
**DOMESTIC HYGIENE: POSSIBLE LINK
BETWEEN ANTIBIOTIC RESISTANT *SALMONELLA*
AND *E. COLI* AND RESISTANCE TO HOUSEHOLD
ANTIMICROBIAL AGENTS**

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A dissertation submitted to the Faculty of Science, University of the
Witwatersrand, Johannesburg, in fulfilment of the requirements for the
degree of Master of Science

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DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Catherine Ann Thorrold

Signed this ____ day of _____ 2005

ABSTRACT

Inappropriate use of antimicrobial household agents has been shown to select for organisms with resistance mechanisms (e.g. efflux pumps), which could lead to the development of antibiotic resistance. The objective of this study was to investigate a possible link between antibiotic resistant gastrointestinal pathogens and reduced susceptibility to antimicrobial agents found in commonly used household disinfectants. Tetracycline and ofloxacin resistant and sensitive *Salmonella* and *E. coli* species were isolated from fresh poultry and clinical samples. Ethidium bromide accumulation assays were performed to assess the presence of active efflux pumps. Using spectrophotometric accumulation assays, the extrusion of the active components of commercial household agents by the efflux pumps was tested. To determine changes in the efficacy of these products, in-use disinfectant testing was performed. Active efflux pumps and extrusion of the active ingredients was observed in the resistant but not the sensitive organisms. When the household products were used at the recommended concentration, a significant reduction of both resistant and sensitive bacteria was observed after the in-use disinfectant testing procedure. However, if the household products were used at concentrations below the recommended concentration, the resistant bacteria were not eliminated as efficiently as the sensitive bacteria.

To my family

PRESENTATIONS AND PUBLICATIONS

The following poster presentations arose from work done towards this dissertation.

1. PathSplash 2004 Congress, Stellenbosch, South Africa, 4-7 July, 2004. Efflux pumps- the link between resistance to household antimicrobials and antibiotics? C. Thorrold, E. Marais, A.G. Duse.
 2. The Faculty of Health Sciences Research Day, Parktown, South Africa, 4 August, 2004. Efflux pumps- the link between resistance to household antimicrobials and antibiotics? C. Thorrold, E. Marais, A.G. Duse.
 3. The 2004 Molecular and Cell Biology Group Annual Symposium, Parktown, South Africa, 6 October, 2004. Efflux pumps- the link between resistance to household antimicrobials and antibiotics? C. Thorrold, E. Marais, A.G. Duse.
-

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	acquired immunodeficiency syndrome
°C	degrees Celsius
ATP	adenosine 5'-triphosphate
BHI	brain heart infusion
bp	base pair
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cfu	colony forming unit
DMP	Diagnostic Media Products
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
HIV	human immunodeficiency virus
kb	kilo base
LB	Luria Bertani
MATE	multidrug and toxic extrusion
MFS	major facilitator superfamily
ml	millilitre
MLCB	mannitol lysine crystal violet brilliant green

mM	millimolar
mm	millimetre
NCCLS	National Committee for Clinical Laboratory Standards
NHLS	National Health Laboratory Service
nm	nanometre
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMSF	phenylmethanesulfonyl fluoride
RND	resistance-nodulation-division
rpm	revolutions per minute
SMR	small multidrug resistance
spp	species
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
µg	microgram
µl	microlitre
µM	micromolar
x g	times gravity
XLD	xylose lysine desoxycholate

CHAPTER 1

1 INTRODUCTION

1.1 Domestic Hygiene

For many years it appears that people have believed that the main occupants of a household are healthy adults, with high levels of immunity to infection (Bloomfield 2001). This is however not true in view of the fact that the home can be occupied by individuals of all ages such as children, parents, and grandparents, all having different health statuses (Scott 2001; Anonymous 2001). This is particularly true in sub-Saharan Africa where 25 million people are currently living with the human immunodeficiency virus (HIV) (WHO/UNAIDS 2004). It is this false ‘healthy household’ assumption that has resulted in complacency, within the public and amongst the authorities, leading people to believe that domestic hygiene is not a serious problem (Bloomfield 2001). In recent years however people who work in public health have come to realize that the home is a potentially important source of infection (Bloomfield 2001; Anonymous 2001).

The home plays a very important role in the transmission of infection throughout the community (Anonymous 2001) because it is the base from which people go to all other places in the community, such as work, school, shopping centres, and leisure centres (Scott 2001). The home also functions as a residence, day care facility for young children, restaurant, hospital, and sometimes an animal shelter (Scott 2001).

There are great benefits to be had in terms of reducing infection levels in the home; these include the reduction of healthcare costs and raising the standards of hygiene practice in the entire community (Bloomfield 2001). A particular area where improved domestic hygiene could have considerable impact is the prevention of infectious intestinal disease (Anonymous 2001).

Infectious intestinal disease causes significant financial loss and morbidity in many countries. Data on the South African situation is scanty, but gastrointestinal infections have been the causative agent for more than three hundred deaths and thirty five thousand hospital admissions per annum in England and Wales from 1991 to 1996, as well as affecting approximately 9.5 million people per annum from 1995 to 1996 in the United Kingdom (Wheeler et al. 1999). It has also been a contributory factor in over a quarter of the deaths of children in the world today, most of which occur in the home in developing countries and could have been prevented (Curtis et al. 2000). The organisms responsible for causing infectious intestinal disease (mainly bacteria and viruses) can be introduced into the home by a variety of sources, including humans, contaminated food, animals, and the environment (Anonymous 2001; Scott 2001; Gorman et al. 2002).

It has been suggested that 28 % of domestic outbreaks of infectious intestinal disease take place due to poor domestic hygiene involving cross contamination via hands and other surfaces (Cogan et al. 2002). An example is the household transmission rate of *Escherichia coli* (*E. coli*) 0157 in Wales, which was estimated at 4-15% (Parry &

Salmon 1998). The kitchen is a significant source of infectious intestinal disease as it harbours many potential pathogens (Rusin et al. 1998) with contaminated dishcloths and sponges being a major cross contamination hazard in the kitchen (Scott & Bloomfield 1990b).

1.2 Cleaning Cloths and Sponges

When used correctly, cleaning cloths and sponges play an important role in domestic hygiene as they aid in the detachment of microbes and soil present on the surface (Humphrey 2001). However, by virtue of their use, the fact that they frequently remain damp for long periods of time, contain residual amounts of food debris, and are not changed frequently, they provide optimal conditions for survival and/or growth of microorganisms (Scott & Bloomfield 1990a).

Many investigations have shown that kitchen dishcloths and sponges become heavily contaminated during use in the domestic setting (Scott & Bloomfield 1990a; Rusin et al. 1998), with the average bacterial count of a dishcloth being as high as 10^8 bacteria (Rusin et al. 1998). In addition to providing reservoirs for the bacteria, dishcloths and sponges also act as disseminators of contamination if not used correctly and studies have revealed that bacteria have been transferred to clean surfaces such as kitchen counters, hands, food, and equipment in sufficient numbers to cause disease (Scott & Bloomfield 1990b).

1.3 Antibiotic Resistance

An organism is referred to as resistant when a change in susceptibility takes place that renders a therapeutic agent ineffective against an infection that was previously treatable by that agent. Many microorganisms have an innate resistance to certain groups of antimicrobials and should be termed innately resistant, and not resistant (Gilbert & McBain 2003).

