Evaluation of the optical density reading for the determination of shelf life of whole chicken carcasses by the enumeration of the microbial contamination levels.

BY

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MOTIVATION

Although there are about 550-million broiler chickens slaughtered per year in South Africa, there are no scientifically based standards to determine the shelf life of fresh and frozen chickens at time of slaughter. The quality, safety and storage life of saleable poultry carcasses are of major concern to the consumers and the manufactures. The modern processing plants cannot produce products free of pathogens or free of spoilage microorganisms. These microorganisms result in outbreaks of the foodborne diseases and spoilage of the products if they are not controlled at a minimum level during slaughter. Many proposals have been suggested and applied to reduce the microbial contamination levels on slaughtered products. These included cleaning and sanitation of the equipment, usage of potable water in all water requiring processing steps, addition of preservatives and the application of different packaging methods.

Salmonella spp., *Staphylococcus aureus* and *Escherichia coli* are typical foodborne pathogens associated with poultry. Spoilage bacteria most frequently associated with poultry processing are *Pseudomonas* spp., *Acinetobacter* spp., *Moraxella* spp., *Alteromonas putrefaciens*, *Corynebacterium spp.*, *Flavobacterium spp.*, *Micrococcus spp.* and *Enterococcus spp.* (Bryan, 1980). Yeasts do not play a similar important role as bacteria in the spoilage of poultry carcasses.

Pienaar *et al*. (1994; 1995) established a technique to rapidly enumerate bacteria on hatching eggs, by determining optical density (OD) at a wavelength of 540 nm after 6 h incubation period. It was demonstrated that the OD reading after 6 h incubation correlates to the bacterial plate counts at the start of incubation. The usage of the method resulted in considerable cost saving in media and time when applied on hatching eggs. For chicken carcasses it a different environment for microorganisms as compared to the eggs. It is therefore possible that a different new incubation time might be required to achieve the logarithmic phase and therefore it will be determined in the first part of this project.

The application of optical density as a means to enumerate bacterial contaminants on chicken carcasses was initiated by focusing on the isolation and identification of pre-dominant bacteria species associated with processed poultry carcasses. The primary objective was to establish the OD methods as a microbial quantifying technique. This will be achieved only after the establishment of precise incubation time interval in the broth medium and determination of the repeatability of OD readings, and the correlation of OD to plate counts. Finally, the method will be evaluated on processed chicken carcasses.

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CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

Poultry products are the major dietary items for a large proportion of the South African population (Bok *et al.*, 1986). The meat, however, renders a suitable substrate for microbial growth as the edible portion of the carcass contains ca. 70% water, has a water activity of 0.98 to 0.99, a protein content of ca. 20.5 % and a fat content of ca 9.5% (Bryan, 1980).

The increase in consumption of poultry products has resulted in an increase of poultry associated foodborne diseases (Todd, 1980). The modern processing plants cannot render pathogen or spoilage microorganisms free products. These microorganisms should be minimized before the final product is distributed to avoid outbreaks of the foodborne diseases and shortened shelf life of the products. The spoilage microorganisms cause consumers to reject the product due to, appearance, off odour or undesirable flavour whereas the pathogenic microorganisms may lead to health hazards. Many proposals have been suggested and applied to reduce the microbial contamination levels on slaughtered products; these include mostly the preservation means, which include chilling or freezing, canning (cooked), curing and drying (Brune & Cunningham, 1971; Ingram & Simonsen, 1980).

Spoilage and pathogenic organisms are introduced into the slaughter plant in large numbers on the skin and feathers of the birds. Faecal contamination is also present on the feet, breasts and backs of many birds, contributing to the presence of high numbers of mesophilic bacteria. The muscle tissues of the birds are generally sterile (Bailey *et al.,* 1987; Mead, 1989; Nottingham, 1982). The microorganisms present on the surface of the birds are transferred onto the skin, into both the abdominal cavity and the cut muscle surfaces. In

addition, various undesired organisms may also derive from the processing equipment and environment (Barnes, 1973).

The most serious disease causing microorganisms belong to the genus *Salmonella.* Other undesirable organisms, which also could cause adverse reactions in the digestive systems of humans comprised *Escherichia coli, Staphylococcus aureus, Clostridium perfringens,* other coliforms and *Camphylobacter* spp. (Mead, 1989; 1994). Although these microorganisms are contained in low numbers on processed poultry carcasses, irregular practices such as temperature abuse during storage results in extensive growth of these pathogenic microorganisms (Mead, 1989).

It is essential that the bacterial contamination levels on poultry products be evaluated. Conventional enumeration methods in poultry carcass production are used on a large scale. These techniques include plate count techniques and most probable number (MPN) techniques. The former is mostly employed for routine monitoring of hygiene and the effect of the sanitary process. The drawbacks to these cultural methods include the fact that they are laborious, time consuming and expensive (Wood & Gibbs, 1982).

New rapid techniques such as impedance, conductivity or capacitance have been researched as alternative rapid methods to enumerate bacterial contamination (Chipley, 1987; Swanson, *et al.,* 2001; Wood & Gibbs, 1982). The drawback to these methods includes the cost involved in the purchase and maintenance of machinery. Other methods are ATP-bioluminescence assay, Limulus amebocyte lysate assays (Wood & Gibbs, 1982) and the microscopic method using the direct epifluorescent filter technique (DEFT). It is however essential to establish microbial enumeration methods that will yield microbial loads on carcasses before the product is sold, consumed or spoiled.

Optical density techniques have mostly been used to detect and measure microbial growth by means of determining the absorbency of the inoculated suspension over time (Chipley, 1987). Pienaar *et al*. (1994) used OD readings to determine the microbial load on hatching eggs. In this study, bacterial counts on hatching eggs were evaluated by reading the optical density at 540 nm after a 6 h incubation period. It was demonstrated that the OD reading after 6 h is directly related to the bacterial count at the start of incubation (Pienaar *et al.,* 1994).

2. PATHOGENS ASSOCIATED WITH POULTRY

If pathogenic bacteria are present at all on the processed carcass, they are represented at low numbers with heterogeneous distributions, requiring extensive sampling to have even modest confidence in negative results (Cason *et al*., 1997). The pathogenic organisms use two basic mechanisms to cause disease, by invasion of the body causing an infection and those that produce toxins (Jay, 1992; Mountney, 1976).

2.1 Salmonella

The primary habitat of *Salmonella* spp. is the intestinal tract of birds, reptiles, farm animals, humans and occasionally insects. The organism can be found on wide variety of environments. Organisms on other body parts can be the results of faecal contamination (Garbutt, 1997; Jay, 1992; krieg *et al.,* 1984).

Food of animal origin, especiall raw foods are vehicles of foodborne Salmonellosis, which is a disease resulting from ingestion of food containing appropriate serotypes of *Salmonella* spp. in significant numbers. Poultry and poultry products are mostly associated with this disease (Aho, 1992; Cox & Bailey, 1987; Jay, 1992). High numbers of the organism are required to produce disease symptoms (10^7-10^9cells/g), which last 2-3 days. However, even low numbers of certain *Salmonella* serotypes. can produce acute

gastroenteritis in children and adults, or acute enteritis in infants. The disease symptoms of Salmonellosis include abdominal pain, diarrhoea, dizziness, fever, headache, nausea, and vomiting. These disease symptoms normally occur 48 h after ingestion of food with the sufficient load of organisms (Jay, 1992).

Ingested organisms proceed through the alimentary tract, where interaction with the mucosal surfaces at the Payer's patches may occur, and penetration or adhering into the intestinal epithelial cells takes place. After proceeding through the intestinal wall and into deeper tissues, some *Salmonella* spp. can invade, survive and multiply in the mononuclear phagocytic system and disseminate to other tissues, causing serious systemic diseases by secreting endotoxins (Barrow *et al*., 1987; Garbutt, 1997).

It is essential to control *Salmonella* during chicken breeding (ICMSF, 2000). Dougherty (1976) found that feeds are frequently contaminated with *Salmonella*, but that breeder/multiplier flocks could also pass contamination to their progeny. The presence of any *Salmonella* in a production line represents a consumer risk and is an indicator for high chance of occurrence of more pathogenic serotypes (Palmu & Camelin, 1997).

Salmonella tends to concentrate in the chicken caeca without causing any symptoms of disease in the birds. The species are also introduced into a slaughter plant by the viscera and intestinal content, or on the outside of the bird by means of faecal contamination. *Salmonella* serotypes isolated from chickens before entering processing plant differ from those isolated from the processed carcasses, showing that carcasses are mostly contaminated by processing equipment (Fanelli *et al*., 1971; Linton *et al*., 1985; Mead, 1989; Mulder, 1995). The organisms show little tendency to multiply in the processing plant under normal circumstances and, in the view of their lower growth limit of *ca* 7° C this shows that they will be unable to grow on a chilled carcass (Mead, 1989). At 10°C the number of *Salmonella* spp. will double in about 11 h

(Bremner, 1977). These bacteria survive for some time in the scalding tank, depending on the temperature of the water within the scalding tank.

2.2 Listeria

Listeria is a ubiquitous environmental microorganism, often found in the faeces of animals (Gray & Killinger, 1966). *Listeria monocytogenes* causes serious and sometimes fatal diseases in humans. The species is widespread in the environment and is sometimes carried in the intestines of healthy animals. *Listeria* is capable of growth under chilled conditions (Gray & Killinger, 1966; Mead, 1994). In extreme cases of listeriosis, young chicks can develop disorders of the central nervous system as well as gross and histological lesions in the liver, spleen, heart and kidneys (Basher & Fowler, 1984). Healthy carriers of *Listeria* among chickens have been demonstrated (Dijkstra, 1987). This contamination could be carried into the processing plant. Cross-contamination at the mechanized slaughter line plays an important role in the spread of *Listeria* to carcasses (Bailey *et al*., 1989).

The ecological characteristics of the organism, its ability to multiply at low temperatures and over a broad pH range as well as at low water activity values enable it to multiply readily in the environment and in feeds. and foods (Skovgaard and Morgen, 1988).

2.3 Escherichia coli

E. coli is a normal inhabitant of the human intestinal tract and has also been isolated from lower intestinal tract of many warm-blooded animals. The association of *E. coli* as a source of foodborne disease resulting from ingestion of contaminated food appeared in 1970’s (Mehlman & Romero, 1982). Isolation of the organism in food is an indication of faecal contamination and the possibility of *Salmonella* spp. being present (Jay, 1992; Krieg *et al.,* 1984; Palumbo, 1986; Pattison, 1993). Diseases in poultry caused by *E. coli* infection,

are probably the most common and economically significant problems in broilers worldwide.

As a normal inhabitant of the intestinal tract, the numbers of *E. coli* in the environment of the poultry house can build up very rapidly. The organism survives better in dry conditions and is harboured in large numbers in litter and dust (Pattison, 1993). *E. coli* also infects chickens by the respiratory route. These respiratory infections result in haemorrhagic tracheitis and pneumonia, with air sacculitis involving the abdominal and thoracic air sacs (Pattison, 1993). According to Peighambari *et al.* (2000), the most frequent causes of *E. coli* infection are underlying infections with infectious bronchitis. Exposure to excess ammonia resulting from poor ventilation or overcrowding can disrupt mucosal barriers, impairs antibacterial defence systems and interferes with normal immune responses causing secondary *E. coli* infection (Awan & Matsumoto, 1997). Edens *et al*. (1997) indicated that *E. coli* type 1 and type 2 are capable of causing severe diarrhoea, dehydration, and mortality in birds kept on warm wet litter.

In humans, pathogenic *E. coli* can be divided into five groups according to their disease causing mechanisms. These groups include Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC) and Facultatively enteropathogenic *E. coli* (FEEC) (Jay, 1992; Krieg *et al.,* 1984; Palumbo, 1986). EPEC causes infantile diarrhoea; ETEC and EIEC cause diarrhoea in adults and children. Other diseases that take place in the human body, except for in the intestinal tract, include Neonatal meningitis, urinary tract injections and septecea (Krieg *et al.,* 1984). ETEC produces two primary toxins, thermo labile (TL), which is closely related to cholera toxins, and thermo stable (TS) (Krieg *et al.,* 1984; Jay, 1992).

2.4 *Staphylococcus aureus*

Staphylococcus species are normal inhabitants of the skin and mucous membrane of animals. In live birds, *Staphylococcus aureus* causes various diseases which include acute septicemia, chronic osteomyelitis, osteomyelitis and synovitis. Young chicks are more likely to be infected by the two latter diseases (Bremner, 1977; Nairin, 1973; Skeeles, 1997). Chickens enter the processing plant carrying *S. aureus* in the bruised tissue infected lesions nasal site, skin surface and arthritic joints (Bryan, 1980). The scalding processing step during slaughter significantly reduces *S. aureus* levels (Gibbs *et al.,* 1978). The commonest source of carcass contamination is the human food handler during further processing or in the catering establishment, since humans carry the organism in noses and hands and it is difficult to remove all of them by ordinary washing.

In humans, intoxification is caused by ingestion of food that has enterotoxins, produced by growing *S. aureus.* A period for growth in the food, however, is needed before sufficient toxin is produced to cause disease. Symptoms occur 2-6 h after consumption of food consisting of endotoxins. These symptoms include nausea, vomiting, abdominal cramps (mostly severe), diarrhoea, sweating, headache, prostration and sometime a fall in temperature (Garbutt, 1997; Jay, 1992). These organisms can produce toxin at temperatures above 18°C (Kraft, 1986).

Staphylococci will not multiply at a temperature below 7°C and at 10°C multiplication is very slow, the optimum temperature for growth being 35 – 39°C, which is also the optimum for production of toxin (Bremner, 1977).

3. MICROORGANISMS ASSOCIATED WITH SPOILAGE

Microbiological spoilage of poultry results in economic losses to retailers and processors. It is therefore important to increase the product shelf life by proper

handling and processing methods. Spoilage bacteria are able to grow on the surfaces of cut muscle tissue and therefore the temperature of the product must be kept as low as possible (Mead, 1982).

3.1 Bacteria

Mesophilic bacteria dominate the surface of freshly processed carcasses but the reduction in temperature during chilling retards the growth of these microorganisms. Consequently psychrothrophs become dominant after few days of storage at refrigerated temperatures (Nottingham, 1982). Spoilage bacteria most frequently associated with poultry processing are species from the genera *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Vibrio, Corynebacterium*, *Flavobacterium*, *Micrococcus, Enterococcus, Alcaligenes, Cytophaga*, *Aeromonas, Serratia* and *Alteromonas putrefaciens* (Bryan, 1980; Cousin, *et al.,* 2001). *Pseudomonas* has been reported as the major spoilage microorganism (Barnes & Impey, 1968; Cox *et al*., 1975; Hamilton & Ahmad, 1992). The storage environment determines the predominant microorganism on the carcass. In an aerobic environment *Pseudomonas* spp. predominate, in anaerobic conditions slow growing lactic acid bacteria like *Lactobacillus* spp. become more dominant whereas *Brochothrix thermosphacta* predominates in drier parts of the chicken carcass (Eburne & Prentice, 1994; Kraft, 1986; Nottingham, 1982).

Spoilage microorganisms are mainly restricted to the surface of the chickens, and therefore the inner portions are generally considered as sterile or contain few organisms (Jay, 1992). These surface related microorganisms are spread over the skin (during scalding and plucking) and on the inner and outer carcass surfaces (during evisceration and further processing) and may lead to the spoilage of the product (McKeekin, 1982). It is necessary to keep the initial bacterial numbers as low as possible to improve the shelf life of the product, since psychrotrophic bacteria continue to multiply during chilled storage (Mead, 1989). Off-odours become noticeable when total counts reach about 10^7

bacterial cells per cm² of skin (Mead, 1982; Mead, 1989; Mienlik *et al.*, 1999) and slime formation occurs when total counts exceed 10^9 viable bacterial cells per cm² (Mead, 1982).

3.2 Yeasts

Yeasts are generally not considered to be of major importance in the spoilage of meat products since their numbers in these products are highly variable relative to bacterial numbers (Jay & Margitic, 1981). Despite the predominance of bacterial loads, the increases in yeast numbers clearly indicate that the yeasts also contribute to the overall microbial ecology and therefore may also play a substantial role during spoilage (Laubscher *et al.*, 2000). Barnes *et al.* (1978), however, reported a large increase in the numbers and proportions of yeasts present on spoiled, polyethylene-wrapped, air chilled turkey carcasses stored at –2ºC. According to Kobatake *et al.* (1992), species of *Candida*, *Cryptococcus*, *Debaryomyces*, *Yarrowia* and *Trichosporon* are associated with the spoilage of poultry. Laubscher *et al.* (2000) however, added *Bullera*, *Rhodotorula* and *Zygosaccharomyces*. Laubscher *et al.* (2000) reported that the major difference in yeast counts and bacterial counts might be due to competitive interaction with the gut flora and therefore only representing the stable yeasts within the trachea of the adult chicken.

4. POULTRY PROCESSING STEPS AND THEIR CONTRIBUTION TO PRODUCT CONTAMINATION

Various steps in the processing plants are recognized as potential sources of contamination. Unloading, stunning, slaughtering, and bleeding are not considered critical control points, but scalding below 60ºC; defeathering machines and chilling are critical and may be responsible for contamination (Simonsen, 1989). Each step contributes differently to the level of contamination of the birds (Mead *et al.*, 1994). Air, workers, water and equipment surface play a major role in contamination and cross contamination of poultry carcasses. The two latter will be discussed later in this review. From

the public health point of view, it is desirable to limit the proportion of carcasses that become contaminated with food-borne pathogens and minimize any contamination on the final product.

4.1 Air

Microbiological contaminants occur in the air as aerosols, defined as solid or liquid particles suspended in the air. Coliforms and *Salmonella* spp. are frequently observed from air samples taken in the vicinity where live birds are hung, killed, scalded and picked (Zottola *et al.*, 1970). The contaminated air, especially in the dirty area, is attributed to the scattering of feathers at the defeathering units (Patterson, 1972), whereas the air in the clean area is generally less contaminated. *Micrococcus* spp., Enterobacteriaceae and *Corynebacterium* spp. are common air contaminants associated with spoilage (Geornaras *et al*., 1998).

4.2 Workers

Workers shed microorganisms from their skin, hair, nose and throat as they work. Workers with cuts or other lesions are greater sources of pathogenic contamination to the birds as they may become infected and bring the organisms to the processing plant. The hygiene of workers is essential in poultry processing (Bryan, 1980).

4.3 Scalding

The primary purpose of scalding the chicken is to enhance feather removal from the birds. After bleeding, the birds are either scalded by spray scalding, stem or hot water immersion scalding. The hot water immersion is the most widely used method (Bryan, 1980). This scalding method is more problematic because the birds carry large numbers of microorganisms on the skin, feathers and in the faeces, and many of these organisms enter the water as the birds are moved continuously through the tank. Water replenishment controls and the high scalding temperature reduce the number of microorganisms, however,

it does not eliminate all microorganisms (Mulder & Dorresteijn, 1977). Pathogenic microorganisms isolated on poultry carcasses immediately after scalding are *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens* and *Staphylococcus aureus.* The psychrotrophic microorganisms are *Microccocus* spp., *Proteus* spp., *Pseudomonas* spp., and *Streptococcus* spp. (Lillard, 1971; Mead & Imprey, 1970; Surkiewicz *et al*., 1969; Walker & Ayres, 1956).

Scalding temperature determines the immersion time. The temperature is chosen with respect to the way that the product is packed and distributed. Fresh chilled market requires 50-51.5ºC for 3 min (soft scalding) or 56-60ºC for 2-2.5 min (hard scalding). High temperature scalding reduces the bacterial counts significantly, but it removes the cuticle, hence making the carcass more susceptible to contamination during further processing, especially during plucking (Bailey *et al.,* 1987; Lillard, 1973).

4.4 Plucking (defeathering)

Each processing step contributes differently to the amount and the type of organisms, which contaminate the carcasses. Plucking is a major contributor to cross contamination (Kaufman *et al*., 1972). This process involves the use of a plucking machine to remove feathers from the skin.

Salmonella spp. are more often isolated from carcasses after plucking than any other processing steps (Notermans *et al*., 1975). Rubber fingers are the main source of microbial contamination in the “dirty” area due to the warm, humid condition provided by the scalding process, thus allowing microbial growth in channels and cracks (Mead & Dodd, 1990). *Micrococcus* spp. are predominantly associated with the rubber fingers (Geornaras *et al.,* 1998).

4.5 Evisceration

In poultry processing plants, chickens are eviscerated either manually or automatically. Simonsen, (1975) found that manual evisceration and opening of

the abdominal cavity give rise to considerable cross contamination especially if the intestine is accidentally cut or rupture. Intestinal contents can directly contaminate the carcass when opening of the carcass is performed in the wrong manner and pulling on the intestines until the breakage occurs (Notermans *et al.,* 1980). Evisceration equipment and hands transfer microorganisms from carcass to carcass (Galton *et al*., 1955; Wilder & MacCready, 1966).

During evisceration of the carcass the number of *Enterobacteriaceae* and *Salmonella* organisms increase (Notermans *et al.,* 1980). *Salmonella* contamination results from caecal and intestinal colonization in the bird (Ramirez *et al*., 1997). Hargis *et al*. (1995) reported that the crop is an important potential source of *Salmonella* contamination on a broiler carcass. The crop has a higher incidence of rupture than the caeca. Feed withdrawal on the production site, 18 to 24 h before slaughter, is used to clear the intestinal tract of ingest and to facilitate evisceration and reduce microbial contamination of equipment and carcasses (Biligil, 1988; Humphrey *et al*., 1993). This was, however found to increase the chance of *Salmonella* contamination because feed withdrawal increases the number of *Salmonella* in the crop whilst caeca *Salmonella* counts remain almost the same between chickens with feed withdrawal and those chickens that were fed (Corrier, 1999).

4.6 Spray washing after evisceration and picking

Mulder & Veerkamp (1974) reported that although live poultry are contaminated with microorganisms, the slaughtered end product has a comparative low bacterial count, because the carcasses are cleaned during processing. Efficient spray washing, using high-pressure jets, after evisceration and final inspection of the carcasses is carried out primarily to ensure that the birds are hygienically clean.

