

4. Information gathered form the farm questionnaire and investigation identified possible hazards associated with chicken carcasses used as feed. Including whole severely decomposed carcasses in feed may increase the level and species of antibiotic resistant organisms' present. Increasing the probability of disease outbreak.

5. The practical management plan for feeding chicken carcasses to crocodiles can be used or adapted by farmers to implement and improve their current feeding plan and SOPs.

**CHAPTER 7**

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## ADDENDUM

During a sample collection visit to a farm, the farmer noted infected yolk sacs in younger chicks used as feed when eviscerated before feeding. The condition is known as Omphalitis, characterized by infected yolk sacs, often accompanied by unhealed navels in young birds. It is infectious but noncontagious and is associated with poor regulation of incubation temperature or humidity. The infected yolk sacs were collected and sent in for microbiological analysis at Onderstepoort Paraclinical labs. The results from the analysis indicated an abundance of microorganisms including; *Salmonella spp., Staphylococcus saprophyticus, Staphylococcus gallinarum* and *E. coli.*

De Wet, A. H., Myburgh, J. G., Duncan, N.M., (2020) 'Evaluation of the quality of chicken carcasses used as feed for Nile crocodiles on commercial farms' *Exotic Leather Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.* January 2020

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Faculty of Veterinary Science
Animal Ethics Committee

4 August 2020

**Approval Certificate**
**New Application**

| | |
|---|---|
| **AEC Reference No.:** | REC046-20 |
| **Title:** | Evaluation of the quality of chicken carcasses used as feed for Nile crocodiles on commercial farms |
| **Researcher:** | Mr AH de Wet |
| **Student's Supervisor:** | Prof JG Myburg |

Dear Mr AH de Wet,

The **New Application** as supported by documents received between 2020-06-03 and 2020-07-27 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-07-27.

Please note the following about your ethics approval:

1. The use of species is approved:

| Species and Samples | Number |
|---|---|
| Poultry | 45 |

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-08-04.

3. Please remember to use your protocol number (REC046-20) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

**Ethics approval is subject to the following:**

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

**Prof V Naidoo**
**CHAIRMAN: UP-Animal Ethics Committee**

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