In the last ten years there has been a significant increase in the number of bacteria that have developed resistance to numerous antimicrobial agents, resulting in increased concerns for public health (Levy 1998). A major selective pressure for the emergence of resistance is the extensive, frequently inappropriate, use of antibacterial agents, in particular broad-spectrum antibiotics (Struelens 1998). It is estimated that approximately 50 % of antimicrobial usage in humans and 80 % in agriculture in the developed world, and possibly even more in the developing world, is thought to be unnecessary (Wise et al. 1998).

The effects of antimicrobial resistance can already be seen in both the community and in hospitals (Braid & Wale 2002). In the community, resistant organisms cause intermittent infections and outbreaks, whilst in hospitals antimicrobial resistance leads to an increase in the duration of stays, and increases in death rate and morbidity (Braid & Wale 2002). The emergence of bacteria resistant to multiple antibiotics has generated a significant threat with regards to the treatment of infections since it is

improbable that new antimicrobial agents can be manufactured fast enough to combat the ever-increasing number of these multi-drug resistant bacteria (Aarestrup et al. 2001).

There is no question that the inappropriate use of antimicrobial agents in humans has led to the increased rates of multi-drug resistant bacteria (Cruchaga et al. 2001), however the appropriateness of use of antibiotics in livestock agricultural practice has recently come under investigation (Singer et al. 2003).

1.4 Antimicrobial Use in the Livestock Industry

Antibiotics have been used in the rearing of livestock ever since the discovery of their therapeutic properties (Butaye et al. 2003). The introduction of antibiotics in the rearing of livestock coincided with increased demand for food in many countries, including South Africa, and has resulted in a variety of developments in animal husbandry practices (Manie et al. 1998; Butaye et al. 2003). These include the breeding of animals in batteries/pens that are filled to capacity at optimal temperature and low light intensity to enhance growth rates and increase body mass (Manie et al. 1998). In addition, many antibiotics including streptomycin, tetracyclines, virginiamycin, penicillin, avoparcin and fluoroquinolones are administered for disease prevention, but subtherapeutic doses are also administered to improve feed efficiency and accelerate weight gain in poultry, swine, sheep and cattle (Manie et al. 1998;

Chopra & Roberts 2001; Geornaras et al. 2001; White et al. 2001; McEwen & Fedorka-Cray 2002; Butaye et al. 2003;).

The practice of dispensing antibiotics for the treatment of sick animals began in the 1940s (McEwen & Fedorka-Cray 2002; Tollefson & Flynn 2002). During the farming of livestock it is easier and more efficient to treat the entire group than a few sick individuals, and in some groups of animals like fish and poultry, mass medication is the only possible method of treatment. The purpose of mass medication is to treat sick animals as well as to provide prophylactic medication to the rest of the animals. This treatment is usually carried out after weaning or transport of animals as these are high risk periods for acquisition of infectious diseases, and antibiotic administration is usually for short periods of time at therapeutic levels (McEwen & Fedorka-Cray 2002).

Subtherapeutic doses of antibiotics are administered to livestock to enhance feeding efficiency and improve their rate of growth, with the outcome being that the animals need less food to reach marketable weight (Manie et al. 1998; McEwen & Fedorka-Cray 2002; Butaye et al. 2003). These antibiotics are usually administered at low concentrations, early in the animal's development and treatment is generally discontinued as the animals mature (McEwen & Fedorka-Cray 2002). The mechanisms of growth promotion are not entirely understood but appear to include increased intestinal absorption of nutrients, enhanced vitamin production by

gastrointestinal microorganisms, elimination of pathogenic organisms, and reduced production of toxins by intestinal bacteria (Butaye et al. 2003).

Although not everyone is in agreement as to whether subtherapeutic doses lead to the development of antibiotic resistant bacteria, there is extensive evidence that they result in significant selection pressures on animal pathogens and commensals (Quednau et al. 1998; McEwen & Fedorka-Cray 2002). This antibiotic resistance would not be a problem for humans if the resistant bacteria were only animal pathogens, but resistance arises in many zoonotic pathogens such as *Salmonella* species, *Listeria* species and *E. coli* which exist in both humans and animals (Chopra & Roberts 2001; Geornaras & von Holy 2001). These bacteria may then be transferred from food items to humans via the food chain (Parsonnet & Kass 1987). Should these organisms be transmitted to humans they could result in food-borne diseases that are difficult to treat or they could transfer their resistance genes to other bacteria that infect humans (Chopra & Roberts 2001).

1.5 Resistance Mechanisms

The response of many bacteria to the extensive utilization of inhibitory agents such as antibiotics is the survival of progeny with resistance to these substances (Levy 2002). Different microorganisms differ in their response to antimicrobial agents due to their differences in cellular structure, physiology, and composition (McDonnell & Russell 1999), and have a remarkable array of mechanisms to develop resistance to

antimicrobial agents (Livermore 2003). Resistance can be either innate or acquired by mutations, plasmids, or transposons (McDonnell & Russell 1999; Bower & Daeschel 1999; Livermore 2003). Innate resistance is attributed to the natural insusceptibility of an organism, whereas acquired resistance arises from changes in the DNA of the organism (Greenwood et al. 2002).

An example of innate resistance in bacteria is impermeability resulting in reduced uptake of antimicrobial agents. Since Gram-positive bacteria lack an outer membrane (Figure 1.1) the barrier is provided by the peptidoglycan layer. In contrast, Gram-negative bacteria have a thick outer membrane (Figure 1.2), causing them to be more resistant than Gram-positive organisms (McDonnell & Russell 1999). In addition to barriers, bacteria also have a range of other resistance mechanisms which are either innate or acquired. These include, among others, degradation of the antimicrobial agent by enzymes, and modification of the antimicrobial target (McDonnell & Russell 1999; Bower & Daeschel 1999; Livermore 2003).

A further intrinsic resistance mechanism is active efflux (Bower & Daeschel 1999; McDonnell & Russell 1999; Livermore 2003). This process involves the synthesis of an integral membrane protein that extrudes the antimicrobial agent from the bacterial cytoplasm to the outside of the cell before it reaches its intracellular target (Bower & Daeschel 1999).