Washing results in a reduction of between 50 to 90% in the aerobic plate counts, *Enterobacteriaceae*, coliforms and *Salmonella* counts. Notermans *et al.* (1980) found contaminating microorganisms attach to the skin during evisceration and cannot be removed by washing alone even though attachment is a time dependent process. Spray washing at different intervals is beneficial in reducing coliforms and *Salmonella* on carcasses because these organisms require longer attachment time.

4.7 Chilling

The chilling process is the final last step before packaging. This involves the reduction of chicken carcasses temperature to 0-4°C by means of the spin chiller. The temperature reduction is essential for delaying the growth of psychrotrophic bacteria and preventing growth of most foodborne pathogen microorganisms (Veerkamp, 1989).

Carcasses can be chilled by immersion in a water tank chilling system or air chilling system depending on the country's legislation or industrial preference. Most European countries prefer air chilling whilst the U.S.A would rather use the water immersion chilling. Water chilling is also a common practice in South Africa. Each chilling method has different disadvantages as well as advantages (Jones & Grey, 1989). With air chilling, the carcasses are usually sold as fresh chilled products, while water chilled carcasses can be sold either fresh or frozen (Barnes, 1973).

Irrespective of the chilling method used, once the carcass is cooled to about 10°C the pathogenic bacteria will be unable to multiply significantly during the actual chilling process, although the ultimate storage temperature should be less than 5°C. Any increases in bacterial counts will be the result of contamination by environmental factors (Barnes, 1973).

4.7.1 Immersion chilling

Many models of immersion spin chillers are designed and installed in poultry industries. The primitive use of ice as coolant is disappearing, as the method involved chilling the broiler carcasses for 4-24 h. During this time the environment become favourable for psychotrophic growth due to the moisture pickup. Growth can occur both on the carcass and in the water under these conditions. Addition of chlorine can reduce the microbial load during this process and improves the sanitation process in the processing plant (Veerkamp, 1989).

In the immersion chilling system, carcasses are moved through the system by mechanical rakes, rotating paddles and sometimes assisted by water flow provided by circulation pumps whilst injection of air is use to improve the agitation. This is done in order to increase heat transfer and control water up take by carcasses (Veerkamp, 1989).

May (1974) reported that the European Economics Community banned the use of immersion chilling in 1974 based on cross contamination results, only to be reinstated in 1977. May (1974) found a reduction of microbial counts in the continuous immersion chiller of 81 to 91% in a study conducted at three processing plants. With this analysis, May (1974) concluded that immersion chilling resulted in less cross contamination when compared to evisceration and defeathering.

In-plant sanitation, at 5-20 ppm available chlorine can be used to control the spread of microorganisms throughout the processing plant (Barnes, 1973; Mead *et al.,* 1975). The chilling tanks are difficult to clean and disinfect effectively thus making the chilling tank a potential source of contamination.

4.7.2 Air chilling

An alternative method to water immersion chilling is air chilling. Air chilling involves a preliminary rapid chilling in a blast tunnel followed by storing in a chill room. This process has the tendency to dry the skin and decrease water activity, which results in reduction of bacterial counts. Many moulds and yeasts will, however grow at lower relative humidity than bacteria. During air chilling, carcasses may become contaminated from the circulating air, particularly if the tunnels are contaminated (Barnes, 1973; Brant, 1974; Mossel & Ingram, 1955). Air chilling requires scalding at 52ºC, as this temperature prevents outer skin layer removal during plugging, and skin discoloration does not occur (Mienlik *et al.*, 1999). This method was installed as a way to reduce cross contamination (Brant, 1974). Mead *et al.* (2000), however, found cross contamination to occur in the air chilling system. The arrangement of the shackle line, if ran at two levels, allows carcasses on the higher levels to drip on those below thus contaminating the lower level of birds (Mead *et al.,* 2000). The air chilling method involves spraying the carcasses with a thin film of water and blowing cold air at low temperature onto carcasses, causing evaporation and hence chilling. This method is able to achieve quick and effective chilling because of the capacity of water to transfer heat (Mienlik et al., 1999).

5. MEASURES TO REDUCE MICROBIAL CONTAMINANTS ON CARCASSES

Many proposals have been suggested and applied to reduce the microbial contamination levels on slaughtered products. The most important of these methods are regular cleaning and sanitary practices in the processing plants and the use of potable water.

5.1 Cleaning and sanitation of the equipment surfaces

Regular schedules of cleaning and disinfection of the plant and equipment are essential for maintaining sanitary conditions. Cleaning routines must include

cleaning of all interior surfaces of the building, cleaning of all equipment, removal of all waste and garbage (Guthrie, 1972; Bryan, 1980).

5.2 Potable water

The high microbial counts in the scalding tank represents a major site of contamination attributed to the microbial loads on the external surfaces of the birds entering the scalding tank (Mulder & Veerkamp, 1974). Predominant isolates associated with the scalding tank water include *Micrococcus* spp. *Enterobacteriaceae* and lactic acid bacteria (Geornaras *et al*., 1998). According to Mulder & Veerkamp (1974), gram-positive bacteria are the main bacterial group present in the water of the scalding tank and their survival depends on the scalding temperature. The water within the spin chiller should not be a source of contamination if the chlorine levels and temperature of the water are controlled.

The use of heavily polluted or contaminated water in plant maintenance in equipment and utensil cleaning may well be a source of contamination of the plant (Guthrie, 1972).

6. SAMPLING OF POULTRY CARCASSES

Samples can be used for the isolation or enumeration of microorganisms from original environment to laboratory media. Recent sampling methods are focused on recovery of the surface contaminants on chicken carcasses (Chipley, 1987; Notermans *et al.,* 1975). Microorganisms may also be embedded in the feather follicle on the outer skin of carcasses. To release embedded microorganisms form these areas requires preliminary treatment of the carcass or sample into a fluid medium and enough force to dislodge the microorganisms (Thatcher & Clack, 1968). The ideal sampling methods must be simple, non-destructive, reproducible and estimate potential shelf life (Patterson, 1972).

Microbial evaluation at a poultry plant can be done on the equipment surface and on the chicken carcass. Evaluation on the equipment is done primarily for hygiene control and sanitary tests. Swabs, contact plates and adhesive tapes are used for equipment sampling (Bryan, 1980; Simonsen, 1989).

There are numerous methods to remove microorganisms from the carcass surface (Bryan, 1980; Simonsen, 1989). Commonly used methods on chicken carcasses include whole carcass rinsed, stomaching or blending of excised skin techniques and swabbing with cotton or alginate swab. Swabbing and tissue excision are also essential for sampling other meat products (Lillard, 1988; Bryan, 1980; Thomson *et al.,* 1976; Ransom *et al.,* 2002). Other alternative sampling methods for chicken carcasses employ spot plate, direct contact plating, added sampling, drip, spray maceration of skin, agar contact, skin scraping and sampling commuted poultry products (Avens & Miller, 1970; Brune & Cunningham, 1971; Bryan, 1980; Gill *et al.,* 2001). Only methods that are widely used and acceptable are briefly discussed in this review, which include whole carcass rinse, maceration of excised tissue and surface swab techniques.

It is more efficient to take samples at different intervals during the day than sampling a single large sample (Collins *et al.,* 1989). Samples for microbial analysis in a laboratory, which is some distance from the processing plant, are always transported in an insulated container cooled with ice packs and is refrigerated at the laboratory pending examination.

6.1 Swabs

Swab methods are the oldest technique used for sampling food, utensils, equipment, walls and floor in the processing plant (Chipley, 1987). Swabs are sterilized by heat, prior to use. Sterile swabs are now commercially available (Brune & Cunningham, 1971).

The swab method only recovers loosely attached bacteria on the exposed chicken carcass hence some cells are retained and this reduces counts (Avens & Miller, 1970; Bryan, 1980).

The commonly used swab methods employ cotton wool or calcium alginate swabs (Bryan, 1980; Chipley, 1987). The former has higher preference, requiring two different bacterial transfers; the first transfer is from the chicken sample to the cotton swab and the second transfer is from the swab to the diluents. Both transfers do not recover all the bacteria. Approximately 50% of the bacteria are recovered with the first transfer (Chipley, 1987; Fromm, 1959; Patterson, 1972). Alginate swabs release all microorganisms from the swab into the diluents because it dissolves.

The common procedure employs sterile swabs, which are pre-moisten in the diluents. The swab stick is then pressed on the side of the tube to remove excess liquid. The swab is rubbed on a definite area to be sampled in a sterile template. The same area is swabbed three times in each direction, rotating the swab in the process. The swab stick is aseptically broken into 10 ml buffer or saline, then vortexed. The suspension is then serially diluted and platted on a suitable medium (Avens & Miller, 1970; Brune & Cunningham 1971; Chipley, 1987). Alginate swabs are used in the same way as the cotton swabs except that they require 9 ml diluents and 1 ml of 10% sodium hexameta phosphate and shaken until it dissolves.

6.2 Whole carcass rinse

The whole carcass rinse technique is a non-destructive method (Lillard, 1988). This method is practical for sampling the entire surface of relatively irregular shaped samples. Whole carcass rinse technique requires immersion of a whole chicken carcass in diluents within a closed container followed by vigorous shaking. Large samples (turkey) are difficult to rinse because of the large volumes of sterile diluents required and the difficulty encountered during

shaking (Chipley, 1987). Conventional practice requires large volumes of diluents, hence research has been implemented to improve the rinse method by focusing on reducing the amount of the diluents used. Dougherty (1974), used 300 ml of lactose pre-enrichment broth and shaking the carcass for approximately 2 min and using 5-10 ml of the aliquot. Cox *et al.* (1981) reported the lowest volume of 100 ml diluents used for the whole carcass rinse technique. They described the procedure as placing chickens individually into sterile polyethylene bags with 100 ml of sterile diluent and shaken vigorously for 1 min. After this the carcasses are lifted from the fluid in the bag and the aliquot fluid drained for 15 min into a screw cap milk bottle, leaving the carcass in the plastic bag. There after the aliquot liquid is microbiologically analyzed by doing the plate counts. Using mechanical shakers, as suggested by Dickens *et al.* (1985) reduce variations in results obtained when using the whole carcass rinse method.

The whole rinse method is essential for recovery of the microbial population that are present on a carcass in low numbers while other sampling methods have low sensitivity for detection of *Salmonella* spp. (Dougherty, 1974).

6.3 Stomaching

Tissue excision followed by stomaching, estimates the bacterial load with more accuracy and precision because it is able to recover organisms that are firmly attached as well as those that are in the feather follicles (Avens & Miller, 1970; Fromm, 1959). After stomaching, the majority of bacterial cells are not firmly attached to large food particles (Pettipher & Rodriguez, 1982). Stomaching is achieved by the method described by Lillard (1988) where known amounts of skin portions are excised by aseptically holding the skin with sterile forceps and cutting with a sterile scissor. The skin portion is placed in the sterile stomacher bag and mixed with a suitable diluent and macerated for some time. The time and amount of diluent used depend on the amount of skin excised (Avens & Miller, 1970).

The choice of the non-destructive excision area is very important; these include the neck skin and the skin at the rear end. More bacteria are recovered from the neck skin when compared to the lateral skin and peri-cloacal skin when analysis was performed by dip and skin maceration at different processing levels (Noterman *et al.,* 1975). More *Salmonella* spp. were recovered by neck skin samples compared to whole carcass rinse and whole carcass rinse plus whole skin samples. However, no significant difference in *Salmonella* spp. isolation rates from either neck-skin only or carcass-rinse plus neck-skin samples was found (Jorgensen *et al*., 2002).

It is more efficient to take small samples at different intervals per day than sampling one large number of samples. General practice of this method requires emulsification of 10 g sample in a heavy-duty plastic bag homogenizing in 90 ml of diluent (usually 1% peptone water) in a stomacher machine (Collins *et al*., 1989), through the action of paddles.

Collins *et al*. (1989) reported maceration of weighed tissue as a destructive method. Emswiler *et al.* (1977) mentioned few advantages and disadvantages of stomaching over blending, which include less labour, low costs involved in cleaning and sterilization of reusable blender jars and blades.

7. MICROBIAL ENUMERATION METHODS FOR POULTRY PRODUCTS

Microbial detection and enumeration are important for assessing food safety and shelf life determination of meat products. Conventional methods are time consuming and labour intensive to meet the industrial criteria for distributing the quality assured products in time (Qvist & Jakobsen, 1985). The conventional methods have the advantages of being able to detect low cell concentration and are also simple and easy to perform. The conventional methods are reasonably sensitive, accurate and not expensive.

7.1 Cultural enumeration methods for poultry products

The cultural methods that are mostly used for determining microbial loads on poultry carcasses include plate counts and MPN methods. These methods yield counts only after a period of incubation, usually over night, implying that these methods are time consuming. Counting of psychrotrophic microorganisms are even more time consuming as they require between 7-10 days incubation before reliable results are obtained (Kraft, 1986). The method was improved by using 25°C for 24-48 h (Russell *et al.*, 1996). Current research is aimed at finding better methods for the enumeration of bacteria on poultry carcasses than the methods described above.

7.1.1 Plate count methods

Many microbiologists are searching for alternative techniques that can reduce the time and effort involved in microbial enumeration. However, colony counts are still the prevalently used methods (Wood & Gibbs, 1982).

The plate count techniques are based on two assumptions: firstly, each microbial cell in a sample will form a separate, visible colony when mixed with solidified medium that permits growth after sufficient incubation (Chipley, 1987; Swanson *et al.,* 2001). The drawback to this assumption is that microorganisms do not always exist as single cells, but can exist in closely associated pairs, clumps, clusters or chains. Based on these, the counts are reported as colony forming units (cfu). During preparation of the sample, shaking and vortex do not completely disrupt these clumps. Blenders have been found to provide better breakdown of clumps (Swanson *et al*., 2001). Second assumption state: all microorganisms evaluated for total count will grow on a single agar medium incubated under one set of condition (Chipley, 1987). However, microorganisms have different growth requirements, which include nutrients, environmental factors, injured cells, hence there is no universal medium that can support growth of all microorganisms under one set of condition (Chipley, 1987; Swanson *et al.,* 2001).

There are numerous plating methods that allow the counting of separate colonies. Spread plate and pour plate are preferred over other alternative colony counting methods (Meynell & Meynell, 1965; Swanson *et al.,* 2001). The former is preferred because of advantages like the ability to isolate colonies for further identification and the temperature of molten agar for pour plate purposes might injure or kill bacterial cells, especially psychrotrophic microorganisms.

General procedures of the plate count methods include sample preparation, labeling of petri dishes and dilution of the sample. From these steps onwards the methods differ with respect of whether spread plate or pour plate method is used. The spread plate technique employs spreading of a measured amount of the suspension on the surface of a well-dried solid medium using a sterile glass rod. In the pour plate method, the measured amount of the suspension is mixed with molten agar in a petri dish at the temperature slightly above 48°C, and immediately distributed to cover the whole area of a petri dish. After the medium has solidified, it is incubated at appropriate conditions (Harrigan, 1998; Kraft, 1986; Meynell & Meynell, 1965; Swanson *et al.,* 2001).

Inhibitory substances on the glassware may result in underestimation of counts. In case where more than one species are grown in general medium products, excretion of inhibiting substances by competitive microorganisms in the agar medium might also result in an underestimation of the bacterial count. Most of the errors originate from the analyst fatigue and competence. Colony counts also depend on the type of media, time and temperature of incubation (Muller & Hildebrandt, 1990a; Muller & Hildebrandt, 1990b; Swanson *et al.,* 2001).

7.1.2 Most probable number (MPN) method

The MPN technique is an estimation method, which involves mathematical counts of viable cells from a fraction of culture that failed to show growth in a

series of tubes containing a suitable medium (Cochran, 1950; Koch, 1970; Herbert, 1990). This dilution technique indirectly determines the microbial density in a liquid medium. The MPN technique is based on two assumptions: Firstly, the microorganisms are distributed randomly throughout the liquid medium, implying that the liquid mixture is thoroughly mixed. Shaking or vortex is applied to enhance mixing. The second assumption is that an incubation medium will exhibit growth at appropriate conditions, even if there is only one cell in the medium (Cochran, 1950). Diluting samples leads to an uneven distribution of microorganisms within the sample, which means the higher the dilution, the higher the error, which occurs with the estimate (Swanson *et al*., 2001).

Between three to five replicate tubes are prepared, diluted and incubated and the results are obtained by counting the number of positive tubes from each dilution (Herbert, 1990). The MPN method is more sensitive and yields higher counts compared to plate counts, even though the method is more variable (Herbert, 1990; Koch, 1970; Swanson *et al.,* 2001). The method can detect microorganisms at the level below 10 cfu/g (Koch, 1970). Koch (1970) reported that the accuracy of the technique is low at the range of 8-36% and 5-50% (of tube with growth) if the large range of error is acceptable.

The MPN method is laborious, though employing automated machines, such as the machines that fill plastic wells, can reduce labour, by scanning wells with microbial growth microbial growth (Koch, 1970).

The method is preferred compared to the plate count technique in the following situations: i) When the organism of concern cannot be cultured on solid medium, ii) When the organisms in the mixed culture contain organisms that grow at different rates, iii) Where there is no selective method for an organism of interest, provided that the organism can produce detectable products (acid production, antibiotics or coloured materials or iv) When agar consists of

altering contents that would influence the counts (Koch, 1970).

8. RAPID ENUMERATION METHODS

Interest in these methods has grown because they show promise in saving time, although most of these methods are expensive. The rapid methods are normally sensetive over a narrow range of microbial counts (Wood and Gibbs, 1982). The ideal method would also be easy to perfom. Other rapid methods, which involve the use of molecular techniquers are aspects of future study and will be discussed later.
A technique such as DEFT- requires only 35-45 min to obtain the results. However, this technique is laborious and difficult to perform since it require many steps which include, pre-filtration; enzyme and surfactants treatment, filtration an staining; and microscopic counting (Shaw and Farr, 1989). ATP methods are very sensitive and are able to detect between 100 cells/ml and 1000 cells/ml. The method detects the ATP produced by bacteria (Avis and Smith, 1989). Chicken carcasses also, however posess ATP and this will affect the results.

The OD method is rapid, easy to perform and sensitive. This method has the drawback of a deviation from a linearity response at high microbial concentrations and its not suitable for aggetative organisms. Turbit medium and samples with suspended particle are also difficult to process with a OD method.

8.1 Direct epifluorescent filter technique (DEFT)

The technique enumerates the flourochrome stained bacteria collected on a membrane filter. The suspension requires a pre-filtration and pre-treatment with enzymes to minimize debris (Bier *et al.,* 2001; Herbert, 1990; Pettipher & Rodrigues, 1982; Shaw *et al.,* 1987). Membrane filtration is used to concentrate material for analysis by collecting it on the filter surface (Bier *et al.,* 2001).

DEFT methodology differs depending upon the food type, microorganisms of interest and whether cells or micro colonies are to be enumerated. Various food suspensions require different pre-filtration and pre-treatment to allow the passage through the membrane filter (Bier *et al.,* 2001). Shaw *et al.* (1987) divided the procedure of the DEFT into five stages, which include:

A) Preparation of sample suspension – The preparation of the sample depends on the sampling technique used. In most studies of DEFT for microbial assessment on meat and poultry products, excision of skin tissues and stomaching have been the most applied methods (Bier *et al.,* 2002; Pettipher & Rodrigues, 1982; Qvist & Jakobsen, 1985; Rodrigues & Kroll, 1985).

B) Pre-filtration – this achieves removal of large particles from the sample suspension. Qvist & Jackobson, (1985) allowed the sample to stand for 30 min prior to filtering to allow large particle to settle and reduce chances of membrane filter blockages. On the other hand, Shaw *et al.* (1987) waited only 2-3 min. The pre-filter method makes use of 25 mm diameter 5 μm polypropylene filter discs, which are mounted in swinnex filter holders.

C) Enzyme and surfactant treatment, which are trypsin and Triton X-100 respectively, in order to lyses muscle cells, disperse proteins and dissolve fat globules.

D) Filtration and staining – the suspension is filtered for the second time with smaller filters and then stained with acridine orange stain and rinsed with citrate NaOH buffer (0.1M; pH 3) and isopropanol (Shaw *et al.,* 1987).

E) Counting – the acridine-orange stained preparation obtains counts by using the epifluorescent microscope. The number of microscopic fields enumerated depends on the number of cells per microscopic field for accuracy of the results (Shaw *et al.,* 1987). The use of an automated microscopic count usually requires counting about 20 fields. Manual conductance of DEFT is limited to 20 samples per person due to

analyst's fatigue (Qvist & Jakobsen, 1985). DEFT has lower detection limits of 6000 per ml for manual count and 15000 per ml for automated counts (Rodrigues & Kroll, 1985).

With DEFT, the results can be obtained in less than 40 min (Herbert, 1990; Rodrigues & Kroll, 1985). DEFT can be applied as an identification step to determine morphology and differentiation by gram staining. Rodrigues & Kroll (1985) differentiated and enumerated gram positive and negative bacteria by using acridine orange as a counter stain.

8.2 Photometry

This is a non-destructive, accurate, quick and easy to perform enumeration procedure (Chipley, 1987; Madigan *et al.,* 2000). It only requires cuvettes containing aliquots of microbial suspension, be interposed between a unit source of light and photocell, which is attached to a galvanometer. Photometry in microbial studies has been employed mostly for monitoring microbial growth and for construction of growth curves. This is possible because the techniques can be performed without destroying or significantly disturbing the sample, hence this technique can be used to follow microbial growth and can be used to mathematically calculate growth rates and yield coefficient of pure cultures. Microbial growth determination involves inoculation of suitable bacterial suspension in a growth medium, incubation and periodic determination of the increase in absorbance of resulting microbial suspension compared to a sterile control medium (Chipley 1987; Madigan *et al.,* 2000).

Light that reaches a solution can be absorbed, emitted or scattered. The light that is deflected by the suspension is called scattered light and the light that is able to transverse the solution is called transmitted light. Light absorption by the bacterial suspension is negligible, since most of the microorganisms are not pigmented, hence most of the light is scattered. Scattering of light in bacterial suspension is deflected from its original direction with only few degrees of

angle (Koch, 1994; Meynell & Meynell, 1965; Taysum, 1956). Scattering is dominated at cell surface, supported by experiments with strongly stained bacteria, where the amount of light entering the cell and available for absorption, including pigment, are small (Meynell & Meynell, 1965; Taysum, 1956).