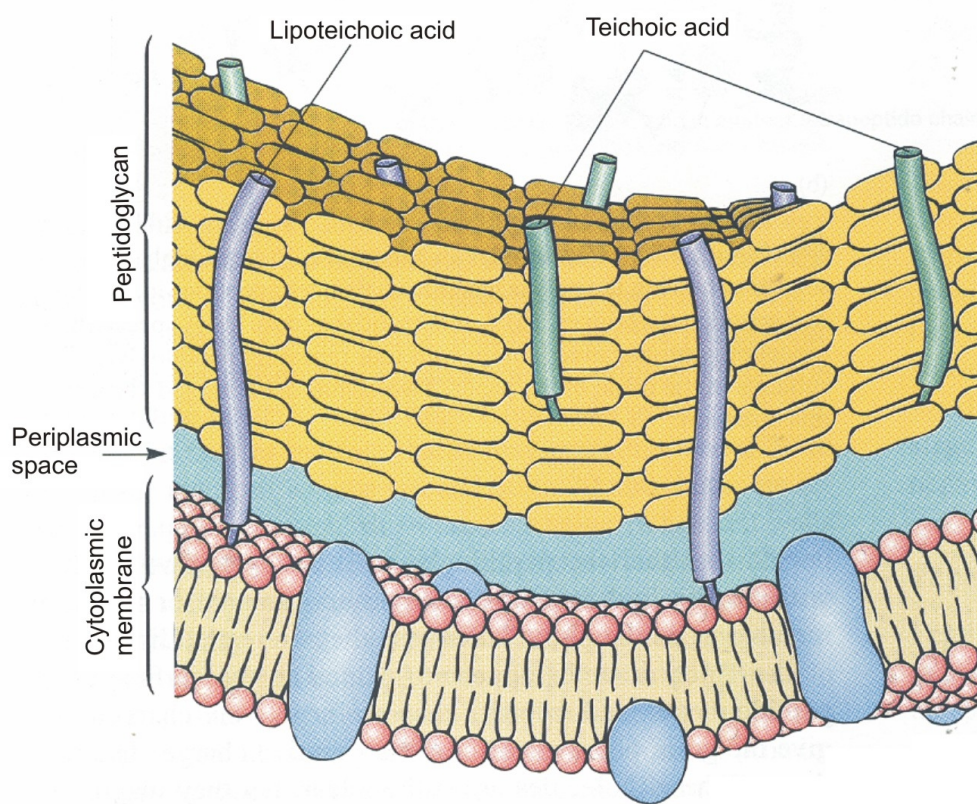


Figure 1.1: Diagram of the Gram-positive cell envelope. Demonstrating the single plasma (or cytoplasmic) membrane, periplasmic space and thick peptidoglycan layer (Adapted from Prescott et al. 1999).

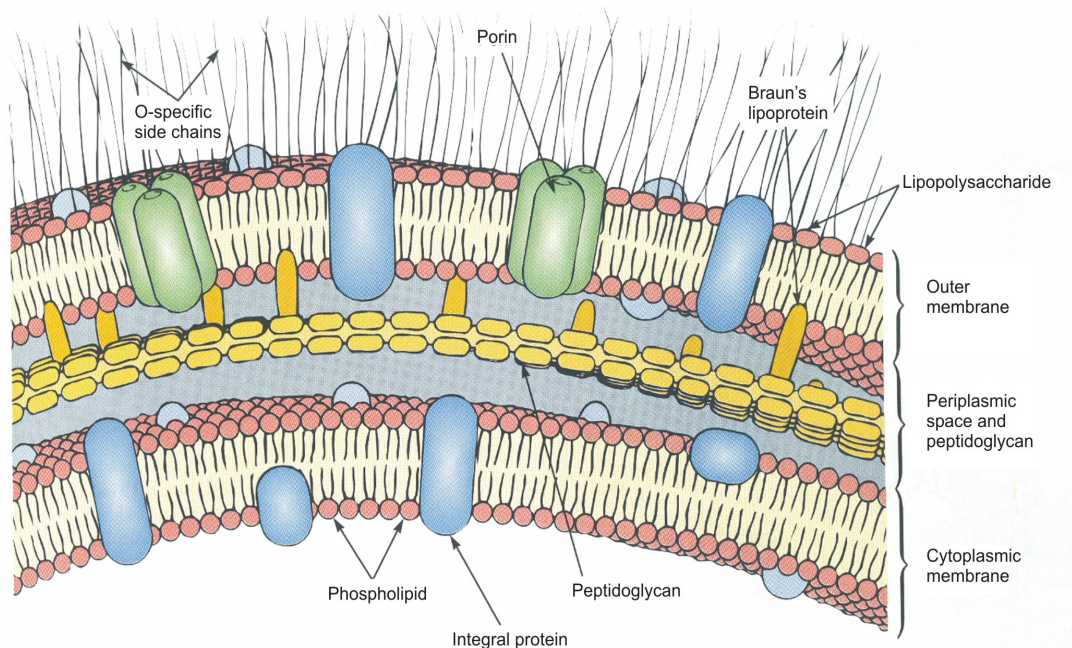


Figure 1.2: Diagram of the Gram-negative cell envelope. Showing the plasma membrane, periplasmic space with peptidoglycan and outer membrane (Adapted from Prescott et al. 1999).

1.6 Antibiotic Efflux Pumps

The occurrence of multiple antibiotic resistant bacteria presents a serious problem in the treatment of bacterial infections (Bolhuis et al. 1995). An underlying mechanism for this multidrug resistance phenotype is the increased activity of efflux pumps (Bohn & Bouloc 1998). Efflux pumps are transport proteins located in bacterial membrane systems which facilitate the transfer of toxic substances from within cells to the external environment (Poole 2000a; Webber & Piddock 2003). Efflux systems that contribute to antibiotic resistance have already been described in a number of

clinically important bacteria, including *Campylobacter jejuni* (CmeABC) (Lin et al. 2002), *Salmonella typhimurium* (AcrB)(Webber & Piddock 2003), *Listeria monocytogenes* (MdrL)(Mereghetti et al. 2000), and *E. coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD) (Poole 2000a).

Transport proteins act by binding to molecules and transporting them across a membrane either actively or passively. In passive transport (facilitated diffusion) the molecule moves in response to a concentration gradient and does not require energy. In active transport the molecule moves against its concentration gradient and therefore requires energy, usually in the form of adenosine 5'-triphosphate (ATP). Whether carrying out active or passive transport, membrane transporters can carry out uniport, symport, or antiport exchange. Uniport is the transport of a single type of solute across the membrane (Figure 1.3A), symport is the transport of two different molecules across the membrane in the same direction (Figure 1.3B), and antiport is the transport of two different molecules across the membrane in opposite directions (Figure 1.3C) (Horton et al. 1996).

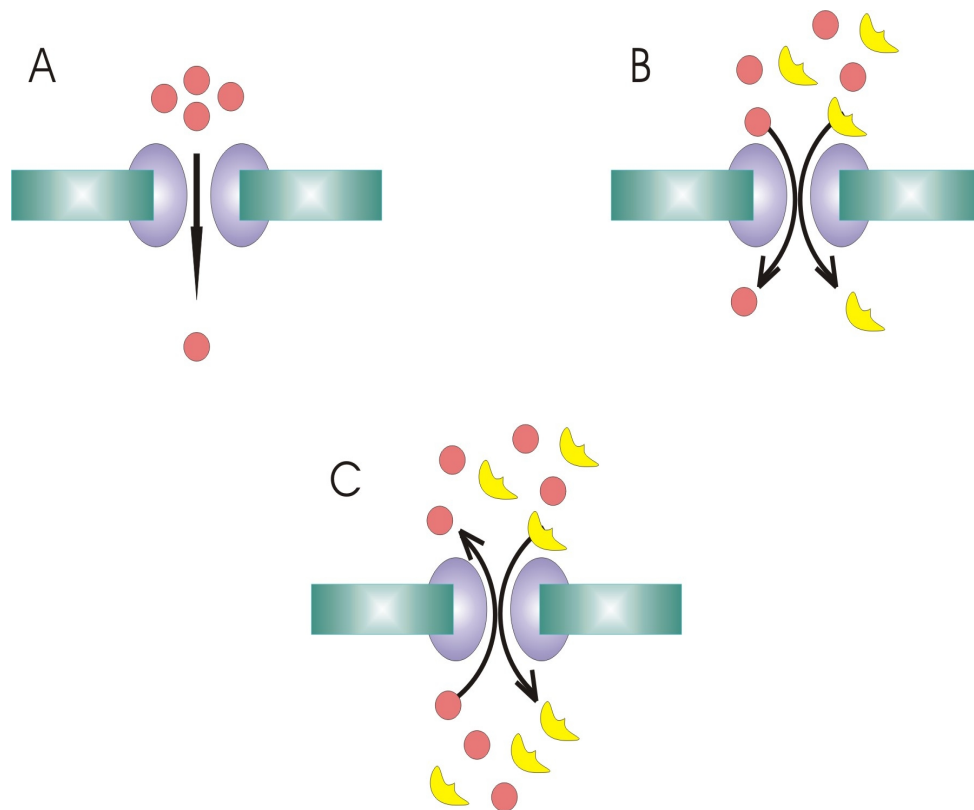


Figure 1.3: Schematic representation of the transport of molecules across bacterial membranes. (A) Uniport, (B) symport, and (C) antiport. (Adapted from Horton et al. 1996).

Efflux pumps can transport a variety of structurally dissimilar compounds (including antibiotics) or may be specific for a single substrate. Efflux pumps were first identified in tetracycline resistant Gram-negative bacteria, but efflux-mediated resistance to a variety of antibiotics including fluoroquinolones was soon discovered in Gram-positive bacteria as well as in eukaryotic organisms (Poole 2000a; Webber & Piddock 2003).

Bacterial antimicrobial efflux transporters have been divided into five superfamilies based on amino acid sequence homology (Poole 2000b). These superfamilies include the major facilitator family (MFS) (Pao et al. 1998), ATP-binding cassette family (Poole 2000b), resistance-nodulation-division (RND) family (Nikaido 1998), small multidrug resistance (SMR) protein family (Poole 2000b), and the multidrug and toxic compound extrusion (MATE) family (Morita et al. 1998; Van Bambeke et al. 2003).

Since Gram-positive bacteria possess only a single membrane (Figure 1.1) they simply require transport proteins that can extrude molecules across a single cytoplasmic membrane layer (Nikaido 1996) (Figure 1.4), and these are predominantly the MFS-type transporters (Poole 2000b).

In Gram-negative bacteria pumps of this design would not be efficient because Gram-negative bacteria have 2 membranes (Figure 1.2). As such pumps of this type would simply move agents from the cytoplasm into the periplasmic space which would be an inefficient operation because these molecules could then diffuse spontaneously back into the cytoplasm through the lipid bilayer domains of the cytoplasmic membrane. Gram-negative bacterial multidrug efflux pumps need to traverse 2 membranes and are therefore entirely different in their construction (Nikaido 1996). Gram-negative efflux pumps fall into the MFS, RND, SMR, and MATE groups (Poole 2000b; Yang et al. 2003).

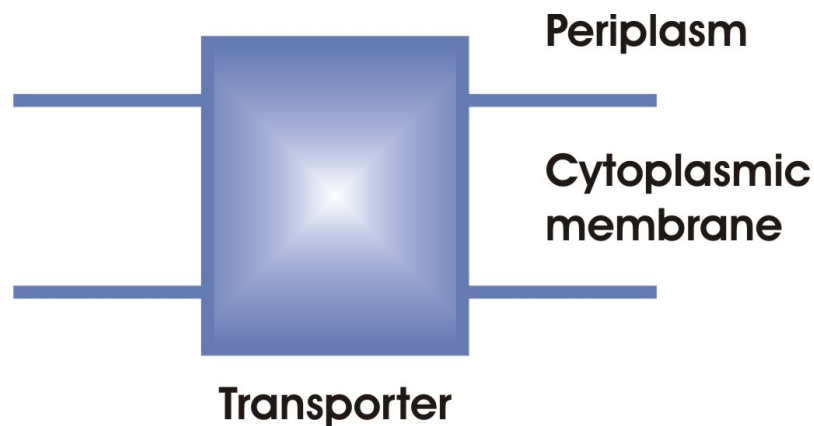


Figure 1.4: Schematic representation of a single component transporter that catalyses drug efflux across the cytoplasmic membrane in Gram-positive bacteria.

The MFS is the oldest superfamily and is found in bacteria, archaea and eukaryotic organisms. They are single polypeptide carriers and are responsible for the transportation of only small substrates (370-820 amino acids) in response to chemiosmotic ion gradients. This family consists of more than 300 sequenced proteins that fall into 17 distinct families, and each is specific for a different type of molecule. Included in the 17 families are those that: are specific for sugars, catalyse the uptake of phosphorylated glycolytic intermediates, catalyse uptake of Krebs cycle intermediates, transport organic and inorganic anions, and two families that catalyse drug efflux (Pao et al. 1998; Saier et al. 1998).

The two drug resistant families in the MSF are topologically distinguishable from each other because the one possesses 12 segments spanning the membrane (Figure 1.5A) while the other possesses 14 segments that span the membrane (Figure 1.5B) (Saier et al. 1998).

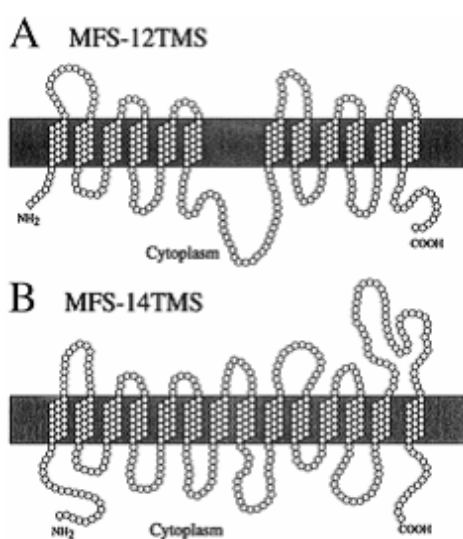


Figure 1.5: Structural model for the drug efflux pumps in the major facilitator superfamily. (A) 12-transmembrane segment and (B) 14-transmembrane segment (Saier et al. 1998).

The RND family is found only in prokaryotes and has been divided into three subfamilies that are specific for the extrusion of drugs, lipo-oligosaccharides, or metal ions. The subunits of the RND permeases are large proteins, usually with more than 1000 amino acid residues that possess a topology consisting of 12 putative spanners and 2 large extracytoplasmic domains (Figure 1.6). At the amino-terminal end of each

protein, the polypeptide chain traverses the cytoplasmic membrane from the cytoplasm to the periplasm once; this segment is followed by a large water-soluble domain that is localized to the periplasmic space. The polypeptide chain spans the membrane six more times and emerges into the periplasmic space as another water soluble domain that is the same size as the first one. The carboxyl-terminal end of the permease is fixed in the membrane with five segments that span the membrane (Saier et al. 1998).