The optical density of scattered light depends on size and shape of the bacterial cell as well as the wavelength used (Meynell & Meynell, 1965; Taysum, 1956). Visible and near infrared light dependence of optical density upon wavelength are slight but finite (Taysum, 1956). Turbidity is inversely proportional to the fourth power of the wavelength (yield a linear relation for many strains) suggesting that scattering agents are small compared to the wavelength of radiation in the medium (Taysum, 1956). Some of the transmitted light might be deflected but still able to reach the photocell as the light is only scattered at a small angle. Hence the use of a narrow photocell is of great significance (Koch, 1970; Meynell & Meynell, 1965).

When optical density values are plotted against standard plate counts during the exponential growth phase, the correlation is linear (Chipley, 1987). The amount of light scattered by bacterial suspension may be proportional to its concentration expressed as mass, number or mean cell length, depending on method used. At high bacterial concentration, transmitted light measurements are more effective while scattered light measurements are effective at low bacterial concentration (Meynell & Meynell, 1965).

Recent instruments, used to measure turbidity, include the spectrophotometer and photometer, which are applied to measure the transmitted light. The difference between these instruments is the methods used to generate incident light of the required wavelength. Photometers use simple filters (usually green, red or blue) while the spectrophotometer employs prisms or diffraction grating (Koch, 1970; Madigan *et al.,* 2000). For photometric methods to measure the

microbial numbers, standard curves are constructed by plotting optical density against cell numbers or dry biomass. High bacterial concentrations lead to loss of linearity due to one cell scattering light and the other cell re-scattering light back to the direction of the incident light (Madigan *et al.,* 2000).

Different photometric instrument have different OD readings for the same sample. Different growth conditions, cell shapes and cell sizes will also yield different OD readings. These factors must be considered during the construction of a growth curve for the rough estimation of microbial quantity (Koch, 1970). The most frequently used wavelength range between 420-600 nm (Koch, 1970; Meynell & Meynell, 1965). This method is subjected to errors such as variations in cell morphology, presence of clumps or clusters, particles in media and non-viable cells or fragments. These methods are quick and easy to perform and have also been used for studying bacterial morphology.

8.3 Impedance

Change in electrical impedance in a medium of growing culture has been implicated for detecting bacterial loads (Cady *et al.,* 1978). Impedance is the resistance to flow of the sinusoidal alternative electrical current through a conducting material, and has a complex entity composed of resistive (conductance) components and reactive (capacitance) components (Entis *et al.,* 2001; Firsternberg-Eden, 1983). The impedance technique is based on the assumption that microorganisms growing in an appropriate medium produce chemical changes that alter the electrical resistance of the solution (Cady *et al.,* 1978). These chemical changes result from microbial metabolism of big molecules to small molecules, resulting in a decrease in conductivity while impedance increases (Entis *et al.,* 2001; Firstenbeg-Eden, 1983). Passing a small electrical current through the culture medium and measuring impedance as the organism grow and metabolize, continuously monitors electrical impedance measurements. The current has negligible interference on growth of the organism (Entis *et al.,* 2001).

Microbial levels of between 10^6-10^7 organisms per ml, are significant to be noted by most impedance instruments (Firstenberg-Eden, 1983; Hardy *et al.,* 1977). Initial microbial estimation in food samples is achieved by recording the time required for the organisms in the sample to replicate to the threshold level (Hardy *et al*., 1977). The time required to reach threshold is called detection time (DT). DT is a function of the initial microbial level, type of medium used and the generation time of the organism of concern (Cady *et al.,* 1978, Hardy *et al.,* 1977). Samples with high microbial levels will reach the threshold rapidly, compared to those with lower concentration. The use of a procedure that involves a cut-off level for rejecting the product is explained and established in the study of Hardy *et al.* (1956) and Cady *et al.* (1978).

The impedance method is easy to perform, it requires placing a sample aliquot into the impedance measuring chamber and recording the DT (Hardy *et al.,* 1977). Instruments that are extensively used to determine impedance include Bactormeter, Rabit, Malthus and BacTrac (Entis *et al.,* 2001). Bactometer is the most used, hence it will be briefly discussed. These computer-monitoring devices measure capacitance, conductivity, or directly measure impedance. The instrument has the following components: computer, printer and Bactometer processing units. The processing units consist of two incubators, with the capacity to monitor 1-128 samples at temperature ranges of 10^oC-58^oC. The results obtained with this deviser can be applied for prediction of shelf life, monitoring environmental functions, perform microbial enumeration of yeasts, moulds, coliforms, lactic acid bacteria, *Escherichia coli* and detection of *Salmonella* and *Listeria* (Entis *et al.,* 2001).

The impedance method was shown to be a rapid alternative to standard plate counts in frozen vegetables (Hardy *et al.,* 1977), different milk products (Cady *et al.,* 1978; Firsternberg-Eden & Tricarico, 1983) and meat (Firstenberg-Eden, 1983).

8.4 Alternative rapid enumeration methods

Most of these rapid methods use structural or metabolic components for microbiological counts. They include limulus assay and bioluminescence (Wood & Gibbs, 1982). These methods are more reliable for detection than enumeration.

8.4.1 Limulus amebocyte lysate assays

The method determines the minute endotoxins content of the Gram-negative microbial cells. Endotoxins coagulate, forming a gel in the presence of a lysate of amoeboctes from horseshoe crab. The method is simple, rapid, accurate and sensitive (Entis *et al.,* 2001; Wood & Gibbs, 1982). Even though most of the microorganisms of processed poultry are predominated by gram negatives bacteria, they all do not produce endotoxins. The method would also not be able to detect Gram-positive bacteria.

8.4.2 ATP-Bioluminescence assay

The method is based on the reaction of adenosine triphoshate (ATP) with luciferin-luciferace, these reactions occur in the presence of magnesium and oxygen (Entis *et al.,* 2001; Strange, 1972; Wood & Gibbs, 1982). The reaction is highly sensitive, allowing detection of microorganisms (Sharpe *et al.,* 1970). The ATP-bioluminescence assays can lose accuracy due to various factors. The most import of these is the detection of ATP from the food sample rather than the microbial ATP (Entis *et al.,* 2001; Sharpe *et al.,* 1970). The non-microbial ATP can be destroyed by physical treatment (centrifugation, ion exchange resin and filtration) or chemical treatments (with nucleotides) or both methods can be implemented at the same time (Wood & Gibbs, 1982). Other factors include the fact that the microbiological population may be metabolically inactive or slow or the ATP level may vary among a group of mixed microbiological population (Entis *et al.,* 2001).

9. CONCLUSION

For the safety of consumers and prediction of shelf life, a rapid enumeration method is required. Most methods have been evaluated for analysis of microbial populations on poultry carcasses with little success. Most of these methods are expensive and therefore the poultry plants still apply the conventional, standard plate count method. The method is laborious and time consuming. The determination of microbial loads by means of the measurement of the OD reading is an easy, rapid and accurate method. With these characteristics the OD method deserves to be evaluated for and established for poultry routine monitoring of critical points.

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CHAPTER 2

ISOLATION AND IDENTIFICATION OF DOMINANT BACTERIAL POPULATIONS FROM CHICKEN CARCASSES.

Abstract

The daily microbial population on chicken carcasses varies in quality and quantity. Different samples collected on the same day have vastly different microbial populations. The microorganisms originate from the feet, feathers, skin and the intestinal tract content. The important microbiological contaminants on carcasses are those, which cause spoilage on chicken carcasses, as well as specific human pathogens. The mesophilic organisms are dominant on the final processed poultry carcasses. In this study samples were randomly collected immediately after evisceration and brought to the laboratory. They were sampled by whole carcass rinse method and bacteria were isolated on Plate Count Agar at 37°C for 24 h. A total of five different species were selected, based on their prevalence in the sample and were isolated and identified by conventional methods. The identification of the isolates was further confirmed by API techniques. The five species consisted of *Escherichia coli, Shewanella putrefaciens, Aeromonas hydrophila, Serratia marcescens* and *Staphylococcus aureus*. Mean counts ($\log_{10}$ cfu/g) of these organisms were 3.08, 1.48, 0.85, 1.74, and 1.21 respectively. Most of these organisms were Gram-negative rods and only the latter was Gram-positive cocci. All isolated species are able to grow at 37°C and this temperature is important for establishment of optical density readings method as an enumeration method on poultry carcasses.

Introduction

Poultry meat products are widely consumed due to their low cost, low fat content and the short period of time required for processing (Alvarez-Astorga *et al.,* 2002). The quantity and quality of hazardous microorganisms on poultry products are important aspects of the hazard analysis critical control point (HACCP) at critical control points (CCP). These microorganisms should be routinely identified and enumerated (Notermans *et al.,* 1994). Expansion of the poultry industries led to more developments in processing plants. These developments might contribute positively or negatively to contamination of the final products, hence the newly improved processing equipment should also be evaluated after installation in order to check their contribution to product contamination. Most of the microbiological control methods that have been implemented, starting from hatcheries to the processing plants, focus mainly on reducing the risk of foodborne outbreaks. The methods applied to reduce microbial contamination start during egg productions and continues until chicken carcass consumption (Corry *et al.,* 2002; Lammerding, 1997;). These measures include; anti-microbial treatments of eggs, feeds, precaution during breeding and transportation to the processing (Corry *et al.,* 2002). The final control points after processing, consists of preservation means, which include chilling or freezing, canning (cooked), curing and drying (Ingram & Simonsen, 1980). The above precautions are achieved by incorporating the use of vaccines and antibiotics to control disease, improved nutrition, and automation of the farms as well as integration of industry. These measures are to meet the high demands of poultry meat products (Bremner, 1977; Todd, 1980;).

Live birds enter the processing plant with microorganisms on their feathers, skin, feet and intestinal tract. Throughout the different processing steps at the plant, contamination occurs when the carcass come into contact with the processing surface that are contaminated with microorganisms (Bremner, 1977; Den Aantrekker *et al.,* 2003; Lindsay *et al*., 1996).

Salmonella spp., *Staphylococcus aureus* and *Escherichia coli* are typical food borne pathogens associated with poultry (Bryan, 1980). Other less common pathogens isolated from poultry include species from the following genus *Campylobacter*, *Shigella, Vibrio, Yersinia, Clostridium*, *Shigella* and *Bacillus* (Bok, *et al*., 1986; Todd, 1980; Tompkin *et al.,* 2001).

Spoilage microorganisms are mainly psychrotrophic in nature and predominate during cold temperature storage (Álvarez-Astorga *et al.,* 2002). These microorganisms are commonly gram-negative rods consisting of *Pseudomonas* spp., *Acinetobacter* spp., *Moraxella* spp., *Shewanella putrefaciens*, *Cytophaga* spp., *Corynebacterium* spp., *Flavobacterium* spp., and gram positive bacteria from the genera *Micrococcus* and *Enterococcus* (Arnaut-Rollier, 1999; Barnes & Thornley, 1966; Barnes & Imprey, 1968; Bryan, 1980; Cousin, *et al.,* 2001; Ingram & Simonsen, 1980, Tompkin, *et al.,* 2001). A major source of psychrotrophic contamination is the immersion washer and chiller water (Thomson & McMeekin, 1980).

The objective of this study was to isolate the dominant microorganisms from processed chicken carcasses. It was important to specifically isolate mesophilic microorganisms, because of their dominance on the surface of the processed chicken carcasses. The isolates will be used in the continuation of this study where optical density will be evaluated as enumeration method.

Aim

To isolate and identify the common, dominant bacteria, based on colony morphology from the surface of chicken carcasses for evaluation of optical density readings as an alternative, rapid method to spread plate counts, for routine microbial enumeration. This is not a complete study of microbial population associated with chicken carcasses.

Material and methods

Description of processing plant

The plant was a Grade A poultry abattoir with a capacity of ca. 7200 birds per hour (ca. 64 800 birds per day). Birds were scalded at 54 - 56°C and feathers removed by three plucking machines in series. The evisceration process was performed manually or automatically. Eviscerated carcasses were spray washed with unchlorinated water followed by pre-washing in a spin washer. Carcasses were subsequently immersion chilled in a counterflow system for 35 min. The temperature of the water varied between 2 - 4°C with a chlorine level of approximately 30 ppm.

Sampling procedure

Twelve chicken carcasses were randomly collected at the abattoir, and sampled in groups of three. The samples were randomly selected and were collected immediately after automatic evisceration. The chickens were transported in a cooler box, and kept at low temperature all the time before being analyzed. The analysis was conducted in less than 1 h of samples arrival to the laboratory

Sample processing and analysis

A total of 12 whole chicken carcasses samples were sampled for microbial contaminants by whole carcass rinse technique as described by Cox *et al.* (1981). Each chicken was aseptically placed in a sterile plastic bag. Thereafter, 100 ml sterile dionized water was added to each bag. The plastic bag was then repeatedly shaken and massaged vigorously for 1 min to ensure adequate contact with the medium. The aliquot water was poured into sterile schott bottles, within 15 seconds to avoid aerial contamination.

A tenfold serial dilution of the aliquot water in 1% Bacto Peptone Water was made and plated in duplicate, by the spread plate technique, onto Plate Count

Agar (PCA, Oxoid, Basingstoke, UK). The plates were incubated aerobically at 25ºC for 48 h for bacterial counts and isolation. PCA is used as a general medium to obtain different organism. The 25ºC incubation temperature in order to allow for the growth of both mesophilic and psyhchrotrops. Plates containing between 30 and 300 colonies were counted and the results were calculated as cfu/g (or the highest number if below 30).

Isolation and identification

Single separate colonies were isolated and streaked out (repeated 3 times) for purification, on to PCA and incubated at 25ºC for 48 h. Each colony was chosen according to morphology difference. Many different species look the same on PCA and the identity of these organisms was confirmed by biochemical tests. The following tests were performed: Gram stain, Morphology, Motility, Catalase, Oxidase, Glucose fermentation, Indole, Urease, Methyl red, Voges Proskauer, Citrate, Beta-galactosidase (ONPG) test, Oxidation and fermentation tests. Additional tests were done for gram-positive isolate.

Oxidase positive and gram-negative organisms were selected for further identification of the API 20 E (bio Merieux Vitek, Inc., Hazelwood, MO) and API 20 NE (bio Merieux Vitek, Inc., Hazelwood, MO) kits according to the manufacturers instructions without any modifications.

Microbial storage

The isolates were stored at –70ºC in Microbank™ Cryovials (Pro-lab. Diagnostics), and were sub cultured every 6 months. This was achieved by inoculating a colony from a 24 h cultured plate and was aseptically transferred to a sterile vial with beads. This was agitated to coat the bead with the suspension and the excess suspension removed with sterile pasture pipette. The vials were closed and placed in trays and stored at –70ºC. Cultures were

also maintained on PCA plates at 4ºC and were routinely sub cultured every month.

Results

The microorganisms were enumerated by means of counting colonies on PCA plates. Additional tests were performed to identify *S. aureus*, since the organism was gram positive. These tests included the coagulase test; growth on NaCl agar (10 and 15% (w/v)); growth at 15ºC and 45ºC; β GAL test; acid formation from ADH, RIB, arabinose, mannose, SOR, lactose, trehalose, which were all positive, while raffinose was negative.

The results of the counts are presented in Table 1. There were other microorganisms on the plates and their counts were represented as others in Table 1. *E. coli* counts were higher than all other isolates. This was observed on plates from all sampled carcasses. The second most prevalent organism was *Serratia marcescens* when compared to the other isolates (See Table 1). There was no significant difference between counts of *Shewanella putrefaciens*, *Staphylococcus aureus* and *Serratia marcescens*.

Table: 1. The average microbial count of microorganisms isolated from 12 chicken carcasses sampled, determined by plate count on PCA in log colony forming units.

	Others	***E. coli***	***Shewanella putrefaciens***	***A. hydrophila***	***Serratia marcescens***	***Staphylococcus aureus***
Counts in log cfu/ g	0.30	3.08	1.48	0.85	1.74	1.23

Only a few different colony morphologies were observed on the PCA plates after incubation. One colony type was found to be smooth and big and was

dominant. These colonies were later identified as *E. coli* (3.08 log cfu/g) and these types of colonies were observed in all samples obtained from all the carcasses. Three other different colonies, which were later, identified as *Shewanella putrefaciens, Aeromonas hydrophila* and *Serratia marcescens* were also found. *Staphylococcus aureus* were isolated as the large yellow colonies on the plates. The lowest counts of the chosen microorganisms were those of *A. hydrophila* that had the average count of 0.85 log cfu/g. The identification results of these organisms are given in Table 2. The identification results were compared to the identification tests in Bergey's Manual of Systematic Bacteriology (Krieg *et al.,* 1984; Sneath *et al.,* 1986). Most organisms isolated were gram-negative rods.

Table 2: Biochemical test to identify the selected isolates. Isolates were incubated at +/- 37°C.

ND= not determined

	E. coli	***Shewanella putrefaciens***	***A. hydrophila***	***Serratia marcescens***	***Staphylococcus aureus***
Gram stain	-	-	-	-	+
Morphology	Rod	Rod	Rod	Rod	Cocci
Catalase	+/-	+	+	+	+
Motility	+	+	+	+	ND
Oxidase	-	+	+	-	-
Only Glc fermented	-	-	+	+	-
Glu/ /Lac/Suc fermented	-	+	-	-	+
Indole	+	-	+	-	-
Urease	-		-	-	+
Methyl red	+	-	+	-	+
Voges proskauer	-	-	-	-	-
Citrate	-	+	-	+	-
(ONPG)	+	ND	+	+	-
Growth in Nutrient Broth	+	+	+	+	+
Fermentative	-	+	+	+	+
Oxidative	-	+	+	+	-
H_2S	-	-	-	-	-

Table 3: Sequential results of API 20 E and API 20 NE, conducted according to manufacture's instruction.

1. **API 20 E**. ONPG=Ortho-nitro-phenyl-β-D-galactopyranoside; ADH=arginine; LDC=lysine; ODC=Ornithine; CIT=sodium citrate; H2S=H2S production; URE=urease; TDA=Tryptophane; IND=Indole production; VP=acetoin production; GEL=Kohn's gelatine; GLU=glucose; MAN=mannitol; INO=Inositol; SOR=sorbitol; RHA=rhamnose; SAC=sucrose; MEL=melibiose; AMY= Amygdalin; ARA=arabinose; OX=oxidase; NO2=NO2 production.
2. **API 20 NE.** (a=assimilation). NO3 =Nitrate reductionto nirites; TRP= indole production; GLU=glucose acidification; ADH= arginine dihydrolase; URE=urease; ESC=esculin hydrolysis; GEL= gelatin hydrolysis; PNPG=p-nitrophenyl-β-D-galactopyranoside; GLU=glucose; ARA=arabinose; MNE mannose; MAN=mannitol; NAGa=N-acetyl-glucosamine; MAL=maltose; GNT=gluconate; CAP=caprate; ADI=adipate; MLT=malate; CIT=citrate; PCA=phenyl-acetate;Oxcytochrome oxidase.

API 20E	***E. coli***	***Serratia marcescens***	**API 20NE**	***Shewanella puteifaciens***	***A. hydrophila***
ONPG	+	+	**NO_3**	+	+
ADH	+	+	**TRP**	-	+
LDC	+	+	**GLU**	-	+
ODC	+	+	**ADH**	-	+
CIT	-	+	**URE**	-	-
H2S	-	-	**ESC**	+	+
URE	-	-	**GEL**	+	+
TDA	-	-	**PNPG**	-	+
IND	+	-	**GLUa**	+	+
VP	-	+	**ARAa**	-	+
GEL	-	+	**MNEa**	-	+
GLU	+	+	**MANa**	-	+
MAN	+	+	**NAGa**	-	+
INO	-	+	**MALa**	-	+
SOR	+	+	**GNTa**	+	+
RHA	+	+	**CAPa**	+	-
SAC	+	+	**ADIa**	-	-
MEL	+	+	**MLTa**	+	-
AMY	-		**CITa**	-	-
ARA	+	-	**PCAa**	-	-
OX	-	-	**OX**	+	+
NO_2	+	-			

The results obtained from API tests mostly gave reasonable identification. Most gave values above 90% correct identification. An exception was *Serratia*, which was identified to the genus level and required complementary test to verify to the specie level. The complementary tests were xylose accumulation and methyl red test. After these tests it was established that the isolated bacteria were *Serratia marcescens*. *E. coli, Shewanella putrefaciens,* and *A. hydrophila* had correct identification of 99.9%, 92.5% and 98.8%.

Discussion

The rise in poultry product production is related to consumer demand for raw chicken and poultry products. To achieve this goal, the whole poultry meat chain has improved its efficiency in procedures and costs (Todd, 1980). High standards are set for the quality and safety of poultry products. The microbial quantity should be minimised, especially for the organisms that have significance in human health. The numbers of these pathogens should be as low as possible to limit the risk of foodborne infections. Improper sanitary measures and mishandling practices in food preparation areas result in outbreaks of foodborne disease and short shelf life of chicken commodities. This can lead to economical losses, loss of reputation and in the worst-case even loss of life (Bryan, 1980; Todd, 1980; Kraft, 1986).

Eviscerated carcasses were chosen for the selection of the most dominate microorganisms (based on colony morphology), because this poultry-processing step has the highest microbial counts, Walker and Ayres, (1956) and Mead, (1989) found eviscerated carcasses had higher microbial load than those from other processing equipment. The chilled carcasses were avoided because the microorganisms on these carcasses might be damaged by chlorine in the form of hypochloride (30 ppm) treatment applied in the chilling system at processing plant.

This study was not intended as a complete study of the microbial population of the chicken carcasses after evisceration. All the selected isolates were able to growth at 37°C, hence this temperature would be applied in the development of OD methods in the future studies
The microbial populations obtained from these samples consisted of total counts in the range of 0.3 log_{10} to 4 log_{10} cfu/g. Tompkin *et al.* (2001), reported similar quantities. Gunderson *et al.* (1954) reported microbial load with mean count of 38000-organisms/cm^2 (3.58 log organisms/cm^2). *E. coli, Shewanella putrefaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus* have previously been isolated from poultry products (Bok et al., 1986; Todd, 1980; Tompkin *et al.,* 2001).