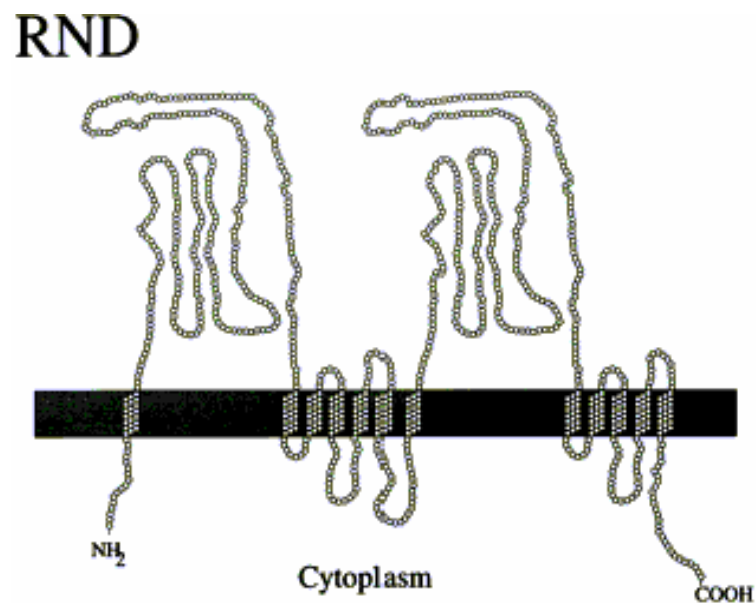


Figure 1.6: Structural model for the members of the resistance-nodulation-cell division family showing the 2 large extracytoplasmic domains and 12 putative membrane spanning segments (Saier et al. 1998).

RND systems predominate in Gram-negative bacteria (Nikaido 1996). They all span both the inner and outer membranes sharing a common three-component organization consisting of a transporter located in the inner membrane, an outer membrane channel, and a periplasmic accessory protein (Nikaido 1996; Zgurskaya & Nikaido 2000) (Figure 1.7).

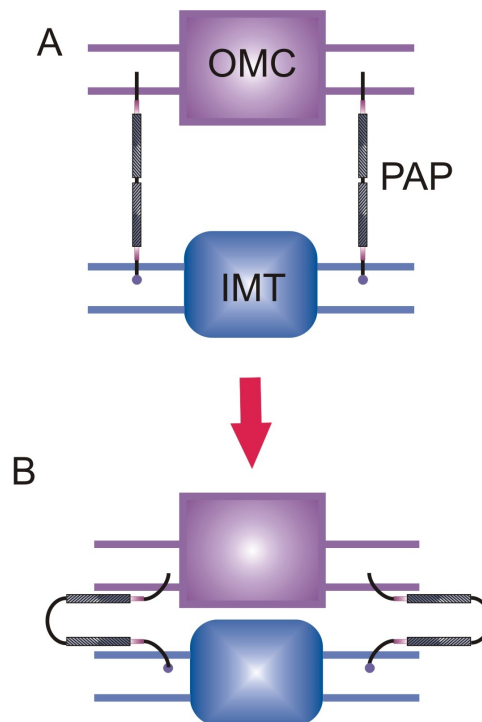


Figure 1.7: Hypothetical structure and mechanism of three component efflux pumps in Gram-negative bacteria. (A) In the non-functioning state the outer membrane channel (OMC) and the inner membrane transporter (IMT) exist as a complex with the periplasmic accessory proteins (PAP). (B) Whilst functioning the IMT and PAP complex folds back on itself bringing the OMC and IMT together allowing efflux across the two membranes (Adapted from Zgurskaya & Nikaido 2000).

The inner membrane transporters function with the periplasmic accessory proteins and outer membrane proteins to facilitate direct passage from the cytoplasm to the external medium of a variety of substrates, including oligosaccharides, proteins, large cations, and small molecules (Aeschlimann 2003). The periplasmic efflux proteins provide the connection between the inner and outer membrane components of the efflux pump (Nikaido 1996). Some periplasmic efflux proteins, such as the hemolysin translocator accessory protein HlyD, have a stretch of hydrophobic residues near the N terminus believed to span the cytoplasmic membrane. Others, like AcrA and AcrE of *E. coli*, have a lipoprotein modification signal and are likely to insert in the cytoplasmic membrane via an attached lipid moiety (Johnson & Church 1999). The outer membrane efflux proteins are often found in the same gene clusters as the inner membrane efflux proteins and periplasmic efflux proteins. However, some outer membrane efflux proteins, like *tolC* of *E. coli*, are not in the same operon and may associate with different inner membrane efflux protein-periplasmic efflux protein complexes (Johnson & Church 1999).

The ATP-binding cassette transporters can be found in bacteria, archaea, and eukaryotic organisms and consist of 30 families, each specific for one of a range of substrates including sugars, amino acids, vitamins, drugs, antibiotics, ions, iron complexes, proteins, peptides, and complex carbohydrates. ATP provides the energy source that drives transport for all bacterial ABC transport systems (Saier et al. 1998).

The ABC-type efflux transporters consist of an integral membrane protein with six α -helical segments spanning the membrane and an energy coupling protein that is embedded in the cytoplasmic side of the membrane. This protein may be either noncovalently associated with the former protein or covalently attached to it in a single polypeptide chain. This structure is then repeated resulting in a system composed of 12 segments that span the membrane (Figure 1.8) (Saier et al. 1998).

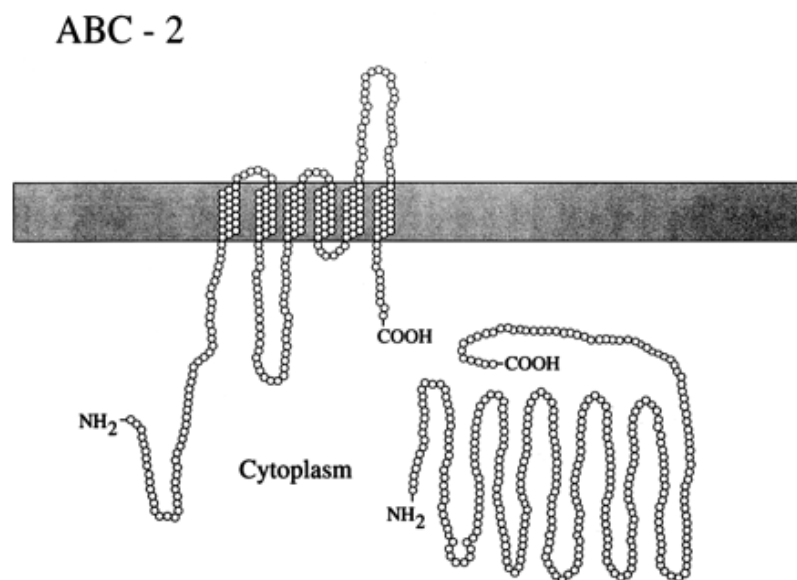


Figure 1.8: Structural model for the efflux pumps found in the ATP binding cassette family (Saier et al. 1998).

The SMR family is a relatively new group of proteins that are found only in prokaryotes and consists of two phylogenetic subfamilies. Members of one subfamily do not confer drug resistance or catalyze drug: H⁺ antiport and the substances of this

group have not yet been determined. Members of the other subfamily all confer multidrug resistance and catalyse drug efflux via a drug: H^+ antiport mechanism.

The subunits comprising the proteins in both subfamilies are extremely small homooligomeric permeases ranging from 100 to 110 amino acids in length. These permeases span the membrane four times as putative α -helices (Figure 1.9) (Saier et al. 1998).

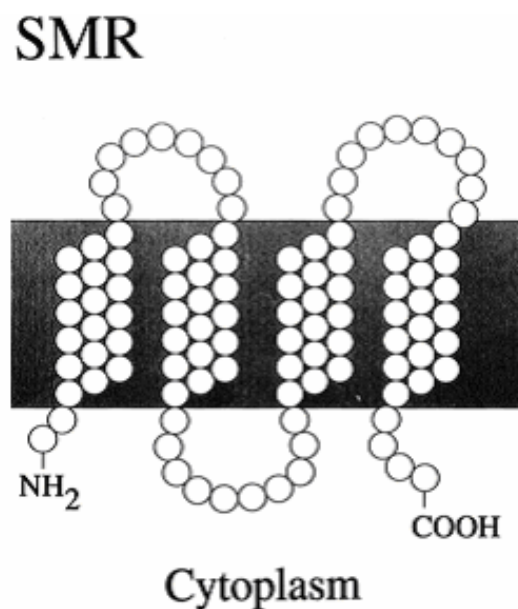


Figure 1.9: Structural model for the members of the small multidrug resistance family (Saier et al. 1998).

Since antibiotic resistant as well as susceptible bacteria carry and express the genes coding for certain efflux pumps there is some debate as to the normal physiological role of these transporters. The fact that both sensitive and resistant bacteria possess

these genes can be explained by the fact that efflux pump genes are part of an operon with a regulatory gene controlling expression. Efflux pumps can also be acquired from plasmids, transposons, or from other bacteria and whether innate or acquired, resistance to a substrate is achieved via increased expression of these genes. This provides bacteria with a mechanism allowing them to survive in hostile environments (Webber & Piddock 2003).

The fact that efflux pumps can extrude a wide variety of compounds is a concern, because over-expression of one pump often results in resistance to many classes of antibiotics as well as some detergents, dyes, and commonly used biocides. Another concern is cross-resistance, as exposure of an efflux pump to any compound belonging to the substrate profile of that particular pump could cause over-expression of that pump resulting in resistance to all other substrates of the pump, including antibiotics, dyes, and biocides (Webber & Piddock 2003). Even though over-expression of a pump does not confer high-level resistance to antibiotics, these bacteria are better equipped to survive antibiotic pressure and develop additional mutations in genes encoding the efflux pumps (Kern et al. 2000).

1.7 Antibiotics Extruded by Efflux Pumps

1.7.1 Fluoroquinolones

Fluoroquinolones are powerful broad-spectrum antimicrobial agents that are used for the treatment of numerous community-acquired as well as nosocomial infections (Kern et al. 2000). The fluoroquinolone agents presently available have high activity predominantly against members of the family Enterobacteriaceae (Kern et al. 2000) and resistance to these antibiotics has been steadily increasing since the 1980s (Pena et al. 1995). High-level fluoroquinolone resistance has recently emerged amongst different members of the Enterobacteriaceae family, including *E. coli* and *Salmonella* (Kern et al. 2000). Even though fluoroquinolones are not recommended for the treatment of listeriosis, they have, due to their wide use for other infections, resulted in the emergence of fluoroquinolone resistance in *Listeria* species (Godreuil et al. 2003).

1.7.1.1 Mode of action

Fluoroquinolones have been shown to kill bacteria by directly inhibiting bacterial DNA synthesis. This inhibition is due to an interaction between the drug and the DNA metabolic enzymes, either DNA gyrase or topoisomerase IV. Both DNA gyrase and topoisomerase IV function by binding to double stranded DNA, breaking both strands of a specific segment of the double strand, passing another segment through the gap, and then resealing the break. In the case of DNA gyrase this reaction removes positive

DNA coils that accumulate in front of advancing replication forks which is essential to initiate DNA replication. For topoisomerase IV, the reaction results in the separation of daughter strands and facilitates segregation of daughter strands into daughter cells (Hooper 2001).

Fluoroquinolones bind to the complexes that form between the DNA and gyrase or topoisomerase IV. This induces a conformational change in the enzyme, the enzyme then cleaves the DNA and the fluoroquinolone prevents re-ligation of the strands. The enzyme is now trapped on the DNA resulting in a quinolone-gyrase-DNA complex which prevents the movement of the replication fork. When the replication fork collides with the trapped complexes, events are triggered that result in cell death (Hooper 2001; Hawkey 2003).

1.7.1.2 Resistance mechanisms

In both Gram-negative and Gram-positive bacteria the mechanisms of fluoroquinolone resistance can be divided into two main categories; namely alterations of drug targets (Vila et al. 1996; Heisig 1996) and decreased cellular accumulation (Heisig 1996). The major alterations in drug targets are a result of point mutations in the DNA gyrase and the quinolone resistance-determining regions of the *parC* gene, which encode the subunits of the target topoisomerases of the fluoroquinolones (Heisig 1996; Kern et al. 2000; Wang et al. 2001; Godreuil et al. 2003). These mutations result in decreased affinity of DNA gyrase and topoisomerase

IV for fluoroquinolones (Hooper 2001). Decreased cellular accumulation can be due to lipopolysaccharide or porin alterations resulting in decreased uptake, or enhanced efflux due to overexpression of endogenous or acquired multidrug resistant pumps (Martinez-Martinez et al. 1998).

1.7.2 Tetracyclines

The tetracyclines were discovered more than 50 years ago and are a class of broad-spectrum, bacteriostatic antibiotics that exhibit activity against a wide range of Gram-positive and Gram-negative bacteria. Due to their broad-spectrum activity, the fact that they are relatively inexpensive, and their lack of major adverse side effects, they have been widely used in the treatment of human and animal infections and as animal growth promoters. Resistance to tetracyclines has emerged in a number of commensal and pathogenic bacteria, including *E. coli*, *Salmonella* and *Listeria* species (Chopra & Roberts 2001).

1.7.2.1 Mode of action

Tetracyclines inhibit protein synthesis in bacteria by penetrating the bacterial membrane systems and preventing the association of the aminoacyl-tRNA with the bacterial ribosome (Chopra & Roberts 2001).

In Gram-positive bacteria the antibiotic is transferred across the cytoplasmic membrane in an electroneutral lipophilic form and accumulates within the cytoplasm (Chopra & Roberts 2001). In Gram-negative enteric bacteria, such as *E. coli* and *Salmonella* spp, tetracyclines cross the outer membrane through porin channels (Figure 1.2) as positively charged cationic complexes and accumulate in the periplasmic space. Once in the periplasmic space the complexes dissociate releasing uncharged tetracycline which is able to diffuse through the lipid bilayer of the inner membrane. In both Gram-positive and Gram-negative bacteria movement of tetracycline across the cytoplasmic membrane is energy-dependent and is driven by the proton motive force. Once in the cytoplasm the tetracycline molecules become chelated by magnesium ions and these chelated tetracycline molecules then bind to the 30S ribosomal subunit. Binding of the tetracycline molecules prevents the aminoacyl-tRNA from associating with the bacterial ribosome. The interaction of tetracycline with the bacterial ribosome is however reversible and as such the tetracyclines exhibit bacteriostatic and not bacteriocidal effects (Chopra & Roberts 2001).

1.7.2.2 Resistance mechanisms

Tetracycline resistance can be attributed to 29 different tetracycline resistance (*tet*) genes that confer resistance via three main mechanisms; efflux proteins, ribosomal protection proteins, and enzymatic inactivation of tetracycline. Eighteen of the 29 *tet* genes have been shown to code for efflux proteins and are found in both Gram-

positive and Gram-negative bacteria. These *tet* genes belong to the major facilitator superfamily and result in the exportation of tetracycline out of the cells, lowering the intracellular concentration of the drug, thereby protecting the ribosome (Chopra & Roberts 2001).