The organism, *Shewanella putrefaciens* has undergone various name changes, from *Pseudomonas putrefaciens* to *Alteromonas putrefaciens* (Ingram & Simonsen, 1980), and recently to *Shewanella putrefaciens* after division of genus *Alteromonas* into two genera by Gauthier *et al.* (1995). *Shewanella putrefaciens* is more prevalent in spoilage of fish than chicken (Ingram & Simonsen, 1980). *Shewanella putrefaciens* is an important spoilage bacterium, and this organism is able to grow in anaerobic condition, at a low growth rate compared to under aerobic condition (Jackson *et al*., 1997) *S. putrefaciens* has been implicated occasionally as a human pathogen, it is most frequently recovered from nonhuman sources, including aquatic reservoirs, natural energy reserves (oil and gas), soil, and fish, poultry, dairy, and beef products. Most human isolates of *S. putrefaciens* occur as part of a mixed bacterial flora, clouding their clinical significance (Brink *et al.,* 1995; Khashe & Janda, 1998; Stenstrom & Molin 1990).

Aeromonas spp. are pathogenic bacteria found mostly in fresh water and seawater (Khandori & Fainstein, 1988; Szewzyk *et al.,* 2000). *A. hydrophila* isolated in this study might originate from the water source in the processing plant, since in literature, low rate of *A. hydrophila* has been isolated from faeces

of chicken carcasses (Akan *et al.,* 1998*;* Janda & Abbott, 1989). Little information is available that associate *A*. *hydrophila* with poultry carcasses. Gastroenteritis is the most prevalent human infection caused by *Aeromonas* spp. (Janda & Abbott, 1989). There is little evidence that connects *A. hydrophila* to these diseases; hence there is no report on the organism as a sole source of foodborne outbreaks. *Serratia marcescens* has been found to be pathogenic, it cause urinary tract infections, wound infections, and pneumonia. The oranism is resistant to a number of antibiotics, which make it significant. This organism is able to grow at 40°C (Krieg *et al.,* 1984; Aucken & Pitt, 1998).

E. coli counts were highest in numbers followed by *Serratia marcescens*. Most organisms isolated from poultry carcasses were gram-negative rods. Similar results have been found in most other research conducted into the bacterial population of poultry carcasses. Bok *et al*. (1986) isolated *A. hydrophila, Serratia marcescens*, *E. coli* and *Staphylococcus aureus.* They found that the E*. coli* isolates were higher in numbers than the other species. These organisms might result from evisceration processing step, as these organisms make up part of the normal intestinal microbiota of chickens and many other animals (Harrison & Hanson, 1950). Evisceration processing step involves the removal of the intestinal organs of chicken during processing and possible rupture of the internal organs could account for much of the contamination found on carcasses.

All species isolated in this study were mesophiles. Because of the temperature used for isolation, psychrotrops and other pathogenic bacteria were present, but were not picked because they were present in low numbers. This corresponds to the study of Ingram and Simonses, (1980) who reported similar results from eviscerated poultry carcasses. *Serritia marcescens, Shewanella putrefaciens* and *A. hydrophila* have been reported to grow at low temperatures (Russell, *et al.,* 1996).

When poultry carcasses enter the processing plant, they are mostly contaminated with gram-positive bacteria, predominantly *Microccocus* spp. (Geornaras, *et al*., 1998; Thomas & McMeekin, 1980). Other gram-positive bacteria such as *Corynebacterium* spp. (which are mostly killed off by soft scalding at 53^0C-56^0C) and lactic acid bacteria are associated with pre-processed poultry carcasses. After the defeathering processing stage, the gram-negative mesophilic microbial populations dominate the surface of the chicken carcasses (Geornaras, *et al*., 1998). This study also showed high number of mesophilic gram-negative bacteria as these chickens were sampled after the evisceration-processing step. The processing environmental conditions, such as the temperature, humidity, and pH of processing allow the proliferation of microorganisms associated with the evisceration step (Simonsen, 1989).

Salmonella spp. were not isolated in this study. This may be a result of the fact that no selective enrichment procedures were conducted in this study. Isolation of *Salmonella* spp. (esp. when low numbers of the organisms are expected) requires enrichment procedures to be conducted. In most studies where *Salmonella* spp. were isolated, the sample was grown in an enrichment and selective medium (Andrews *et al.,* 2001; Bok *et al.,* 1986; Cox *et al.,* 1981). Another possible explanation could be that in this study only dominant organisms were isolated and identified. *Salmonella* spp. normally occur in very low numbers. Routine *Salmonella* spp. detections are mostly qualitative, determining the absence or presence in a sample of interest. (Andrews *et al.,* 2001; Dougherty, 1974; Ingram & Simonsen, 1980). Jorgensen *et al*. (2002) recorded 21% of *Salmonella* spp. positive birds out of 241 samples. Bok *et al.* (1986) also used selective and enrichment procedures and successfully isolated *Salmonella* spp.

E. coli is a dominant isolate of human faeces (Levine, 1985). The organism is a natural inhabitant of the intestinal tract of human and a variety of animals

(Meng *et al*., 2001). Only few strains of these organisms are capable of causing diseases to humans and the disease is associated with *E. coli* and includes toxin, which mediate secondary diarrhoea and invasive dysentery syndrome (Levine, 1985; Meng *et al.,* 2001;).

Staphylococcus aureus is a public health hazard associated with poultry, since many strains of the organism produce enterotoxins that cause food poisoning if ingested. Resulting symptoms from enterotoxins food poisoning includes vomiting and diarrhoea, which occur 2 to 6 h after ingestion of the toxin. *S. aureus* produce enterotoxins at the level of 10^6 cells/g. The organisms originate from skin, nose, and mouth of human food handlers (Lancette & Bennett, 2001). Contamination on poultry carcasses can result from live chicken faeces, feathers, bruised tissues, infected lesions, and skin (Bryan, 1980; Lancette & Bennett, 2001). Even after *S. aureus* elimination on the carcasses, sufficient enterotoxins may remain to cause food poisoning. Low storage temperature and competitive microbial populations, do not favour the growth of these organisms on raw poultry product, hence rendering *S. aureus* of low significance (Bryan, 1980).

Conclusion

The results of this study suggest that microorganisms, which are isolated after evisceration are mostly mesophilic with *E. coli* as the most dominant specie, followed by *Serratia marcescens.* Other species isolated did not differ significantly in number. *Shewanella putrefaciens* and *A. hydrophila* can grow at low temperatures. These 5 isolates were selected to be used in further experimentation on the establishment of the OD readings as alternative method to standard plate counts for bacterial quantification. *E. coli* was found to be the most dominant species and this isolate was selected as the species to be used for most of the further work on the development of the OD method for enumeration of bacteria on chiekens.

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CHAPTER 3

ESTABLISHMENT OF OPTICAL DENSITY (OD) READING METHOD AS A RAPID, ALTERNATIVE METHOD FOR MICROBIAL ENUMERATION FROM CHICKEN CARCASSES.

Abstract

The plate count method, especially the spread plate method, has been the method of choice for the enumeration of contaminating microbial populations in the poultry industry. However, these methods are laborious and time consuming. These drawbacks result in the final poultry product having been distributed or consumed without knowing the initial microbial quality of processed poultry products. In this study optical density (OD) reading as a microbial enumeration method on poultry carcasses was established and evaluated for its application as a rapid, alternative method to standard plate counts. Organisms used to develop this technique were isolated from eviscerated chicken carcasses. These bacterial species were *E. coli, Shewanella putrefaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus*.

During the development of the method, the optimal wavelength was selected as 420 nm. Incubation time of 4 h was chosen by construction of growth curve and choosing the time where all isolates were in the exponential growth phase. Once these two parameters were established, it was essential to determine the repeatability of the OD readings and the correlation of OD readings to standard plate counts. Repeatability was determined by inoculating the media with different concentration of cell cultures (pure culture and mixed cultures), incubation under the above conditions and the determination of the OD reading of 5 test tubes inoculated from the same inoculum. Most of the OD readings obtained from pure cultures had coefficients of variation less than 15%. Similar coefficients of variation values was obtained with mixed cultures and most

values were even below 10%. These results show that the method is highly repeatable. The OD readings after 4 h incubation were correlated to standard plate counts of the samples. Correlation of OD reading to plate counts had a positive strong correlation, which was above 0.9 for all isolates, except for *Serratia marcescens,* which had the correlation coefficient of 0.886.

The OD method evaluated after 4 h of incubation is a repeatable, rapid and simple method to quantify microorganisms.

Introduction

Poultry carcasses are the most suitable substrates for microbial growth as the edible portion of the carcass contains ca. 70% water, has a water activity of 0.98 to 0.99, a protein content of ca. 20.5 % and a fat content of ca 9.5% (Bryan, 1980). Increase in consumption of poultry has resulted in an increase of poultry associated foodborne diseases, particularly salmonellosis (Todd, 1980).

Shelf life and hygiene standards of perishable food products are determined by the time required for spoilage microorganisms to reach a critical level, which makes the product unsuitable for consumption. Another reason for microbial quantification of poultry products is to monitor the effectiveness of sanitary process on processing equipment. Microbial monitoring is evaluated at critical processing steps, mostly after chilling and before packaging. Newly installed processing equipment should also be microbiologically tested for their contribution to contamination of the product. Through the regular quantification of the microbial populations on chicken carcasses, any decrease in microbial quality standards can be detected earlier and rectified before there are problems (Bremner, 1977). Initial microbial load is useful for predicting the shelf life of the organisms. This is achieved by creating predictive models. Large numbers of growth curve are required to evaluate growth rates, which are the core aspects in creating the predictive models (Dalgaard *et al.,* 1994).

Most of these microorganisms are of no significance to human health or spoilage. Other isolates are pathogenic and can cause gastroenteris diseases to human after consumption (Patterson, 1972). Most birds enter the processing plant with contamination on their feet, feathers, skin and intestines, contributing contamination of the carcasses with mesophilic and spoilage bacteria. These microorganisms are dispersed to other body parts of the same carcass or other carcasses during processing (Bremner, 1977; Mead *et al.,* 1994; Mead, *et al.,* 2000; Mulder & Veerkamp, 1974), though none of the typical spoilage bacteria

has been isolated from the intestines. Different processing steps in the poultry processing plant can either reduce or increase bacterial loads (Bremner, 1977; Den Aantrekker *et al.,* 2003; Lindsay *et al*., 1996; Thatcher & Clack, 1968). Scalding reduces most of the spoilage microorganisms and the effectiveness of reduction depends on the scalding temperature. On the other hand, the plucking machine is a major source of contamination and cross contamination (Bremner, 1977).

Many microbial quantification methods are available and commercialised. Conventional methods commonly used, involve the use of plate count methods and the most probable number (MPN) determination. These methods are very sensitive and one could increase specificity by using selective medium.

These methods yield counts only after a period of incubation, usually overnight. These methods are expensive, laborious, and time consuming, especially for psychrotrophic microorganisms, which require 7-10 days incubation before reliable results are available (Bredie & De Boer, 1992; Edmiston & Russell, 1998; Kraft, 1986). Temperature of 25°C for 24-48 h has been suggested for the isolation and enumeration of psychrotrophic microorganisms with plate count methods (Russell *et al*., 1996). These methods require use of dilutions, and the use of many plates (plate counts) or many tubes (MPN). Numerous plating methods allow countable separate colonies to be obtained. Spread plate and pour plate are preferred over other alternative colony counting methods (Meynell & Meynell, 1965; Swanson *et al.,* 2001). The factors involved in choosing the quantitative method involves acceptance of the method by regulatory agencies, feasibility for food, costs, time, personnel, equipment, space, accuracy and reproducibility (Bremner, 1977; Chipley, 1977; Harrigan, 1998).

Rapid methods have been proposed in order to replace the time consuming and expensive conventional methods. Rapid enumeration methods that have received attention in poultry include the direct microscopy by direct epifluorescent filter techniques (DEFT); flow cytometry; measurement of electrical impedance, and the estimation of microbial constituents in ATP-Bioluminescence assay and Limulus amebocyte lysate assays has been evaluated (Bier *et al.,* 2001; Cady *et al.,* 1978; Edmiston & Russell, 1998; Entis *et al.,* 2001; Firsternberg-Eden, 1983; Harrrigan, 1998; Herbert, 1990; Pettipher & Rodrigues, 1982; Sharpe *et al.,* 1970; Shaw *et al.*, 1987; Wood & Gibbs, 1982). Enumeration methods like flow cytometry and impedance requires expensive equipment. Limulus amebocyte lysate assay only count gram-negative bacteria, while the ATP from chicken carcass would make ATP-Bioluminescence assay to lose its accuracy. The determination of OD as a method was evaluated to reduce the labour, time, and cost (Chipley, 1987; Madigan *et al.,* 2000).

Optical density (OD) reading of organisms is evaluated in this study for routine microbial load monitoring on chieken carcasses. It is done to overcome the drawback encountered when the conventional method (standard plate counts) are applied (Chipley, 1987; Madigan *et al.,* 2000). The method is based on a suspension blocking the light beam by scattering, causing suspension to look turbid. The greater the concentration of the organisms, the lesser the light that passes through the suspension and the more the light is scattered (Harrigan, 1998). The use of OD readings for microbial enumeration is widely applied as it is non-destructive, time efficient and less labour intensive when compared to other techniques (Chipley, 1987; Madigan *et al.,* 2000).

Aim

The aim of this study was to establish the methodology of the optical density readings as an enumeration method by establishing the inoculation time,

determination of repeatability of the method and determine correlation of OD reading to standard plate counts.

Material and methods

Microorganisms used in the development of OD reading method

Chicken carcasses were sampled by whole carcass rinse, as described by Cox *et al.* (1981), and identified by biochemical test and API (bio Merieux Vitek, Inc., Hazelwood, MO) techniques (described in Chapter 2). *E. coli, Shewanella putrefaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus* were isolated and identified as dominant species on automatically eviscerated, chicken carcasses (Chapter 2). Most of these organisms were gram-negative rods and one isolate, *Staphylococcus aureus*, was a gram-positive cocci. This temperature was used in the establishment of optical density readings method as a possible enumeration method on poultry carcasses.

Obtaining and storage for application of the OD microbial storage

The isolated microorganisms were stored by different methods depending on the duration of storage. Storage of selected species at the temperature of –70°C was done in Microbank™ Cryovials (Pro-lab. Diagnostics). The microorganisms were recovered from Microbank Cryovials by removing one bead from the vial. The bead was placed in a sterile Nutrient Broth (Difco) and incubated at 25°C for 48 h. The short-term storage of the isolates was done on PCA plates and fresh cultures were streaked out every month on PCA agar and stored at 4°C. The organisms were first grown on PCA agar before they can be used in for any developmental steps.

Wavelength selection

The optimal wavelength was determined by scanning 1 ml of sterile Nutrient Broth over a wide range of wavelength (200-700 nm) using the computerized

spectrophotometer (Beckman, DU 650). The appropriate wavelength was chosen as the area next to the peak for high sensitivity.

Determination of optimal incubation time for establishing OD reading as a microbial enumeration method.

Optimal incubation time for establishing OD reading as an enumeration method was determined by construction of growth curves for the isolated bacterial species. The 18 h cultures (with the bacterial concentration of approximately 10^8 cfu/ml) were prepared for construction of growth curve. The 18 h cultures were obtained by inoculating a colony of each isolate in 10 ml Nutrient Broth (Difco) and incubating for 18 h at 37°C. Nutrient Broth medium is a complex medium and it has the necessary nutritional components to support growth of all the isolates.

A 250 μl sample of the 18 h old culture, were each of the cultures was inoculated into sterile 50 ml Nutrient Broth in shake flask. The flask was incubated in a shaker at 37°C with a rotation speed of 165 rpm. Small volumes of 1 ml were aseptically removed hourly and transferred into disposable cuvettes. The OD was read spectrophotometrically (Spectronic^R Genesis$^-$ 5) at a wavelength of 420 nm. The above sampling procedure was repeated for a period of 9 h.

The OD readings were plotted against time on a logarithmic scale. The optimal incubation time was chosen as the time when all the isolates were in exponential growth phase. These were repeated three times and in duplicate for each isolate.

Repeatability of OD readings, using the selected incubation time of 4 h.

An 18 h old culture was prepared as mentioned above. Ten fold serial dilutions were made in 1% Bacto Peptone Water (Difco) from the 18 h culture. From each dilution, 100 μl of the suspension were inoculated into five different tubes

each containing 10 ml Nutrient Broth and incubated at 37°C for 4 h in a shaker (~165 rpm). After incubation a small volume of 1 ml was transferred into disposable cuvettes and the OD reading were spectrophotometrically (Spectronic^{R} Genesis^{-} 5) evaluated at wavelength of 420 nm.

The mean OD reading, standard deviation and coefficients of variations were calculated for each set of 5 test tubes. This was repeated 3 times for each isolate.

Repeatability of mixed cultures using the selected incubation time

Different volumes of two or three different isolates of separately prepared 18 h old culture were used to make mixed cultures. The various volume, to the sum of 100 μl, from the mixed culture were inoculated into five tubes containing 10 ml Nutrient Broth in 5 test tubes and were incubated for 4 h in a shaker (165 rpm). After incubation, a small volume of 1 ml from each test tube was transferred into disposable cuvettes and OD obtained spectrophotometrically at a wavelength of 420 nm.

The mean OD reading, standard deviation and coefficients of variation were calculated for each set of 5 test tubes. This was repeated 3 times for each isolated.

Correlation of OD readings to the total plate counts

Standard plate counts

The same dilutions used for evaluation of the repeatability of the OD reading method were used for correlation studies. At the same time when the repeatability sample were inoculated, 100 μl from the same ten fold serial dilutions which were made in 1% Bacto Peptone Water (Difco), was inoculated onto Plate count agar (PCA, Oxoid, Basingstoke, UK), in triplicate, by the spread plate technique for total bacterial counts. The plates were aerobically

incubated at 37°C for 24 h. Plates containing between 30 and 300 colonies were counted and the results were calculated as colony forming units (cfu) (or the highest number if below 30) were counted and the means determined from duplicate plates.

Optical density

The optical density readings determined above were used to conduct the correlation graphs.

The values of standard plate counts were plotted against OD readings on a scatter plot graph.

Results

Determination of optimal incubation time for establishing OD reading as a microbial enumeration method

The area next to the peak was chosen for highest sensitivity; 420 nm was chosen (Figure. 1).

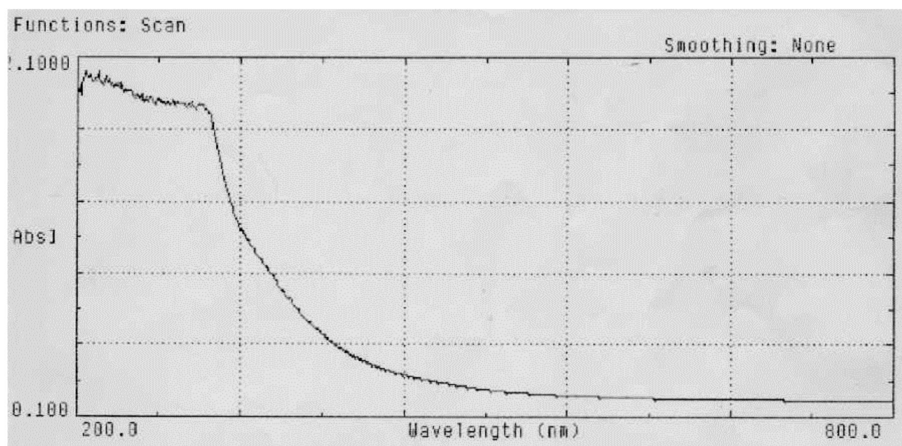

Figure 1: A wavelength scan of nutrient broth over 200-800 nm range

Figures 2-6 show the growth curves obtained for the different isolates. All the isolates had a short lag phase, with the duration of approximately 1 h. *A.*

hydrophila (Figure. 4) had the shortest lag phase, which can almost not distinguished from the exponential phase. After 9 h of inoculation, all the species reached the stationary phase. At 4 h most of the evaluated species were in their late log phase.

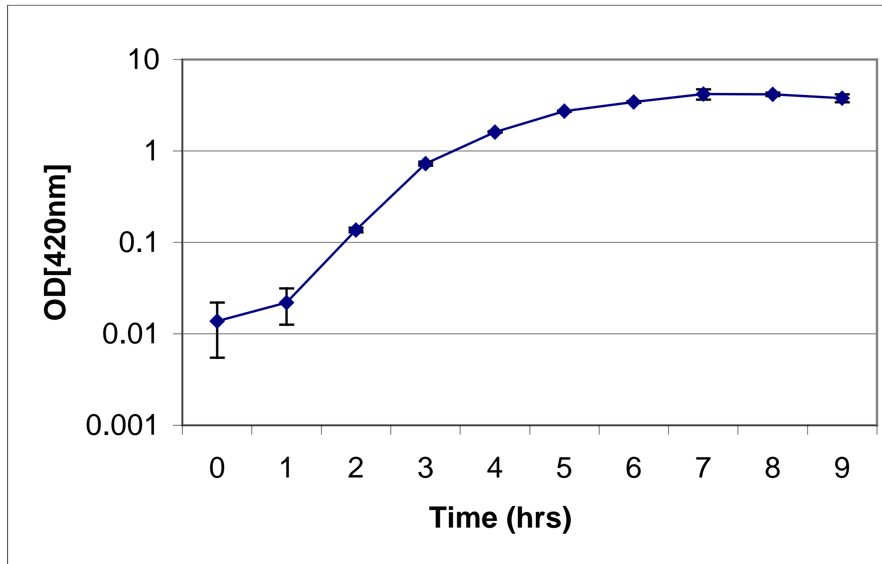

Figure 2: Growth curve of *E. coli,* incubated at 37°C for 9 h in Nutrient Broth.