Tetracycline efflux proteins function by exchanging a proton for a tetracycline-cation complex against a concentration gradient. The efflux proteins have been divided into six groups based on amino acid homology. Group 1 includes Tet (A), Tet (B), Tet (C), Tet (D), Tet (E), Tet (G), Tet (H), Tet (Z), and possibly Tet (I), and Tet (J), all of which are found in Gram-negative bacteria except Tet (Z) which is found in Gram-positive bacteria. Group 2 contains Tet (K) and Tet (L) which are principally found in Gram-positive bacteria. Gram-negative efflux genes are widely distributed and are associated with large plasmids; Gram-positive genes on the other hand are usually found on small plasmids. Tetracycline efflux proteins are very important as they share structural similarities with other efflux proteins that confer resistance to quaternary ammonium compounds, quinolones, and chloramphenicol (Chopra & Roberts 2001).

Nine of the 29 *tet* genes have been shown to code for ribosomal protection proteins. The most comprehensively characterised being the Tet (M) and Tet (O) proteins. These proteins are found in the cytoplasm and prevent tetracycline from interacting with the ribosome thereby allowing the aminoacyl-tRNA to bind to the acceptor site on the ribosome. The only *tet* gene known to confer resistance via enzymatic alteration of tetracycline is the *Tet (X)* gene. This gene is a 44-kDa cytoplasmic

protein that chemically alters tetracycline in the presence of oxygen and NADPH (Chopra & Roberts 2001).

1.8 Household Antimicrobial Agents

Mounting concerns of pathogens in the food and general consumer markets has led companies to increase the use of active chemical agents (biocides) in a variety of domestic products (Braid & Wale 2002). These products have been produced for the decontamination of areas that are thought to be microbially contaminated such as food preparation surfaces and toilets, and to improve general hygiene in the home (Fraise 2002). Researchers have however recently started to express concern regarding the use of antimicrobial chemicals in the domestic and industrial setting, especially with regard to the addition of antibacterial agents to many items (McDonnell & Russell 1999).

Although not extensively researched, the main concerns arising from the inclusion of these agents are that:

- Indiscriminate use of biocides may give rise to the evolution and selection of multidrug-resistant strains through mechanisms like efflux pumps, and
- Commonality between target sites on the antibiotic and biocide could lead to the selection of mutants with altered target sites and hence result in cross-resistance (McDonnell & Russell 1999).

1.8.1 Triclosan

Triclosan (Table 1.1) has been used for more than 30 years (Levy et al. 1999; Slater-Radosti et al. 2001) and belongs to a class of compounds known as the 2-hydroxyethers (Chuanchuen et al. 2001). This class of compounds is known to exhibit broad-spectrum antimicrobial and antifungal activity with triclosan being the most potent and commonly used member of the class. Since triclosan is active against a wide range of Gram-positive and Gram-negative bacteria, it is included in many products such as mouthwashes, toothpastes, soaps, and lotions as well as chopping boards, fabrics (e.g. tea towels), and plastics (McMurry et al. 1998; Levy et al. 1999; Chuanchuen et al. 2001).

For a long time triclosan was thought to act as a non-specific biocide by disrupting bacterial membranes (Fan et al. 2002) and, that because it had no discrete mechanism of action, the acquisition of resistance was regarded as improbable (Heath et al. 1999). Studies have however shown that triclosan acts on a defined bacterial target in the fatty acid biosynthetic pathway, the enoyl-acyl carrier protein (ACP) reductase (FabI) (McMurry et al. 1998). Missense mutations in the *fabI* gene confer resistance to triclosan (McMurry et al. 1998), especially in organisms such as *Staphylococcus aureus* and *E. coli* that rely on this enzyme to perform the last step in the elongation cycle of bacterial fatty acid biosynthesis (Heath et al. 1998; McMurry et al. 1998; Heath et al. 1999; Heath et al. 2000; Slater-Radosti et al. 2001).

The Fab pathway is a prime target for antibacterial agents because it plays a crucial role in cell growth and function (Schweizer 1998). A particular concern is that resistance to triclosan can lead to cross-resistance to other antimicrobial agents (Heath et al. 1999) as some mutations leading to triclosan resistance have been shown to cause resistance to isoniazid (an antibiotic used to treat tuberculosis) (Chuanchuen et al. 2001).

1.8.2 Salicylic acid

Salicylic acid (Table 1.1) has many applications in humans, plants, and bacteria. In humans it can be used as an anticoagulant, antipyretic, analgesic, and anti-inflammatory drug. It is involved in the induction of flowering and plays a role in signal transduction pathways that confer resistance to systemic diseases in plants (Adilakshmi et al. 2000). In bacteria it has been shown to have significant antimicrobial action by itself as well as increase or decrease the action of certain types of antibiotics when the antibiotic and salicylic acid are used in combination (Polonio et al. 2001).

The drug has been shown to have effects on many bacteria including *Salmonella*, *E. coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (Rosner et al. 1991; Adilakshmi et al. 2000; Polonio et al. 2001). The enhanced activity of antibiotics when used in conjunction with salicylic acid can be attributed to a variety of actions. The first being that since salicylate affects bacterial adhesions necessary for bacterial

colonization and propagation in host tissues, it inhibits biofilm formation, allowing the antibiotic direct access to the bacterial cells (Polonio et al. 2001; Kupferwasser et al. 2003). The second mechanism is the induction of the *mar* operon in *E. coli* (Cohen et al. 1993; Alekshun & Levy 1997). The *marAB* operon mediates intrinsic resistance of *E. coli* to many antibiotics by efflux (Webber & Piddock 2001) and salicylic acid has been shown to stimulate expression of this operon thereby increasing efflux-mediated antibiotic resistance (Cohen et al. 1993).

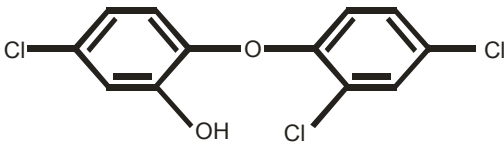
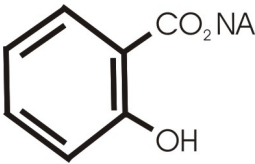
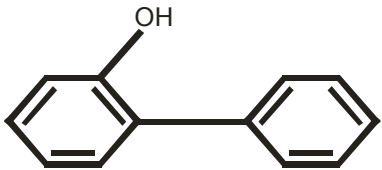
Decreased activity of certain antibiotics has been shown in *Salmonella* and *E. coli* when used together with salicylic acid (Rosner et al. 1991). This is due to the fact that salicylic acid decreases the amounts of porins in the outer membranes of these bacteria resulting in decreased permeability and, as such, reduced uptake of the antibiotic (Rosner et al. 1991).

1.8.3 *Ortho*-phenylphenol

Ortho-phenylphenol (Table 1.1) is used as an antibacterial and antiviral agent as well as a fungicide (Murata et al. 1999) and belongs to the phenolic group of antimicrobial agents (McDonnell & Russell 1999). These agents have been used for many years in several compounds as they possess preservative, disinfectant, and antiseptic properties (McDonnell & Russell 1999).