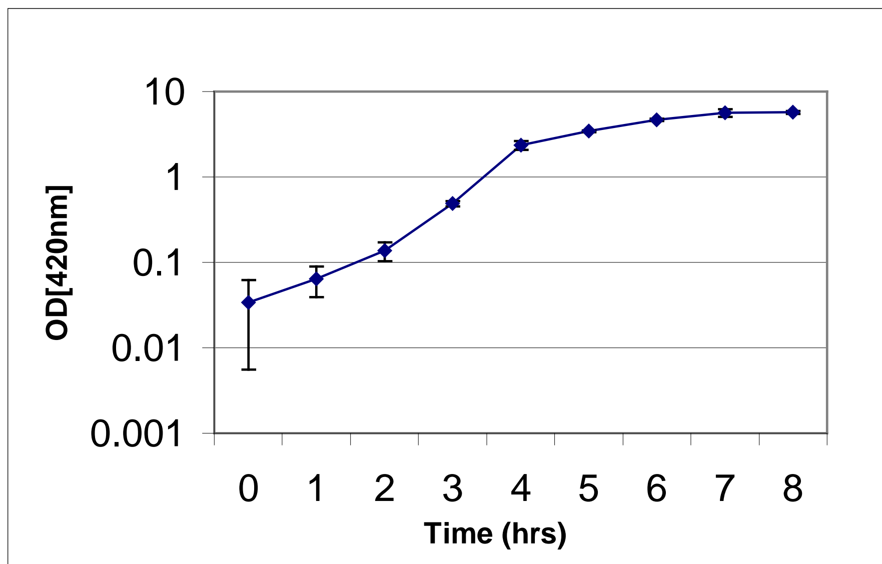

Figure 3: Growth curve of *Serratia marscesens*, incubated at 37°C for 9 h in Nutrient Broth.

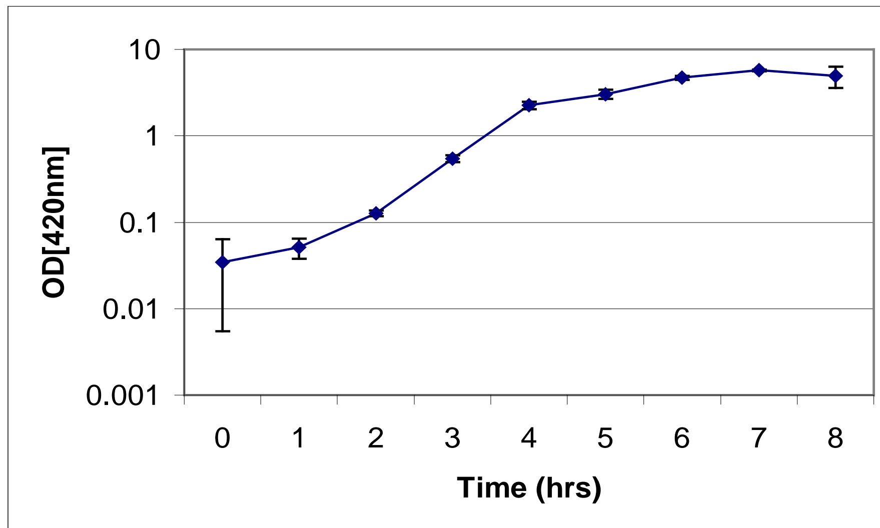

Figure 4: Growth curve of *A. hydrophila*, incubated at 37°C for 9 h in Nutrient Broth.

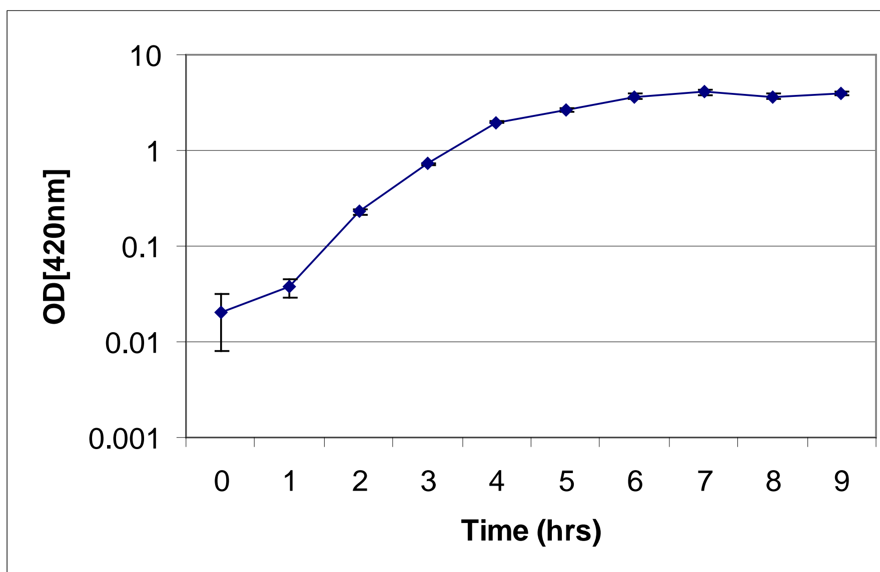

Figure 5: Growth curve of *Staphylococcus aureus,* incubated at 37°C for 9 h in Nutrient Broth.

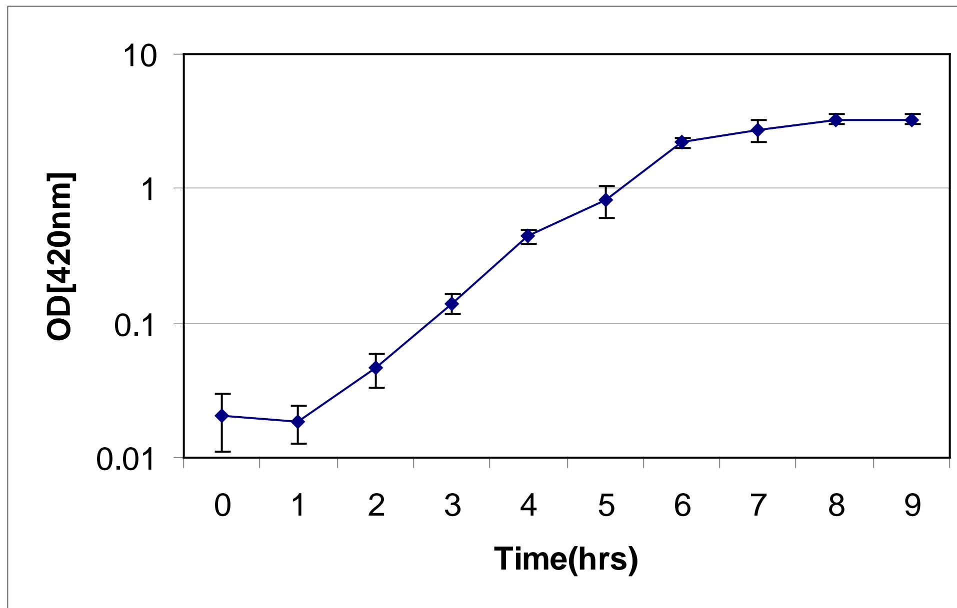

Figure 6: Growth curve of *Shewanella putrefaciens*, incubated at 37°C for 9 h in Nutrient Broth.

Repeatability of OD readings determination for pure culture after 4 h incubation time.

In 70% (21/30) of all cases of pure cultures determinations, coefficients of variation lower than 15 %. High coefficient variance values ranged between 30-45%, mostly from values with mean OD reading less than 0.1 (Table 1-5). *Shewanella putrefaciens,* had the highest values of coefficients of variation, which had high value between 70-88%, obtained with OD readings of 0.01 (Table 5).

Table 1: Repeatability of OD readings evaluated with different concentrations of *Serratia marcescens* suspension, after 4 h incubation in a shaker.

Mean OD	*SD	**CV%
1.659	0.140	8.414
1.676	0.043	2.540
2.959	0.083	2.812
0.442	0.042	9.413
0.072	0.010	13.272
1.660	0.139	8.414

*Standard deviation **Coefficients of variation

Table 2: Repeatability of OD readings evaluated with different concentrations of *E. coli* suspension, after 4 h incubation in a shaker.

Mean OD	*SD	**CV%
2.088	0.069	3.345
1.261	0.140	10.95
1.099	0.055	4.902
0.909	0.035	4.072
0.250	0.021	9.181
0.047	0.021	43.15

*Standard deviation **Coefficients of variation

Table 3: Repeatability of OD readings evaluated with different concentrations of *A. hydrophila* suspension, after 4 h incubation in a shaker.

Mean OD	***SD**	****CV%**
1.678	0.185	11.02
1.453	0.158	10.87
0.806	0.09	12.28
0.179	0.046	24.98
0.030	0.014	44.87
0.013	0.004	41.28

*Standard deviation ** Coefficients of variation

Table 4: Repeatability of OD readings evaluated with different concentrations of *Staphylococcus aureus* suspension, after 4 h incubation in a shaker.

Mean OD	***SD**	****CV%**
2.1959	0.141	7.214
1.6227	0.056	4.258
1.3704	0.049	3.647
1.0002	0.033	3.769
0.5816	0.052	10.89
0.1196	0.032	36.76

*Standard deviation **Coefficients of variation

Table 5: Repeatability of OD readings evaluated with different concentrations of *Shewanella putrefaciens* suspension, after 4 h incubation in a shaker.

Mean OD	*SD	**CV%
0.42	0.02	4.98
0.16	0.02	12.93
0.07	0.01	16.36
0.03	0.01	77.21
0.01	0.01	88.19
0.02	0.01	70.09

*Standard deviation ** Coefficients of variation

Repeatability of OD readings at 420 nm after 4 h incubation with mixed cultures.

Coefficients of variation values higher than 15% were only obtained when OD readings were above 1.94. This was evident, especially, when 70 and 75% of the mixture was made up of *E. coli* suspensions. Most of the OD reading evaluations had coefficients of variation that were less than 15%, with 67.67% (12/18) of all cases were less than 10% (Table 6-8). The three isolates mixed cultures (Table 9-11) had a coefficients of variation 85.19% (24/29) of coefficients of variations, all were less than 10%, and none of coefficients of variation values were above 20%.

Table 6: Repeatability of OD readings, evaluated with different concentrations of mixed culture consisting of *Staphylococcus aureus* (B) and *E. coli* (A). The mean OD, *standard deviation and **Coefficients of variation were calculated.

B (µl)	A (µl)	Mean OD	*SD	**CV%
5	95	1.39	0.10	6.88
10	90	1.54	0.12	7.53
15	85	1.55	0.05	3.03
20	80	1.64	0.05	2.86
25	75	1.97	0.40	19.96
30	70	2.11	0.37	17.36

Table 7: Repeatability of OD readings, evaluated with different concentrations of mixed culture consisting of *Serratia marcescens* (C) and *E. coli* (A). The mean OD, *standard deviation and **Coefficients of variation were calculated.

(C) (µl)	(A) (µl)	Mean OD	*SD	**CV%
5	95	1.56	0.12	7.39
10	90	1.70	0.13	7.62
15	85	1.59	0.11	6.69
20	80	1.71	0.06	3.40
25	75	2.00	0.38	19.10
30	70	2.19	0.30	13.53

Table 8: Repeatability of OD readings, evaluated with different concentrations of mixed culture consisting of *A. hydrophila* (D) and *E. coli* (A). The mean OD, *standard deviation and **Coefficients of variation were calculated.

(D) (µl)	**(A) (µl)**	**Mean OD**	***SD**	****CV%**
5	95	1.64	0.13	7.76
10	90	1.61	0.11	7.02
15	85	1.63	0.05	3.00
20	80	1.72	0.09	5.29
25	75	1.93	0.34	17.41
30	70	1.90	0.32	16.66

Table 9: Repeatability of OD readings, evaluated with different concentrations of mixed culture consisting of *Serratia marcescens* (C), *E. coli* (A) and *Staphylococcus aureus* (D). The mean OD, *standard deviation and **Coefficients of variation were calculated.

(C) (µl)	**(A) (µl)**	**(D) (µl)**	**Mean OD**	***SD**	**CV%**
5	90	5	1.35	0.09	6.38
5	85	10	1.47	0.06	3.81
5	80	15	1.44	0.08	5.55
10	85	5	1.38	0.03	2.07
10	80	10	1.23	0.02	1.39
10	75	15	1.25	0.03	2.29
15	80	5	1.11	0.05	4.52
15	75	10	1.26	0.08	6.73
15	70	15	1.25	0.10	7.67

Table 10: Repeatability of OD readings, evaluated with different concentrations of mixed culture consisting of *Shewanella putrefaciens* (E), *E. coli* (A) and *Staphylococcus aureus* (B). The mean OD, *standard deviation and **Coefficients of variation were calculated.

(E) (µl)	(A) (µl)	(B) (µl)	Mean OD	*SD	**CV%
5	90	5	1.50	0.07	4.44
5	85	10	1.51	0.23	14.92
5	80	15	1.62	0.08	5.02
10	85	5	1.46	0.06	4.05
10	80	10	1.45	0.06	4.21
10	75	15	1.56	0.04	2.66
15	80	5	1.59	0.05	2.98
15	75	10	1.84	0.34	18.51
15	70	15	2.06	0.40	19.26

Table 11: Repeatability of OD readings, evaluated with different concentrations of mixed culture consisting of *Staphylococcus aureus* (B), *E. coli* (A) and *A. hydrophila* (D). The mean OD, *standard deviation and **Coefficients of variation were calculated.

(B) (µl)	(A) (µl)	(D) (µl)	Mean OD	*SD	**CV%
5	90	5	1.84	0.10	5.65
5	85	10	1.78	0.06	3.10
5	80	15	1.74	0.03	1.56
10	85	5	1.58	0.09	5.69
10	80	10	1.47	0.13	9.14
10	75	15	1.80	0.21	11.52
15	80	5	1.53	0.10	6.22
15	75	10	1.69	0.13	7.62
15	70	15	1.84	0.10	5.65

Correlation of OD readings to the total plate counts.

In the current study, the scatter plots of OD readings were plotted against log cfu/ml. Correlation coefficient of greater than 0.9 was obtained for most organisms except for *Serratia marcescens*, which had correlation coefficient of 0.886 (Figure. 7). The best correlation was obtained with *Staphylococcus aureus*, which had correlation coefficient of 0.979. Other organisms evaluated, which include *A. hydrophila, Shewanella putrefaciens* and *E. coli* had the correlation coefficient of 0.949, 0.916 and 0.959 respectively (Figure 8-11).

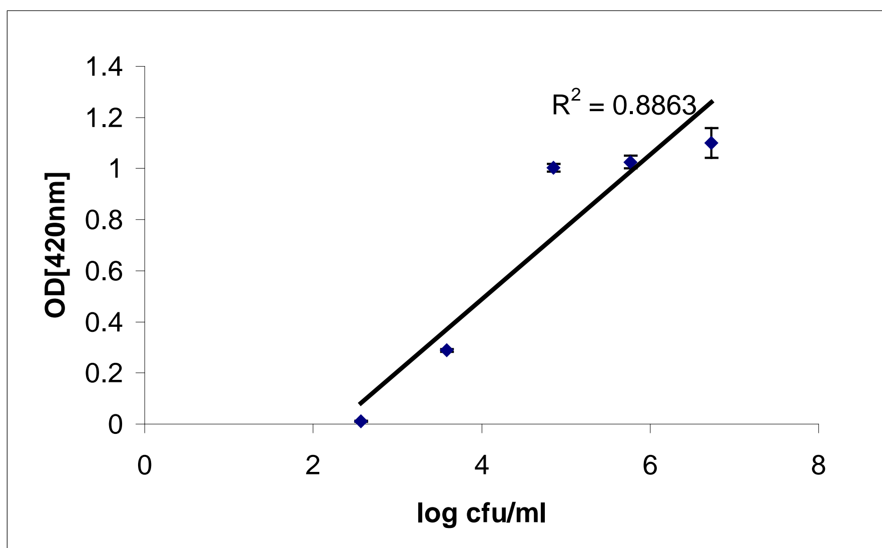

Figure 7: Scatter plot graphic, presentation of the correlation between the mean OD readings (at 420 nm) after 4 h incubation at 37°C and a log mean of the standard plate count, for *Serratia marcescens.*

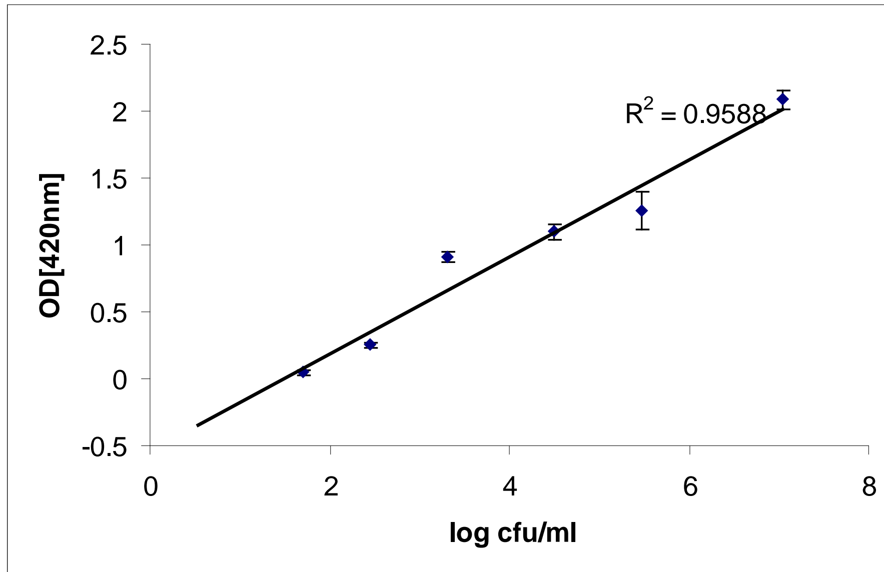

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Figure 8: Scatter plot graphic, presentation of the correlation between the mean OD readings (at 420 nm) after 4 h incubation at 37ºC and a log mean of the standard plate count, for *E. coli*

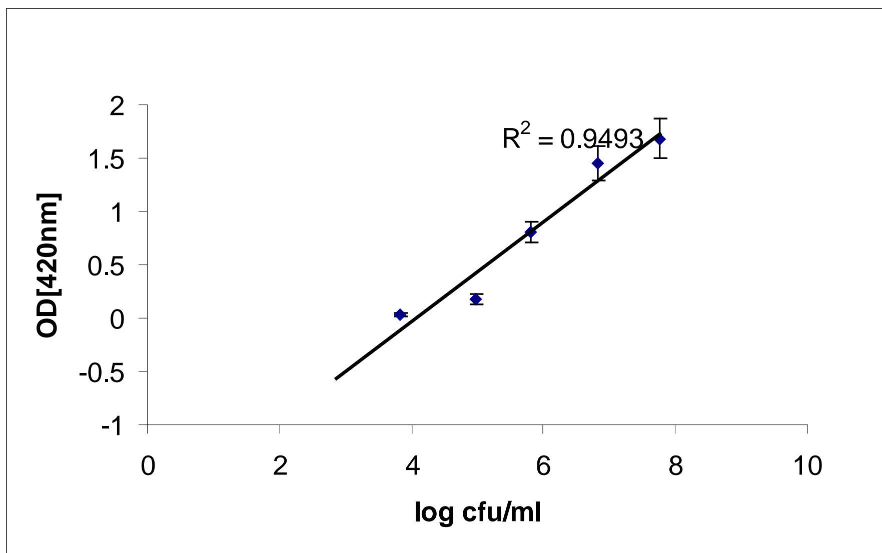

Figure 9: Scatter plot graphic, presentation of the correlation between the mean OD readings (at 420 nm) after 4 h incubation at 37ºC and a log mean of the standard plate count, for *A. hydrophila*.

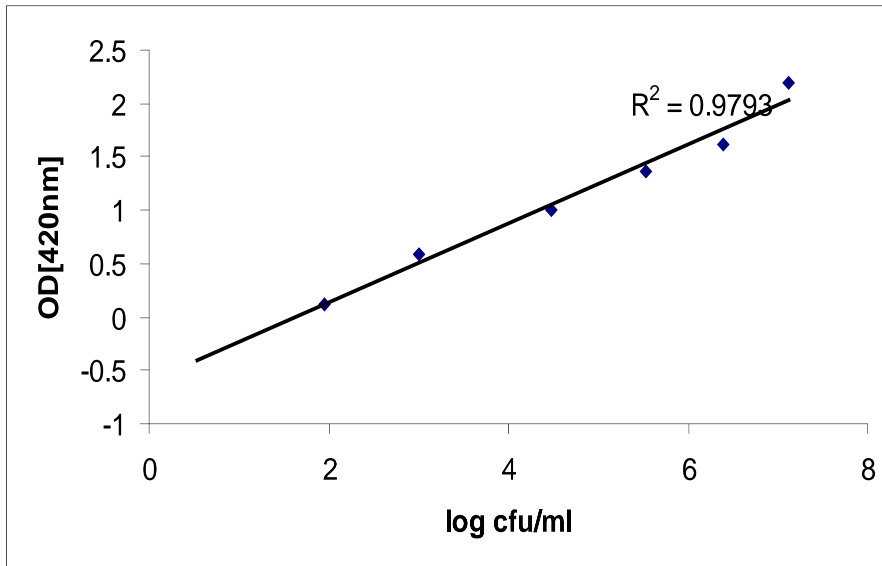

Figure 10: Scatter plot graphic, presentation of the correlation between the mean OD readings (at 420 nm) after 4 h incubation at 37°C and a log mean of the standard plate count, for *Staphylococcus aureus.*

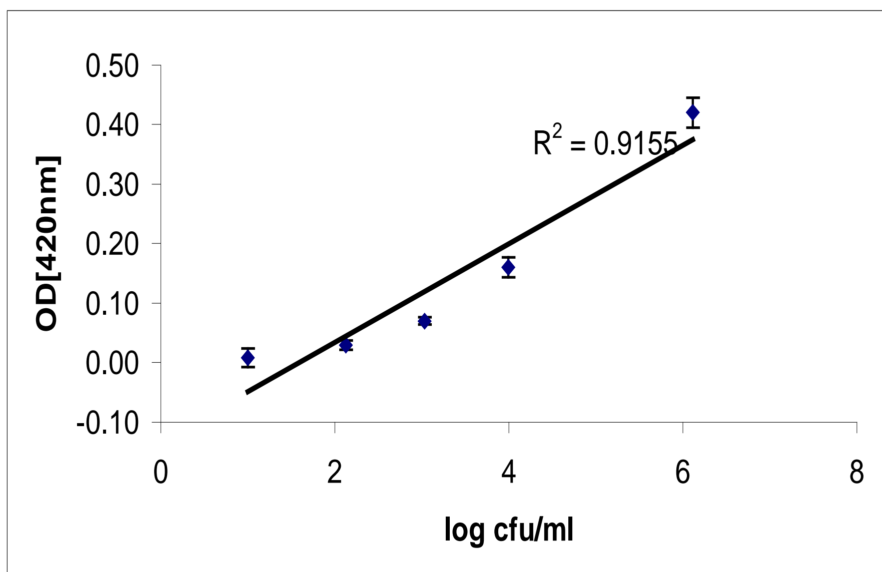

Figure 11: Scatter plot graphic, presentation of the correlation between the mean OD readings (at 420 nm) after 4 h incubation at 37°C and a log mean of the standard plate count, for *Shewanella putrefaciens.*

Discussion

In suspensions where high bacterial concentration are determined by OD methods, the predicted OD readings might be lower than the experimental values due to two factors. Firstly, light scattered away from the incident direction by one cell might be re-scattered back to the original direction by another cell in the same suspension. Bacterial cells are seldom distributed evenly throughout a sample and are not always found as single cells as they can clump. Vortex and shaking of the solution when making dilution may break or add to the formation of clumps (Collins *et al.,* 1989; Harrigan 1994; Koch, 1994).

The OD method is rapid for construction of growth curves by following microbial growth in controlled situations. Other alternative methods, which are laborious, expensive and time consuming, employ plate counts and dry biomass determinations (Entis *et al.,* 2001; Koch, 1994;). During the construction of growth curves all isolates were allowed to grow until they were in the stationary phase. In the study of Pienaar *et al.* (1994) only one isolate was allowed to reach the stationary phase, it was not clear for the other organisms to distinguish between the exponential phase and stationary phase. In the current experiment, 4 h was chosen as the appropriate incubation time. During application of OD method 4 h inoculation time would allow viable cells to grow to the level that is accurately detected. The longer incubation time is required in cases there are conditions that lead to ‘ injured’ cells. Incubation time also makes the OD method a viable count method, since the number of viable cells are allowed to increase and makes the dead cell content in the suspension negligible. It is important to count viable bacterial cells because they are responsible for causing diseases and spoilage.

In the study where the OD readings were established for microbial enumeration of species isolated from eggs, exponential phase was used to establish the optimal incubation time. An incubation time of 6 h was selected in the study

(Pienaar *et al.*, 1994). Pienaar *et al.* (1994) used a wavelength of 560 nm instead of 420 nm as used in this study. Pienaar *et al.* (1994) also did not incubate in a shaker.

All growth curves obtained in this study had a short lag phase which, are similar to those obtained by Dalgaard *et al.* (1994) in the establishment of OD a microbial predictive model, using specific growth rate determination. Dalgaard *et al.* (1994) found the correlation of OD readings to plate counts, with spoilage and pathogenic bacteria to be over a narrow range. In this study the growth curve was used to establish the suitable incubation time for establishing the OD method. The exponential phase as the stage where nutrients are in excess and microorganism are actively growing, will give more reliable and accurate OD readings (Koch, 1994).

Powell (1963) reported that in a suspension of bacteria, particles are not always homogeneous, and have different shapes and sizes. These make it essential to evaluate the repeatability of OD readings, by evaluating five Nutrient Broth test tubes inoculated with similar inoculums. The OD readings had coefficients of variation that were mostly less than 15% for individual isolates, which indicate that, the OD readings were reliable and repeatable. The OD method as shown had low sensitivity for low-density suspensions when OD readings were below 0.1 (especially the pure cultures, in Tables 2-5) resulting in higher coefficients of variation. This can be clearly seen with results obtained from *Shewanella putrefaciens* (Table 5). Pienaar *et al.* (1994) also found values that went beyond 15%. High coefficients of variation values were obtained when low OD readings were obtained.
With all this results, the repeatability was found to be very high, considering the low coefficients of variation obtained for the pure cultures.

The OD readings from mixed cultures gave better coefficients of variation as compared to the pure culture suspension. The coefficients of variation did not

exceed 20% (Table 6 -11). With the 3 isolate mixed culture, coefficients of variation of less than 10% and low standard deviation were obtained. A mixed culture suspension consisting of *Shewanella putrefaciens, E. coli* and *Staphylococcus aureus* (Table 10) had high coefficient variance as the *E. coli* content reach 70 to 75 µl. This is similar to two-organism mixed culture (Table 6,7 & 8). High coefficients of variation was obtained when the mean OD readings exceed 1.94. The dilutions performed on these high OD readings might have slight effect, since the values were diluted if the OD reading were above 1. The requirement for dilutions when the concentration reach a level where the linearity deviate from Beer-Lambert law, resulting in falsely low estimates of the cell density (Dalgaard *et a*l., 1994). This drawback can affect the accuracy of OD reading when used to evaluate suspensions with high microbial population. *E. coli* was used in most of the OD reading evaluations, as compared to other isolates. This is because it was the predominant specie when the microorganisms were enumerated in Chapter 2.

The strength of the correlation between OD readings and plate counts was measured by determining correlation coefficient. A positive and perfect correlation has the value of 1. The value of -1 has negative and linear correlation and zero have no correlations (Viljoen & Van der Merwe, 2000). The correlation of OD to plate counts had values ranging from 0.886-0.979, (Figure 7-11) which indicates a positive and strong relationship between OD readings and plate counts.

Comparison of results in the current study to those found in literature is complicated, since most of the literature reports correlated the standard plate counts to detection time (DT). DT is the measure of the required time for initial bacteria load to adapt to growth medium and to grow at selected incubation conditions until specific signal is produced (Jorgensen & Schulz, 1985; Mattila, 1987). In the current study the organism were incubated for a fixed time of 4 h.

High correlation coefficient (R^2=0.91) was obtained when the correlation of OD and standard plate count was evaluated with *Debaryomyces hansenii*, (Sorensen & Jakobsen, 1997). Jorgensen & Schulz (1985) with *E. coli, Streptococcus faecalis* and *Staphylococcus aureus* isolated from retail mince meat found a perfect correlation coefficient of 1 for all isolates, from his study with pure culture when standard plate counts were correlated to the DT determined by automated OD method.

Conclusion

A 4 h incubation time was chosen from the growth curve as all isolates were in the exponential phase of growth. The repeatability of the OD reading determined after 4 h incubation was found to be very high using five tubes for pure cultures and mixed culture. This can be used to conclude that the method is reliable for microbial enumeration. The low variation in results leads this method to be acceptable. The correlation coefficient of the values obtained in the correlation of OD readings to standard plate count with this high correlation coefficient obtained with the scatter plot were mostly above 0.9, showing the reliability of the correlation between the OD and standard plate counts. The OD method evaluated after 4 h of incubation is a repeatable, rapid and simple method to quantify microorganisms.

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CHAPTER 4

EVALUATION OF OPTICAL DENSITY READING AS A MICROBIOLOGICAL ENUMERATION METHOD ON ARTIFICIALLY INOCULATED CHICKEN CARCASSES.

Abstract

In order to evaluate OD readings as a rapid, alternative microbiological enumeration method on chicken carcasses, samples of carcasses were prepared by eradicating all microorganisms. This was done using a non-toxic disinfectant called Virukill, which completely removed all bacteria from the carcasses. A concentration of 0.3 v/v% of Virukill applied for 2 h eradicated all organisms and had no residual effect. The effectiveness of this concentration was confirmed using three sampling methods, which are neck skin, swab method and whole carcass rinse.

Virukill treated carcasses were re-infected with various concentrations of either pure cultures or mixed cultures. Pure cultures of *A. hydrophila, Serratia marcescens* were individually tested. Mixed cultures consisted of *E. coli, Shewanella putrifaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus*. Inoculated carcasses were sampled by whole carcass rinse and neck skin methods. Samples were serially diluted and aliquots of 100 μl were inoculated into five 10 ml Nutrient Broth and incubated at 37°C for 4 h for the determination of OD readings. Samples from the same dilutions were inoculated onto PCA which were incubated at 37ºC for 24 h, after which time the colonies were counted. The results obtained from both methods were correlated.

The correlation of OD readings to the standard plate counts was evaluated by plotting a scatter plot graph. A correlation coefficient of 0.903 and 0.968 was found for mixed culture determined by whole carcass rinse and neck skin

sampling methods, respectively. *A. hydrophila,* had correlation coefficient of 0.849 and 0.985 for whole carcass rinse and neck skin respectively. Similarly *Serratia marcescens* had 0.993 and 0.940 for whole rinse and neck skin respectively. There is not a significant difference between samples collected by two evaluated methods. Positive high correlation coefficients obtained shows that a strong correlation exists between standard plate counts and OD readings.

Introduction

It is essential to enumerate viable microorganisms on chicken carcasses, because growth of pathogenic microorganisms result in foodborne diseases to humans after consumption of contaminated products. High levels of psychotrophic microorganisms shorten the shelf life by causing spoilage.

The microbiological evaluations on poultry carcasses are done by detection methods or enumeration methods. Microorganisms, significant to quality of the product, are expected to be in low numbers and are simply detected while those that are present in high numbers are enumerated. Both detection and enumeration methods depend on the sampling method used. The ideal sampling method should be able to recover all microorganisms on the poultry surface. This includes recovering microorganisms from the water film, strongly attached to the surface, and those that are in the feather follicles (Notermans *et al.,* 1975). The most applied methods are swab sampling, whole carcass rinse and neck skin excisions (Bryan, 1980; Tompkin *et al.,* 2001).

Conventional microbial enumeration methods are both time and labour intensive, but are still applied routinely (Entis, *et al.,* 2001; Wood & Gibbs, 1982). Many microbiologists are aware of various available, rapid techniques, which have had high interest in research (Wood & Gibbs, 1982). The conventional method includes the plate count method. The method is preferred because of the low cost involved, accuracy and repeatability (Mattila, 1987; Wood & Gibbs, 1982), though the method is laborious and time consuming. Among other factors that contribute to the need for alternative methods include that conventional methods requires preparation of large numbers of petri dishes, serial dilutions and the results are obtained after 24 h to 2 weeks (Edmiston & Russell, 1998; Kraft, 1986).

One of the rapid enumeration methods that have received attention in poultry processing is direct count using light microscopy counts. This method cannot

differentiate between viable and dead microbial cells. It is also difficult to count microbial concentrations below 10^6 (Harrigan, 1998; Meynell & Meynell, 1965; Swanson, *et al.,* 2001). Common microscopic methods includes direct epifluorescent filter techniques (DEFT) and membrane filtration.

Other rapid enumeration methods for poultry meat products are available although they have some limitations. These include electrical measurements applied in the form of impedance method, and the estimation of microbial constituents in ATP-Bioluminescence assay and limulus amebocyte assay (Bier *et al.,* 2001; Cady *et al.,* 1978; Edmiston & Russell, 1998; Entis *et al.,* 2001; Firsternberg-Eden, 1983; Herbert, 1990; Pettipher & Rodrigues, 1982; Sharpe *et al.,* 1970; Shaw *et al.,* 1987; Wood & Gibbs, 1982).

Sampling methods primarily affects the accuracy and effectiveness of recovery of microbial quantity (Fromm, 1959; Patterson, 1972). The accuracy for quantifying methods relies on the state in which cells occurs as some might appear singly and in pairs, chains, clusters or clumps (Bremner, 1977; Chipley, 1987). Other factors to consider in microbial recovery include the media, incubation conditions, and other organisms require enrichment, depending on the method applied.

OD readings, as an enumeration method, is promising for reduction of cost and time. The method is based on the assumption that when the beam of light falls upon a solution or particles, the electrical field associated with the light polarizes the electron clouds of the atoms in the particles. The oscillatory clouds then serve as a secondary source of light and emit light in various directions. The scattered light has the same wavelength as the incident light and is merely redirected from the incident light. The important factor with the scattered light is the intensity and the angular distribution (Madigan, 2000; Meynell & Meynell, 1965).

Microbial growth in a liquid medium changes a clear liquid medium to a turbid medium. This can be measured and quantified by using a spectrophotometer (Jorgensen & Schultz, 1985). Application of photometric measurements in bacterial studies includes determination of optical properties, which are more difficult. These are rapid determination of size and concentration (Powell, 1963). OD measurements have also been used to monitor microbial growth for construction of growth curves. The use of Optical density readings in growth curve construction improved the time, labour and costs involved when the alternative method are used, such as plate counts and dry biomass determinations. The results obtained from the OD method can vary depending on the various cell sizes in the suspension (Sokatch, 1969). Jorgensen & Schultz (1985) and Mattila (1987) determined the use of OD readings as a rapid method for determining microbial load of meat samples.

Pienaar *et al.* (1994) used the optical density to determine the regular, predominant species isolated from the eggs. In the preliminary study, the incubation time for OD readings, of the dominant microorganism was determined by choosing the period when all microorganisms were in the exponential phase during growth. Repeatability of the method was also determined by evaluating the pure culture and mixed culture. OD readings were correlated to standard plate counts (Pienaar *et al.,* 1994; Pienaar *et al.,* 1995). These finds support the use of optical density readings for the determination of microbial contamination levels of poultry carcasses.

The primary objective of this study was to evaluate optical density readings as rapid alternative to standard plate count on poultry carcasses. In chapter 3 it was demonstrated that OD readings after 4 h incubation is repeatable and has a high correlation to standard plate counts on pure cultures *in vitro*. Chicken carcasses have varying quantities of microorganisms on their surface, which have been acquired during breeding and processing. This makes it difficult to evaluate OD methods over a wide range of microbial load on chicken

carcasses. To evaluate and correlate the efficacy of OD readings to standard plate count methods, the first objective is to remove all contaminating microorganisms from the surfaces of chicken carcasses, so that they can be re-inoculated with known concentrations of bacteria. Different concentrations of a non-toxic, highly effective disinfectant, called Virukill, were evaluated for the complete eradication of microorganisms on chicken carcasses.

Aim

The aim of this study was to evaluate OD readings as a rapid, alternative enumeration method for evaluation of initial microbial quantity of the artificial inoculated chicken carcasses. Also to evaluate the effective sampling method between whole carcass rinse and neck skin sampling methods

Materials and methods

Description of processing plant

The poultry processing plant that was selected for this study was a Grade A poultry abattoir with a capacity of ca. 7200 birds per hour (ca. 64 800 birds per day). Birds were scalded at 54–56°C and feathers removed by three plucking machines in series. The evisceration process was performed manually or automatically. Eviscerated carcasses were spray washed with water (not chlorinated water), followed by pre-washing in a spin washer. Carcasses were subsequently immersion chilled in a counterflow system for 35 min. The temperature of the water varied between 2–4°C with a chlorine level of 30 ppm.

Preparation of chicken carcasses for artificial inoculation, by sterilization.

Evaluation of different concentrations of Virukill treatments for eradiation of all microbial organisms on chicken carcasses

Six chicken carcasses were randomly selected after the chilling process step, and then brought to the laboratory in a cooler box containing ice. Upon arrival,

chicken carcasses were immediately evaluated for initial microbial load by the neck skin sampling method. The carcasses were then soaked in a Virukill™ solution. A volume of 4 l of diluent per carcass was used, as this is the same water volume standard used at processing plant for chilling. Both high and low Virukill concentrations were evaluated. The former treatments involved 1, 1.5 and 2 v/v% Virukill, for a 30 min contact time and for the later 0.1, 0.2 and 0.3 v/v% Virukill, for a 2 h contact time. Carcasses were rubbed after every 30 min during the 2 h contact time and every 10 min for the 30 min contact time treatments. This was done to reach remote areas, such as behind the thighs and wings. Sterile gloves were used in every situation when handling the carcasses. After treatment the carcasses were allowed to drip for 10 sec followed by rinsing with 1000 ml sterile distilled water, to reduce residual Virukill concentrations from the skin surface.

Sterilisation tests for the efficiency of Virukill treatment and evaluation of residual effect of Virukill on poultry carcasses after treatment.

After Virukill treatment each individual chicken carcass was placed into a sterile polyethylene plastic bag. From all six carcasses a total of 10 g of neck skin (collected as described below) and microbiologically evaluated on PCA plates incubated at 37°C and 25°C. After incubation the plates were evaluated for the absence of colonies.

Immediately after the Virukill effectiveness test, a total of 500 ml of the diluted suspension of 18 h culture of *E. coli* was added to the plastic bag and tightly closed, to artificially inoculate the carcasses. A contact time of 30 min was allowed. After this, another 10 g neck skin sample was removed and microbiologically (on PCA plates) examined in order to determine residual effects of the Virukill treatment on the carcasses.

Evaluation of neck skin, whole carcass rinse, and swabs sampling methods for the reliability in determining the effectiveness of Virukill treatment.

Low Virukill concentration treatments were further evaluated by three common poultry sampling methods, i.e. neck skin, whole carcass rinse and swab sampling. All six carcasses were used for neck skin sampling as described below. The same carcasses were divided into two groups of three carcasses. One group was evaluated by the swab sampling method and the second group was sampled by the whole carcass rinse method. Sterile gloves were used and each test had four repetitions.

Neck skin

A total of 10 g (approximately 1.7 g/carcass) of the chicken neck skins were removed from all 6 (3 in correlation studies) treated chicken carcasses. This was achieved by aseptically holding the neck skin with a forceps and cutting the neck skin with a pair of scissors. The neck skins were placed into sterile Whirl pak bags (Nasco, USA), homogenized for 1 min in 90 ml 1 % Bacto Peptone Water (Difco) using a Colworth 400 Stomacher (London, UK). The mixture was then used for microbial analysis.

Whole carcass rinse

This method was completely elaborated by Cox *et al*. (1981). Each carcass was placed into a polyethelyne plastic bag and weighed. A volume of 100 ml of 1% Bacto Peptone Water (Difco) was added to each plastic bag. The plastic bags were tightly sealed and vigorously shaken for 1 min. The aliquot water was rapidly poured into a sterile Schott bottle. The sampled aliquot was then used for microbial analysis.

Swab technique

This method was described by Evancho *et al.* (2001) and Chipley (1987). The cotton wool swab was firstly pre-moistened by immersion in the 10 ml of 1% Bacto Peptone Water (Difco) and the excess liquid on the swab was pressed

out against the interior wall of the test tube. The area of 5 cm × 5 cm (25cm^2) was swabbed using a sterile paper template as a guide for the area to be sampled. This was achieved by slowly rubbing the swab three times over the marked area, reversing direction between strokes. Once the selected area was covered with the swab, the swab was placed into a test tube with 10 ml of 1% Bacto Peptone Water (Difco), and the top end broken. The mixture was vortexed for 5 seconds and used for microbial analysis

Microbial analysis

Ten-fold serial dilutions in 1% Bacto Peptone Water (Difco) was plated in triplicate by the spread plate technique onto Plate Count Agar (PCA, Oxoid, Basingstoke, UK) for total bacterial counts, and incubated aerobically at 37°C for 24 h. Complete eradication was observed when the plates showed no colonies after inoculation of duplicated plates.

Correlation of optical density to standard plate counts on artificially inoculated chicken carcasses

Inoculation of sterilised chicken carcasses for correlation studies

A total of three chicken carcasses were treated with 0.3 v/v% Virukill as described above. After Virukill treatment each chicken carcass was individually placed in the 500 ml bacterial suspension, consisting of an 18 h microbial culture (with the bacterial concentration of approximately 10^7 cfu/ml). The 18 h culture was prepared by inoculating a colony of an isolate in 10 ml nutrient broth and incubating for 18 h at 37°C. Different concentrations of bacterial suspension were acquired by mixing the 18 h cultures with water to make a 500 ml volume. Chickens were suspended in the bacterial solution for 30 min to allow the bacteria to attach to the chicken surface, and then the aliquot suspension was discarded by holding the carcass upside down, inside the plastic bag, by one leg for 10 seconds.

The chicken carcasses were sampled using neck skin and whole carcass rinse method. Both these methods have been described in detail in the above sections. From both sampling methods the plate count and OD reading were conducted as described below. The results were obtained and plotted on a scatter plot graph, for correlation analysis.

Optical density reading determinations

Samples collected by neck skin and whole rinse method were used. A 0.1 ml sample was inoculated into two sets of five test tubes consisting of 10 ml Nutrient Broth (Difco). The OD reading of one set of test tubes was determined immediately at 420 nm using a spectrophotometer. The other set of 5 test tubes were incubated for 4 h at 37ºC in a shaker (165 rpm). After 4 h incubation, a 1 ml sample from each test tube was placed into disposable cuvettes and OD readings determined at 420 nm by using a spectrophotometer. OD values of the first set of test tubes were subtracted from the value obtained from the second set, which was incubated for 4 h. The difference of mean OD values obtained between these the two sets of test tubes were used for correlation to plate counts. Sterile Nutrient Broth treated as inoculated tubes were used as control.

Standard plate counts

The same sample used for OD readings (before incubation), were used for bacterial counts. This was done by making ten fold serial dilution in 1% Bacto Peptone Water and 0.1 ml aliquot was pipetted and plated in duplicate on Plate count agar (PCA, Oxoid, Basingstoke, UK) by spread plate technique for total bacterial counts. The plates were aerobically incubated at 37ºC for 24 h. Plates containing between 30 and 300 colony forming units (cfu) (or the highest number if below 30) were counted and the mean count determined from the duplicate plates.

Results were also presented in tables to evaluate the standard deviation for OD readings and plate counts.

Results

Preparation of chicken carcasses for artificial inoculation

Evaluation of different concentrations of Virukill for eradication of all microbial organisms on chicken carcasses and residual effect detection

Immersion of carcasses in high concentrations of Virukill of 1, 1.5 and 2 v/v%, for a 30 min contact time eradicated all the initial microbial population on the chicken carcasses. The PCA plates had no colonies after incubation of 24 h at 37°C (Table 1). All three of these Virukill concentrations, however showed residual effects resulting in chicken carcasses remaining sterile after re-infection with ~10^3 /ml of 18 h *E. coli* culture. PCA plate from neck skin samples of re-infected carcasses showed no growth after 24 h incubation at 37°C (Table 1).

Table 1: Evaluation of high concentrations of Virukill treatments for eradication of microorganisms on processed poultry carcasses. Treatment applied for 30 minutes.

Virukill [v/v%]	Sterility achieved	Microbial recovery after re-inoculation
1.0	+	-
1.5	+	-
2.0	+	-

Low concentrations of Virukill at 0.1 and 0.2 v/v% were only able to reduce the microbial load. The initial bacterial load averaging 10^4 cfu/g was reduced to less than 10 cfu/g at these low Virukill concentrations. On the other hand a 0.3 v/v% Virukill solution eradicated all microorganisms on the chicken carcasses (Table

2). The low concentration Virukill treatments did not have any residual effects as the re-inoculated bacteria could be re-isolated from treated chicken carcasses (Table 2). The concentration of 0.3 v/v% Virukill was chosen as the appropriate Virukill concentration for further studies, because it eradicated all microorganisms on the processed carcasses without any residual effect.

Table 2: Evaluation of low concentrations of Virukill treatments for eradication of microorganisms on processed poultry carcasses. Treatment applied for 2 h.

Virukill [v/v%]	**Sterility achieved**	**Microbial recovery after re-inoculation**
0.1	-	+
0.2	-	+
0.3	+	+

Evaluation of neck skin, whole carcass rinse, and swabs sampling methods for the reliability in determining the effectiveness of Virukill treatment.

When using the swab sampling method, complete microbial eradication with low concentration of Virukill (0.1 and 0.2 v/v%) as seen by the absence of growth on PCA plates after appropriate incubation in two different experiments (Table 3).

When similar Virukill concentrations (0.1 and 0.2 v/v%) were evaluated by whole carcass rinse and neck skin sampling methods, bacterial growth was found after treatment (Table 3). Complete bacterial eradication was obtained in 2 out of 4 repetitions from both neck skin samples and whole carcass rinse, of chickens treated with 0.2 v/v% Virukill. All repetitions with the 0.3 v/v% Virukill treatment resulted in complete eradication of all microorganisms from the

chicken carcasses when using all three sampling methods, as the PCA plates showed no colonies after appropriate incubation for 24 h at 37°C.

Table 3: Effectiveness of low Virukill treatments for eradication of microorganisms on processed poultry carcasses, by comparing swab sampling, whole carcass rinse and neck skin methods.

Virukill [v/v%]	Swab sampling [cfu/ml^2]	Whole rinse [cfu/g]	Neck skin [cfu/g]
0.1	- (0.000)	+ (0.010)	+ (0.800)
0.2	- (0.000)	+ (0.005)	+ (0.200)
0.3	- (0.000)	- (0.000)	- (0.000)

Correlation of optical density to standard plate counts on artificially inoculated carcasses

The scatter plots presentations of the results obtained from mixed cultures and individual organism as pure culture mostly had positive, significant values of the correlation coefficient, which were mostly greater than 0.900%. Carcasses inoculated with pure culture of *A. hydrophila* sampled by whole carcass methods (Figure. 3) had a correlation coefficient of 0.848, while carcasses sampled by neck skin method had a significant correlation coefficient of 0.914 (Figure. 4). The correlation coefficient for *Serratia marcescens* was 0.993 and 0.940 for evaluation of samples obtained by whole carcass rinse and neck skin sampling methods respectively (Figure. 5 & 6). The mixed culture had correlation coefficient values of 0.903 and 0.978 for whole carcass rinse and neck skin sampling methods respectively (Figure. 1 & 2). Similar results were also presented in tabular forms, additional information incorporated in the tables, (which is not included in the graphs) included coefficients of variation for OD, and standard deviation for plate counts. The highest plate counts for neck skin were above 10^6 cfu/g, and for whole carcass rinse the values were 10^4cfu/g (Table 4 & 8), with the exception of *A. hydrophila* where 10^6 cfu/g

(Table 6) was obtained. (Table 4-10) indicate that the higher the microbial load, the larger the standard deviation.

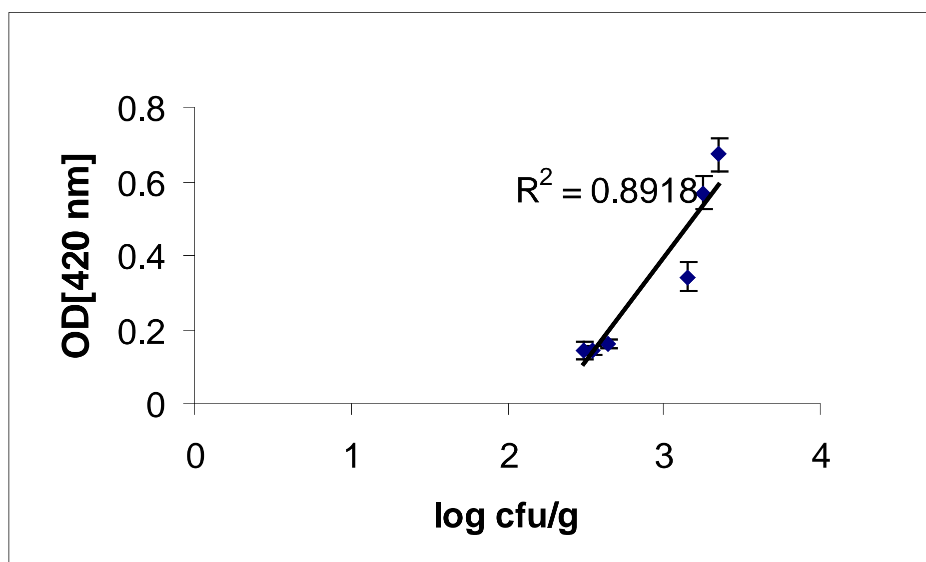

Figure 1: Correlation of standard plate counts to OD reading for mixed culture (*E. coli, Shewanella putrifaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus*) sampled by whole carcass rinse sampling method.

Table 4: OD readings and standard plate count of the correlated value by evaluation of *standard diviation and **coefficients of variation for mixed culture (*E. coli, Shewanella putrifaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus*) sampled by whole carcass rinse sampling method.(Same highlighted changes were done for all correlation tables)

OD readings			**Plate counts**		
Mean OD	***SD**	****CV%**	**LOG (cfu/g)**	***SD**	****CV%**
0.142	0.022	15.468	2.495	0.816	32.71
0.145	0.012	8.2873	2.545	0.079	3.105
0.163	0.013	7.9755	2.637	0.169	6.408
0.341	0.039	11.444	3.152	0.075	2.38
0.568	0.045	7.9225	3.246	0.184	5.669
0.672	0.046	6.8452	3.345	0.156	4.664

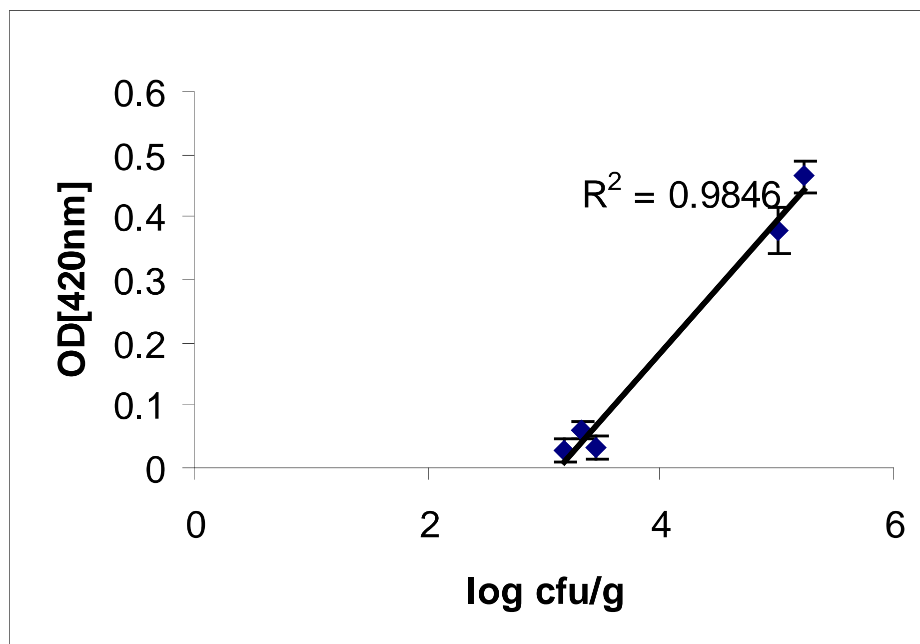

Figure 2: Correlation of standard plate counts to OD reading for mixed culture (*E. coli, Shewanella putrifaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus*) sampled by neck skin sampling method.

Table 5: OD reading and standard plate count of the correlated value by evaluation of *standard deviation and **coefficient of variance for mixed culture (*E. coli, Shewanella putrifaciens, A. hydrophila, Serratia marcescens* and *Staphylococcus aureus*) sampled by neck skin sampling method.

OD readings			**Plate counts**		
Mean OD	***SD**	****CV%**	**LOG (cfu/g)**	***SD**	****CV%**
0.027	0.0195	71.527	3.179	0.025	0.786
0.032	0.0173	53.634	3.455	0.045	1.302
0.061	0.0142	23.156	3.332	0.074	2.220
0.378	0.0357	9.414	5.017	0.169	3.368
0.465	0.0257	5.533	5.238	0.595	11.359

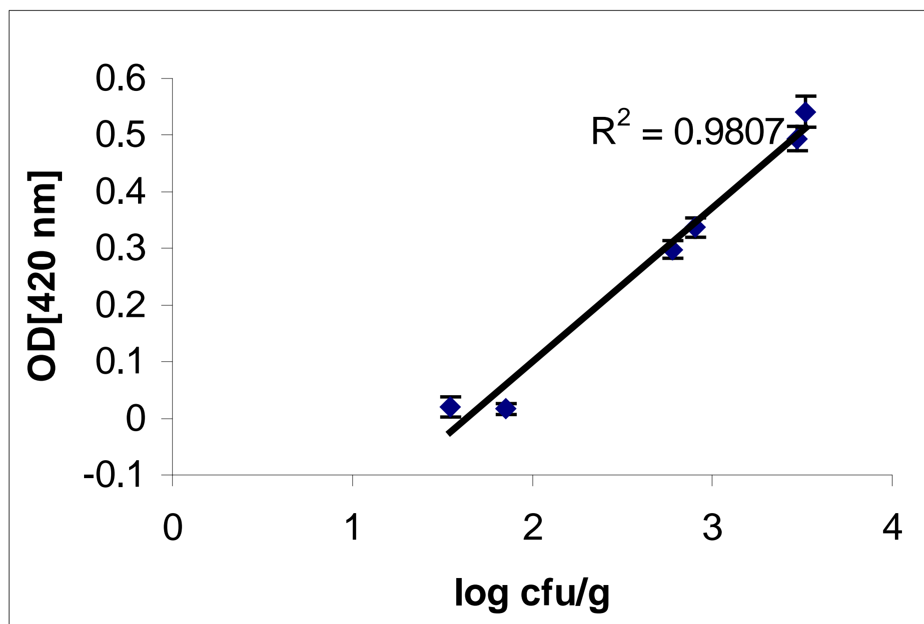

Figure 3: Correlation of standard plate counts to OD reading for *A. hydrophila* sampled by whole carcass rinse sampling.

Table 6: OD readings and standard plate count of the correlated value by evaluation of *standard deviation and **coefficients of variation for *A. hydrophila* sampled by whole carcass rinse sampling.

OD readings			Plate counts		
Mean OD	*SD	**CV%	LOG (cfu/g)	*SD	**CV%
0.0166	0.0095	57.331	1.8513	0.34	18.366
0.0202	0.0180	88.911	1.5441	0.214	13.860
0.2977	0.0156	5.259	2.7782	0.300	10.799
0.337	0.0176	5.2077	2.9031	0.150	5.167
0.4933	0.0215	4.3606	3.472	0.679	19.556
0.5412	0.0278	5.1362	3.5185	0.123	3.496

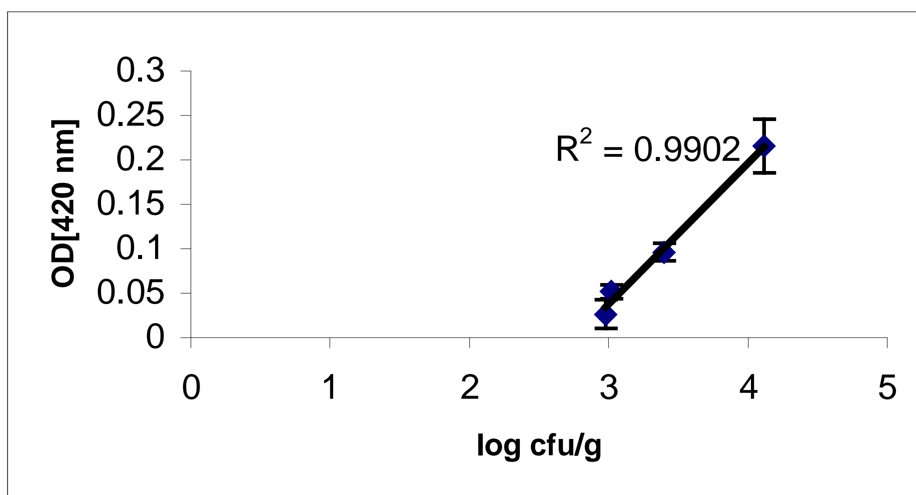

Figure 4: Correlation of standard plate counts to OD reading for *A. hydrophila* sampled by neck skin sampling method.

Table 7: OD readings and standard plate count of the correlated value by evaluation of *standard deviation and **coefficients of variation for *A. hydrophila* sampled by neck skin sampling method.

OD readings			**Plate counts**		
Mean OD	***SD**	****CV%**	**LOG (cfu/g)**	***SD**	****CV%**
0.0264	0.0159	60.383	2.98	0.512	17.208
0.0518	0.008	15.415	3.02	0.366	12.131
0.0963	0.0098	10.129	3.40	0.074	2.178
0.2154	0.0305	14.160	4.11	0.04	0.972

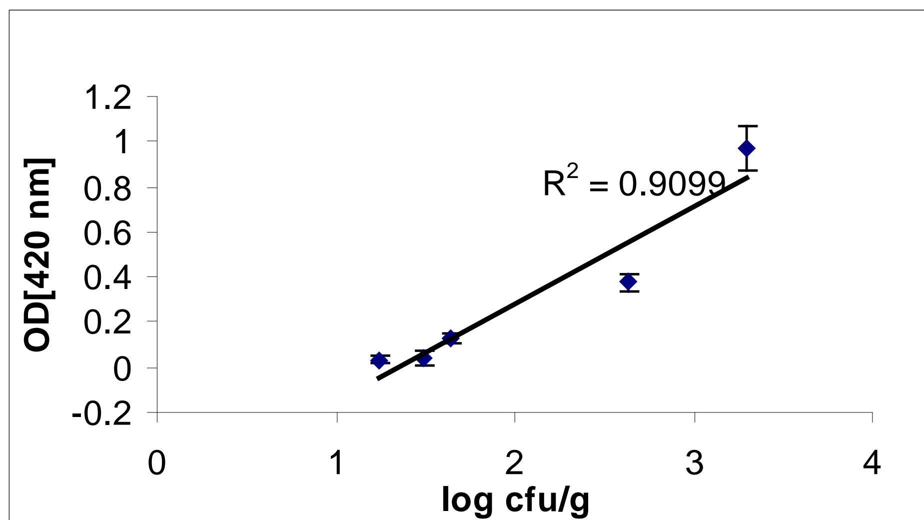

Figure 5: Correlation of standard plate counts to OD reading for *Serratia marcescens* sampled by whole carcass rinse sampling.

Table 8: OD readings and standard plate count of the correlated value by evaluation of *standard deviation and **coefficients of variation for *Serratia marcescens* sampled by whole carcass rinse sampling.

OD readings			**Plate counts**		
Mean OD	***SD**	****CV%**	**LOG (cfu/g)**	***SD**	****CV%**
0.0326	0.0179	54.755	1.236	0.017	5.7453
0.0432	0.033	76.435	1.490	0.1033	6.9329
0.1258	0.021	16.669	1.638	0.3887	23.735
0.3749	0.034	13.863	2.638	03887	14.736
0.9703	0.0967	9.966	3.301	0.0155	0.4695

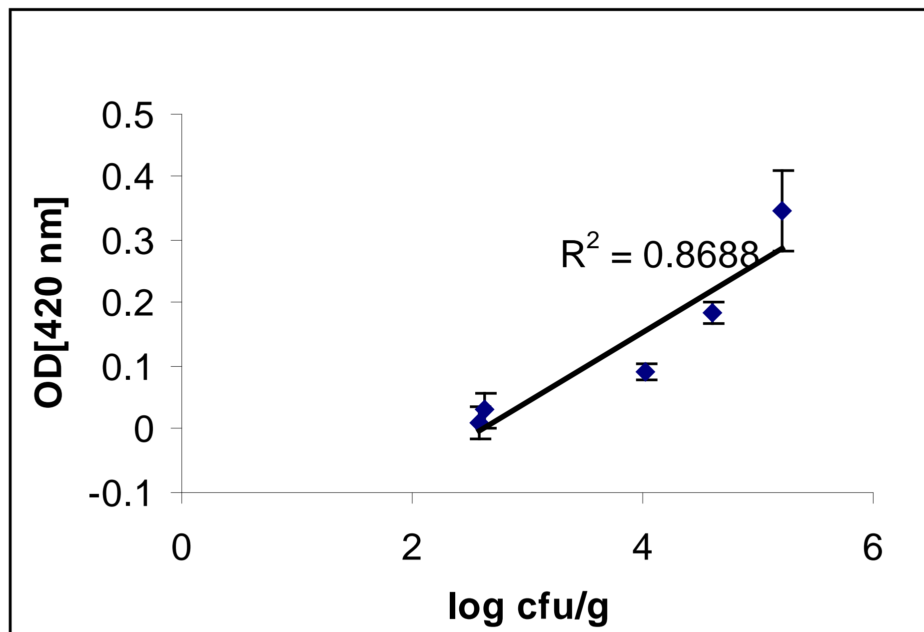

Figure 6: Correlation of standard plate counts to OD reading *Serratia marcescens* sampled by neck skin sampling method.

Table 10: OD readings and standard plate count of the correlated value by evaluation of *standard deviation and **coefficients of variation for *Serratia marcescens* sampled by neck skin sampling method.

OD readings			**Plate counts**		
Mean OD	***SD**	****CV%**	**LOG (cfu/g)**	***SD**	**CV%**
0.0109	0.027	247.706	2.574	0.213	8.275
0.0302	0.027	89.404	2.6284	0.0212	0.8066
0.0918	0.0112	12.200	4.0233	0.151	3.7532
0.1851	0.0167	9.022	4.6128	0.149	3.2302
0.3468	0.0659	19.002	5.2148	0.056	1.0739

Discussion

Chicken carcasses from the poultry processing plant are contaminated with vast numbers of microorganisms. Microorganism-free carcasses are essential because these studies are based on the re-infection of carcasses with known numbers of specific microbial species. A chemical method was used to eradicate all microorganisms on chicken carcasses. This was done to prepare carcasses for the primary objective of this study, which was to evaluate OD readings as a possible enumeration method by establishing the correlation between OD readings after 4 h incubation to standard plate counts.

It was demonstrated in the study that a 0.3 v/v% solution of Virukill completely removed all bacterial contaminants on poultry carcasses, without leaving any residual effects as demonstrated by the fact that bacteria could be re-isolated from treated chickens, which were re-infected with bacteria. The use of a 0.3 v/v% Virukill solution was thus selected for all further studies in which bacterial free chicken carcasses were required for inoculation with known bacterial concentrations to determine if there is a relationship between OD readings and plate counts to determine bacterial contamination levels.

Virukill was selected to remove all bacterial contamination from these carcasses, as this product has been shown to be highly effective at very low concentrations. The advantages of Virukill include being non-corrosive, it's not an environmental hazard, it's able to work at wide range temperatures and it is biodegradable after 40 h. Only small concentrations are required for complete microbial eradication. According to the manufacturers, Virukill disinfectant brought great improvements in animal hygiene by decreasing chances of animals being infected with pathogenic microorganisms. Virukill is able to inactivate viruses, bacteria, fungi, yeast and *Mycoplasma*. Virukill can be safely applied on walls, floors and equipment. International respected institutes have tested low concentrations of Virukill, for its effectiveness as microbial disinfectant. This institutes include the South African Bureau of Standards; the

Department of Tropical Diseases at the University of Pretoria; and Irene Agricultural Research Council (ARC). Virukill has also been applied to living animals by continuously dosing the drinking water or fogging the chickens.

Low Virukill concentrations (0.1 and 0.2 v/v%) greatly reduced microbial populations on poultry carcasses (Table 2 & 3). These results show great potential for other applications of Virukill in the poultry processing industry, which includes its use in a chilling system. For the Virukill to be successfully applied in this system, it should be first tested for its effectiveness under chilling conditions (2-4°C for 35 min).

Low concentration of alternative disinfectant cannot eradicate all microorganisms on poultry carcasses. Applications of high concentrations are expensive, toxic, produce carcinogenic compounds or change the carcass's sensory factors (Smulder & Greer, 1998). Important chemical disinfectants that have received great attention are chlorine and ozone. Chlorine in the form of hypochlorite has been implemented in the chilling system of most poultry processing plants (Lillard, 1979; Sofos & Smith, 1998). Corry *et al.* (1995) reported a complete microbial eradication after chicken carcasses treated with high dose of irradiation, though high irradiation dose alters flavour, odour and appearance. Irradiation is expensive and requires large space (Kampelmacher, 1984).

In this study the most researched and applied sampling methods to recover microorganisms from chicken carcass were evaluated during the sterility analysis. The swab method was found to be unreliable for microbial enumeration studies because of its low recovery of microorganisms from the surface of the carcasses (Table 3). The method was thus eliminated from further studies of the correlation of OD readings after 4 h incubation to plate counts. The swab sampling method has previously been reported to have a low recovery as compared to neck skin and whole carcass rinse methods. The low

recovery of microbial populations on chicken carcasses is because this method removes mostly the weak attached microorganisms (Fromm, 1959).

There was no significant difference between neck skin and whole carcass rinse sampling methods when detecting bacteria from chicken carcasses (Table 3). The method of choice for further experimentation between these methods was based on other factors, which include how rapid and easy the method, is to perform. The neck skin sampling methods is able to recover microorganisms that are firmly attached, as well as those embedded in the feather follicles (Avens & Miller, 1970; Emswiler *et al.,* 1977; Fromm, 1959). This method is easy, accurate and requires less time for collection of samples from carcasses. The whole carcass rinse is useful for recovering organisms that are present in low numbers on the carcasses, such as *Salmonella* spp. (Dougherty, 1974; Cox *et al.,* 1981). Jorgensen *et al.* (2002) however, recovered more *Salmonella* spp. by using the neck skin collection methods than through whole carcass rinse. The whole carcass rinse, on the other hand is a non-destructive method (Lillard, 1988). Recent developments of automating the shaking step of this method reduce the labour and variability in this method (Dickens *et al.,* 1985).

Homogenized food samples have high amount of debris that would interfere with accuracy of the OD readings. Mattila (1987) suggested the use of dilution to reduce background effect, and subtraction of the initial OD readings (before incubation) from OD reading after DT is reached. Jorgensen & Schultz (1985) suggested centrifuging mixed meat samples at 300x g for 1 min to eradicate major food debris correlation conducted similar to Mattila (1987)

Significant low (<15%) values of coefficients of variation were obtained for OD readings from samples obtained from carcasses, though low OD readings values were higher. The microbial loads in this study were evaluated up to below 100 cfu/g (Table 6). A commercially, processed carcass consist of higher than 10^3 cfu/g total bacterial counts, after processing (Al-Mohizea *et al.,* 1994;

Sakhare *et al.,* 1999). Lower microbial count evaluation is essential for future methods, which include finding a method that would greatly reduce the initial load of processed poultry carcasses (this is a major concern in poultry industry).

Most studies of OD readings correlated to plate counts are very difficult to compare to the current study. In these studies detection time (DT) was correlated to plate counts (Mattila, 1987; Jorgensen & Schulz, 1985). A brief description of DT was given in Chapter 3. Jorgensen & Schulz (1985) found a perfect correlation coefficient of 1 from his study with pure culture and 0.98 with samples directly take from mincemeat, when standard plate counts were correlated to the DT determined by automated OD method.

Direct correlation exists on dominant microorganisms isolated from eggs both from pure culture and bacteria sampled directly from eggs (Pienaar *et al*., 1994; Pienaar *et al.,* 1995). OD readings, as an enumeration method are cost effective, reliable and have wide industrial applications (Mattila, 1987).

Conclusion

The use of Virukill at the concentration of 0.3 v/v% for 2 h is effective for microbial eradication of all microorganisms determined by PCA plate at 37° for 24 h. This treatment does not have high residual effect. The use of high concentrations of Virukill treatments resulted in microbial elimination with Virukill residue left on the carcasses. This residue is sufficient to kill the microorganisms after re-infection of carcasses. Though this would be good for eradication of microorganisms, in cases where the primary purpose of the experiment was to eradicate microorganisms indefinitely. The primary reason for eradication of microorganisms in this study was to prepare chicken carcasses for re-infection with known microbial species. This is done to correlate OD reading to the standard plate counts.

The OD methods have significant correlation coefficients with both sampling methods; hence both methods can be applied. The use of neck skin sample method is advantageous over the whole carcass method for routine evaluation, because of the time and labour required to do the latter technique. In cases where the detection of particular species which, are significant to human life but are present in low concentration on the chicken carcasses, the whole carcass rinse method is advisable.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION.

Birds enter the processing plants with microorganisms on many parts of their body, indicating that most live birds are contaminated during hatching and breeding (Aho, 1992; Walker & Ayres, 1956). The processing steps facilitates the dispersion of these microbial contaminates to other carcasses and body parts on the same carcass (Mulder & Veekamp, 1974; Notermans, *et al.,* 1980). The most important microorganisms isolated on poultry products are various human pathogens, with major concern primarily based on the health risk of the consumers. The other important groups of microorganisms in the production of poultry products are the psychrotrophic organisms. Although the latter group of microorganisms is present in low numbers after processing, they are significant because they are able to grow at low storage temperatures and cause spoilage (Cousin *et al.,* 2001; Russel *et al.,* 1996). Yeasts are isolated only in small numbers on processed poultry (Hinton *et al*., 2002).

The initial microbial population on the processed chicken carcasses consists mostly of mesophilic microorganisms (Russell *et al.,* 1996). The enumeration of this initial microbial load on chilled carcasses is essential in determining the safety and shelf life prediction of the final products (Ayres *et al.,* 1950). The final spin chilling processing step is important as it involves the final washing of carcasses with the objective of cooling the carcasses and reducing the microbial load.

Conventional microbial enumeration methods for hygiene and safety evaluations are time consuming. The most preferred method is the plate count method as they are accurate and cost effective. Various other enumeration methods have been evaluated for monitoring microbial load on poultry carcasses. The conventional method of preference in poultry industry, involves

standard plate counts or most probable number (MPN) determinations. Obtaining results with these conventional methods ranges from 24 h to 7 days. The main problem with these methods is that when the results are available, the poultry product have already been distributed, sold or consumed (Swanson *et al.,* 2001). One way to overcome this would be storing the products at the processing plant until the manufacturer is sure of microbial status of the carcass. This would require a large storage facility, which could lead to great financial loss because of the space required and power to keep the products refrigerated. The delays in obtaining microbial quantity complicate matters. This means that if the pathogenic and spoilage microorganisms are high in numbers on the chicken carcasses, this could result in an outbreak of foodborne disease or early spoilage. It could have a detrimental effect on the manufacturer, in that it could lead to great economical loss. The reputation of the manufacturer can be jeopardised and in the worst-case scenario this could end up in the company going bankrupt. Serious damage to the credibility of the product can also result from consumption of contaminated products which have a serious negative impact on the industry as a whole and the damage is not only limited to that particular supplier. Rapid methods for the evaluation of microbial load detection could eliminate these problems, and finding such a method that is both rapid and highly reliable would be most desirable in the poultry processing industry.

As there is such a demand for rapid microbial detection methods, many rapid detection methods have been developed and tested. Methods that were evaluated include DEFT, Impedance, Limulus amebocyte lysate assays and ATP- Bioluminescence assay (Jorgenson *et al.,* 1973; Sharpe *et al.,* 1970; Wood and Gibbs, 1992). Disadvantages associated with the implementation of these techniques range from being expensive, laborious, and to a lesser extent some are discriminative against gram staining test, specifically Limulus amebocyte lysate assays, which only can estimate gram-negative microorganisms (Jorgensen *et al.,* 1973). Finding a rapid method, which is

reliable, as well as time and labour efficient is of the greatest essence. Hence, in this study we evaluate the determination of OD readings after a specific incubation time as an alternative, rapid method for microbial enumeration on poultry carcasses in order to reduce these drawbacks encountered with other methods, especially time consuming by conventional methods. Implementation of such an OD reading method could also rapidly detect problems in the sanitary system in the processing plant, thus allowing the manufacturer to rectify this problem before the distribution of the products.

Another major problem in obtaining accurate microbial status of poultry carcasses is the sampling methods, which are needed to obtain microorganism from the carcasses for laboratory evaluation techniques. These methods include whole carcass rinse, swab and neck skin sampling methods (Aven & Miller, 1970). In this study, some focus was placed on the evaluation of different sampling methods.

Chicken carcasses were sampled by whole carcass rinse, and the predominant microorganisms were identified by conventional biochemical tests as well as with the API (bio Merieux Vitek, Inc., Hazelwood, MO) techniques. Cox *et al.* (1981) and Dougherty, (1974) found that whole carcass rinse was effective for the isolation of microorganisms from poultry carcasses. In these experiments it was established that the whole carcase rinse method was acceptable as a sampling method.

The current study was not a complete evaluation of the microbial population on whole chicken carcasses. The first step of this project was to isolate the dominant (depending on isolation media (PCA) and incubation conditions (25°C for 48 hrs)) common species from automatically eviscerated chicken carcasses. These isolates were required for the establishment and evaluation of the OD reading method as a rapid enumeration method. *E. coli, Serratia marscecens, A. hydrophilia, Shewanella putrifaciens* and S*taphylococcus aureus* were

isolated in this study. It can be seen from these results that most of the organisms isolated are primarily mesophilic. These results are similar to those obtained by Ingram & Simonsen, (1980) and Russell *et al.* (1996). The temperature of 37°C is optimal for the selected isolates; hence it was used during the development of OD reading method. The total counts found on the carcasses were in the range of between 10^3-10^4 cfu/g. These counts were found to be similar to those reported by Tompkin *et al.* (2001).

The major objective of this study was to develop the procedures and evaluation of OD reading after a short incubation period as a rapid enumeration method to determine bacterial load on the chicken carcasses. This was done in order to use OD readings as an alternative method to plate counts for bacterial enumeration. The method was tested on both pure culture on organisms in nutrient broth and organisms on artificially inoculated chicken carcasses.

Preliminary tests involved the selection of the optimal wavelength for the procedure. Scanning the media through a range of wavelengths achieved this. A wavelength of 420 nm was selected as the most efficient wavelength. The next stage in the development of the procedure was the determination of a suitable incubation time. This was done by construction of growth curves of the selected isolates. The ideal incubation time is one in which all of the selected isolated are found to be the exponential phase of growth. An incubation time of 4 h was chosen as the incubation time in Nutrient Broth inoculated with the above-mentioned bacterial cultures. The 4 h incubation period was determined by construction of growth curves as the time where most of the isolates were in exponential phase of growth.

The repeatability values obtained from pure cultures and mixed cultures in this work showed low standard deviation and low coefficient of variation. This clearly demonstrates that this method is highly repeatable.

The next step in the establishment of this procedure was to determine if there is a correlation between OD readings after the selected 4 h of incubation and the plate count of the initial sample. This was achieved by inoculating different concentrations of the 18 h cultures of the selected isolates into 10 ml Nutrient Broth test tubes and incubated for 4 h at 37°C. The OD readings obtained after incubation were correlated to standard plate counts, determined on PCA plates that were inoculated from the same samples as those used to determine OD readings, before incubation. Based on the high correlation between standard plate counts and OD readings, it might be possible to replace the conventional methods with the OD reading method.

It has been demonstrated that the OD reading at 420 nm after an incubation period of 4 h, is a possible test method to determine the bacterial counts in pure cultures done *in vitro*. The next phase of this experiment was to determine if the technique could be used on chicken carcasses.

It was essential in this study that chicken carcasses be sterile before they are artificially inoculated, and the non-toxic disinfectant called Virukill was used to achieve this objective. Virukill has been shown to be highly effective for the removal of viruses, bacteria, fungi, yeast and M*ycoplasma*. Virukill can be safely applied on walls, floors and equipment. Virukill has been extensively tested by a number of different institutions, for its effectiveness as microbial disinfectant. These institutes include the South African Bureau of Standards; the Department of Tropical Diseases and the Department of Poultry Health at the University of Pretoria; and Irene Agricultural Research Council (ARC). Virukill has also been applied to living animals by continuously dosing the drinking water or fogging the chickens and is registered in South Africa through Act 36 of 1947 for this application.

Different concentrations of Virukill were evaluated in order to select a dilution, which would achieve sterility and would not have residual effects on the

carcasses when they were re-infected with the desired bacterial cultures. The concentration of 0.3 v/v% of Virukill with the contact time of 2 h at ambient temperature achieved microbial free carcasses without any residual effects. Most chemical disinfectants that have already been researched are only able to achieve microbial free carcasses at high concentrations and have a high residual effect. These chemicals at high concentrations change the sensory characters of the carcasses (Kotula *et al.*, 1974). (Deleted part of this)

The sterilized carcasses were artificially infected with known species at different concentration and evaluated for correlation of OD readings to standard plate counts. It was demonstrated that the correlation between the two methods was high. This indicates that the determination of OD reading after only 4 h of incubation could be used for monitoring microbial contamination on chicken carcasses. Comparison of results in the current study to findings in literature is complicated; since most literature correlated the standard plate counts to detection time (DT) (Mattila, 1987; Jorgensen and Schulz, 1985). DT is the measure of the required time for initial bacteria load to adapt to growth medium and to grow at selected incubation conditions until specific signal is produced (Jorgensen & Schulz, 1985). In the current study the organisms were incubated for a fixed time of 4 h. Jorgensen & Schultz, (1985) and Mattila, (1987) found good correlation when they correlated the plate count method and turbidity, but in that study automated machines were used.

The application of the OD method in monitoring the chicken carcasses reduces the time required in obtaining the results from chicken carcasses. This method is also easy to perform.

During this study, it has been demonstrated that the OD reading after 4 h is highly repeatable when the same inoculum is used. It has also been shown that there is a good correlation to plate counts when using pure cultures. A method

was established during this work for the complete removal of bacterial contaminated from chicken carcasses, using the non-toxic disinfectant Virukill. It was also established that at the dose of Virukill used for the removal of bacteria, contaminants did not result in any residual activity of Virukill on the carcasses.

The results from artificially inoculated carcasses show that there is a high level of correlation between the OD readings after 4 h incubation and the plate counts made directly from the carcasses samples. With the OD reading method, results could be obtained after only 4 h, where plate count method required overnight incubation.

This work has clearly demonstrated that the OD reading method is a repeatable and rapid method for determination of bacterial contamination on chicken carcasses. The technique is easy to perform and rapid. The method also requires equipments, which should be available in most microbiological laboratories (spectrophotometer, shake incubators and test tubes). This technique does not require expensive dedicated equipment. The major drawback to this method is that it requires dilution to be done when the OD reading is above 1.

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CHAPTER 6

SUMMARY

Key terms: poultry carcasses, mesophiles, optical density, plate count, sampling, isolation, identification, repeatability, correlation, virukill.

Increased media and public interest over meat contamination have highlighted the need for continual improvements in this regard in the poultry industry. In spite of all the effort and money placed into research on microbial contamination of poultry carcasses, it is still not possible to produce carcasses, which are free of spoilage and pathogenic microorganisms. Carcasses are mostly contaminated during processing, contributing to the presence of high number of mesophilic microorganisms. The spoilage microorganisms cause consumers to reject the product due to appearance, off odour or undesirable flavour whereas the pathogenic microorganisms may lead to health hazards.

The Standard plate counts methods have been the method of choice for the enumeration of contaminating microbial populations in the poultry industry. However, these methods are laborious and time consuming. In this study, optical density (OD) reading as a microbial enumeration method on poultry carcasses was established and evaluated for its application as a rapid, alternative method.

In chapter 2, the dominant microorganisms were isolated and identified from eviscerated poultry carcasses. The carcasses were sampled by whole carcass rinse method and bacteria were isolated on Plate Count Agar at 27°C for 48 h. A total of five different species were identified, based on their prevalence in the sample and were identified by conventional methods. The identification of the isolates was further confirmed by API techniques. The dominant species were *Escherichia coli, Shewanella putrefaciens, Aeromonas hydrophila, Serratia*

marcescens and *Staphylococcus aureus*. Mean counts (log_{10} cfu/g) of these organisms were 3.08, 1.48, 0.85, 1.74, and 1.21 respectively.

In chapter 3, OD readings as an enumeration method was established. This required the establishment of a number of parameters, which include:

1) Determining the wavelength to be used throughout this study. This achieved by scanning the medium through the spectra of 200-700 nm and a wavelength of 420 nm was selected as the optimal wavelength.
2) Determination of incubation time, which was achieved by the construction of separate growth curves for each isolates and determine the time when all isolates were in the exponential phase of growth. A time of 4 h was selected.
3) Evaluation of the repeatability of the OD readings of each isolate. Both pure culture and mixed culture were evaluated. The coefficients of variation for repeatability evaluations had coefficients of variation less than 15% for pure culture. Similar coefficients of variation values was obtained with mixed cultures and most values were even below 10%.
4) The correlation of OD reading evaluated after 4 h incubation at 37°C to standard plate counts (incubated in PCA at 37°C for 24 h). The scatter plots graph obtained in this study had had a positive strong correlation, which was above 0.9 for all isolates, except for *Serratia marcescens,* which had the correlation coefficient of 0.886. The high repeatability and correlation showed high potential of the OD in enumeration, hence the further objective of the involved evaluating the method on chicken carcasses.

In chapter 4, carcasses were sterilized, followed by artificially inoculation with known microorganisms at different load. Sterilization was evaluated by examining different concentrations of Virukill solution at different concentration.

Virukill is a non-toxic, highly effective disinfectant. High concentration (1, 2 & 3 v/v%) of this disinfectant was effective in eradicating all microbial population under the applied conditions. It was established that at these high levels, substantial residual effects were found which prevented the re-inoculation of chicken carcasses. With lower concentrations, it was established that a 0.3 v/v% of Virukill eradicated all microorganisms and did not have residual effect, thus allowing re-inoculation of the carcasses with known concentrations of bacteria. A correlation coefficient between standard plate counts and the OD reading method of 0.903 and 0.968 were found for mixed cultures determined by whole carcass rinse and neck skin sampling methods, respectively. *A. hydrophila,* had correlation coefficient of 0.849 and 0.985 for whole carcass rinse and neck skin respectively. Similarly *Serratia marcescens* had 0.993 and 0.940 for whole rinse and neck skin respectively.

The results found in this study clearly show that bacterial enumeration through the use of OD readings is capable of reducing time and labour required to obtain the initial microbial load after processing of the carcasses. The OD method evaluated on artificially contaminated carcasses is promising. The method shows great potential for enumeration of bacteria during routine evaluation at the poultry processing plant.

CHAPTER 7
OPSOMMING

Toenemende belangstelling deur die media en publiek na vleis-kontaminasie het die nodigheid vir toenemende verbetering in hierdie verband in die pluimvee industrie aan die lig gebring. Ten spyte van al die moeite en geld aan navorsing na mikrobiese kontaminasie van pluimvee karkasse is dit steeds nie moontlik om karkasse, vry van bederf en patogeniese mikroörganismes, te produseer nie. Karkasse word meestal gekontamineer gedurende die ontwikkeling, wat bydra tot die teenwoordigheid van hoë getalle mesofiliese mikroörganisme. Die bederf mikroörganismes veroorsaak dat verbruikers die produk verwerp weens voorkoms, af-reuke of onaanvaarbare geur, waar patogeniese mikroörganismes kan lei tot gesondheidsgevare.

Die standaard plaat-telling metodes was die metode van keuse vir die bepaling van kontaminінerende mikrobe populasies in die pluimvee industrie. Maar hierdie metode is arbeid intensief en tydrowend.

In hierdie studie is optiese digheidslesings (OD) as ‘n mikrobiese bepaling metode op pluimvee karkasse ontwikkel en geëvalueer as ‘n vinnige alternatiewe metode.

In hoofstuk 2, is die dominante mikroörganismes geïsoleer en identifiseer uit geslagte pluimvee karkasse. Monsters is geneem vanaf karkasse deur die heel karkas-spoel metode en die bakterieë is geïsoleer op “plate count agar” (PCA) by 37°C vir 24 uur, ‘n totaal van vyf verskillende spesies is geselekteer, gebaseer op voorkoms in die monster en is geïdentifiseer deur konvensionele metodes. Die identifikasie van die isolate is verder bevestig deur API tegnieke. Die dominante spesies was *Escherichia coli, Shewanella putrefaciens, Aeromonas hydrophila, Serratia marcescens* en *Staphylococcus aureus*. Die

gemiddelde tellings (log_{10} cfu/g) van hierdie mikroörganismes was 3.08, 1.48, 0.85, 1.74, en 1.21 respektiewelik.

In hoofstuk 3, is die OD lesings as bepalings metode ontwikkel. Dit was dus nodig om ‘n aantal parameters vas te stel:

1) Bepaling van die golflengte wat deur die studie gebruik is. Dit is gedoen deur media te skandeer deur die spektrum 200-700 nm en ‘n golflengte van 420 nm is geselekteer as die optimum golflengte.
2) Bepaling van inkubasie tydperk wat gedoen is deur afsonderlike groeikurwes vir elke isolaat te doen en vas te stel wanneer alle isolate in die eksponensiële groei fase is. ‘n Tyd van 4 ure is geselekteer.
3) Bepaling van die herhaalbaarheid van die OD lesings van elke isolaat. Beide pre-isolasie suiwer kulture en gemengde kulture is geëvalueer. Die koëffisiënt van variasie van minder as 15% is getoon vir suiwer kulture. Soortgelyke koëffisiënt van variasie waardes is verkry vir gemengde kulture en die meeste waardes was minder as 10%.
4) Die korrelasie van OD-lesings (geëvalueer na 4 uur inkubasie by $37^{o}C$) met standaard plaattellings (geïnkubeer in PCA by $37^{o}C$vir 24 ure). Die “scatter plots” grafiek verkry het positiewe korrelasie getoon, wat hoër as 0.9 was vir alle isolate behalwe *Serratia marcescens* wat ‘n korrelasie koëfisiënt van 0.886 gehad het. Die hoë herhaalbaarheid en korrelasie toon baie potensiaal vir die OD-lesings in die bepaling, waarna die metode geëvalueer is op hoender-karkasse.

In hoofstuk 4 is karkasse gesteriliseer, gevolg deur kunsmatige inokulasie met bekende mikroöganismes teen verskillende hoeveelhede. Sterilisasie is geëvalueer deur verskillende konsentrasies Virukill oplossings te ondersoek. Virukill is ‘n nie toksiese, hoogs effektiewe ontsmettingsmiddel. Hoë konsentrasies (1, 2 en 3%) van die ontsmettingsmiddel was effektief om alle mikrobiese populasie te ontwortel onder die gegewe kondisies. Daar is vasgestel dat by hierdie hoë konsentrasie daar substansieele residuele effekte

was wat her-inokulasie van die hoenderkarkasse verhoed het. By laer konsentrasie is daar vasgestel dat in 0.3% van Virukill alle mikroörganimes verwyder is en dat daar geen residuele effekte was nie, dus was her-inokulasie van die karkasse met bekende konsentrasies bakterieë moontlik. ‘n Korrelasie koëffisiënt tussen die standaard plaat-tellings en die OD lesing metode was 0.903 en 0.968 vir gemengde kulture bepaal deur heel karkas spoel en nekvel monstering metode respektiewelik. *A. hydrophila* het ‘n korrelasie koëffisiënt van 0.849 en 0.985 vir heel karkas spoel en nekvel respektiewelik getoon. So ook het *Serratia marcescens* ‘n 0.993 en 0.940 vir heel karkas spoel en nekvel respektiewelik gehad. Die resultate gevind in hierdie studie wys duidelik dat bakteriese bepaling deur die gebruik van OD-lesings dit moontlik maak om die tyd en werk lading nodig om die aanvanklike mikrobiese lading na prosessering van die karkasse te verminder. Die evaluering metode van OD op self gekontamineerde karkasse is belowend. Die metode wys groot potensiaal vir die bepaling van bakterie gedurende roetine evaluasie by die pluimvee plantasie.