The antiseptic and disinfectant activities can be attributed to the membrane active properties of this chemical. Relatively low concentrations of phenolic compounds result in damage of the bacterial membrane causing leakage of intracellular constituents, together with the release of potassium ions. These compounds have also been shown to produce an increase in permeability to protons resulting in an influx of protons and the abolishment of the proton motive force rendering the cells incapable of energy dependent activities including active efflux. It has been proposed that these compounds act only during separation of daughter cells during mitosis and that young bacteria are more susceptible than older bacteria. High concentrations have however been shown to result in coagulation causing irreversible cellular damage (McDonnell & Russell 1999).

Table 1.1: Chemical structures of biocides added to commonly used household cleaning products (McDonnell & Russell 1999; Dobashi 2004; Wood 2004).

Biocide	Chemical Structure
Triclosan	
Salicylic acid	
<i>Ortho</i>-phenylphenol	

1.9 Rationale for this Study

It has been reported that the inappropriate use of antimicrobial household agents selects for organisms with antimicrobial resistance mechanisms such as efflux pumps, and that this could in turn lead to the development of resistance to other substances such as antibiotics (Levy 2001).

The reverse hypothesis, that antibiotic resistant organisms could become resistant to antibacterial agents due to the action of efflux pumps, has however not been extensively examined.

The latter phenomenon could be of particular concern in the domestic setting if:

1. The antibiotic resistant organism is resistant to a high concentration of the biocide, e.g. that recommended by the manufacturer, or
2. Even if the antibiotic resistant organism is susceptible to the high concentration of biocide, it may have reduced susceptibility to lower concentrations of biocide.

If this scenario is true the antibiotic resistant organisms could also possess active efflux mechanisms, resulting in additional resistance to antimicrobial agents. If these bacteria were to infect humans they could result in diseases that would be extremely difficult to treat.

Due to the fact that dishcloths are used extensively in the domestic setting, remain damp for long periods of time, contain residual amounts of food debris, and are not changed frequently, they provide favourable conditions for the survival and/or growth of microorganisms (Scott & Bloomfield 1990a), for these reasons they were chosen to observe the effects of household antimicrobial agents in the domestic setting.

1.10 Aim

The aim of this study was to establish whether there is a link between antibiotic resistant organisms isolated from livestock and reduced susceptibility to the antimicrobial agents found in commonly used household disinfectant products, and whether this was likely to be due to the action of efflux pumps.

1.11 Objectives

The specific objectives of the study were to:

- Isolate two groups of potential human gastrointestinal pathogens, one sensitive to, and the other resistant to, antibiotics known to be extruded by efflux pumps,
 - Determine whether the bacterial strains showed efflux activity that could pump out ethidium bromide (a substrate for bacterial efflux pumps), or whether they possessed inactive efflux pumps that could be induced upon the addition of ethidium bromide,
 - Establish whether the active/activated efflux pumps could extrude the functional components (antimicrobials) found in commonly used household disinfectant products, and
 - Determine the effect, if any, the related household disinfectant products had on the survival of sensitive and resistant bacteria in artificially contaminated dishcloths in an in-use disinfectant test.
-

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Media

All reagents were of analytical or molecular grade. For a complete list of the composition of all media and buffers/solutions used, see Appendix A.

2.2 Bacterial Strains

Bacteria known to cause infectious intestinal disease were obtained from the following sources:

- Isolated from chicken products
 - Selected from the National Health Laboratory Services (NHLS) culture collection compiled from previous studies
 - Diseased chickens (a kind gift from Dr S. Bisschop and Dr J. Picard from the Onderstepoort Veterinary Institute, University of Pretoria)
 - Isolated from humans with infectious intestinal disease (from the NHLS)
 - Ethics clearance was obtained for the use of patient samples and no patient data was collected. Ethics # MO1-05-07.
-

2.3 Microbiological Procedures for the Isolation of Bacteria from Chicken Products

2.3.1 Sample Collection

Twenty four fresh chicken products were obtained from retail outlets and abattoirs and analyzed for the presence of tetracycline and ofloxacin resistant as well as susceptible *Salmonella* and *E. coli* species.

2.3.2 Preparation of the test sample

25 g of the chicken sample was weighed, placed into a sterile plastic packet, and 225 ml of sterile buffered peptone water (Diagnostic Media Products [DMP], Rietfontein, South Africa) was added. The mixture was homogenized for approximately 30 seconds, divided into three 50 ml aliquots, which were centrifuged at 3000 g for 15 minutes in a bench-top centrifuge and the supernatant discarded. This process was repeated for each organism.

2.3.3 Isolation of *Salmonella* species

The pellets from the three aliquots were resuspended in 10 ml of sterile buffered peptone water containing either 160 µg of tetracycline (Oxoid, Basingstoke, England), 80 µg of ofloxacin (Oxoid), or no antibiotic. These concentrations of antibiotic were used as they were high enough to exclude the sensitive bacteria, but

low enough to allow for the isolation of bacteria with intermediate resistance (NCCLS 2001). All three suspensions were incubated at 36 ± 1 °C for 24 hours.

1 ml of each of the resuspended samples was then added to 10 ml of selenite cystine broth (DMP) and incubated at 36 ± 1 °C for 24 hours. 100 µl from each of the resuspended samples was also added to 10 ml of Rappaport broth (DMP) containing 100 µl of novobiocin stock solution and the broths incubated at 42 ± 1 °C for 24 hours.

From the selenite cystine and Rappaport broths a loop of culture was inoculated onto xylose lysine desoxycholate (XLD) agar (DMP) and mannitol lysine crystal violet brilliant green (MLCB) agar plates (DMP), streaked for single colonies, and incubated at 36 ± 1 °C for 24 hours. After 24 hours the plates were examined for typical colonies of *Salmonella* according to Table 2.1.

All presumptive *Salmonella* colonies were selected and inoculated into Singers broth (DMP), and incubated at 36 ± 1 °C for 4-5 hours. If the colour of the broth remained green (confirming the presence of *Salmonella*) one drop was placed on a nutrient agar plate (DMP) to check for purity. Isolates were also typed using an API 10S (bioMerieux, Durham, USA).

Table 2.1: Visual characterization of certain *Salmonella* spp. on selective media.

Media	Colony morphology	Organism
XLD agar	Translucent red colonies with or without a black centre.	<i>Salmonella</i>
	Yellow colonies with a black centre.	Possible lactose fermenting <i>Salmonella</i>
	Orange or slightly opaque with or without a black centre.	<i>Salmonella typhi</i> (Xylose positive strains)
MLCB agar	Purple to black large colonies.	<i>Salmonella</i>
	Mauve-greyish colonies with or without a black centre.	Atypical strains of <i>Salmonella</i>

2.3.4 Isolation of *E. coli*

The pellets from the three aliquots were resuspended in 10 ml of sterile buffered peptone water containing either 160 µg of tetracycline, 80 µg of ofloxacin, or no antibiotic. All three suspensions were incubated at 36 ± 1 °C for 24 hours; these were used as the initial suspensions. From the initial suspensions serial dilutions from 10^{-1} to 10^{-8} were prepared.

The top film of the 3M Petrifilms™ for coliforms and *E. coli* (3M Microbiology products, USA) was lifted and 1 ml of the required dilution of sample transferred to the centre of the bottom film by holding the pipette perpendicular to the film. The top film was slowly rolled onto the sample to prevent air bubbles being trapped under the

film, the sample evenly distributed on the film by applying gentle downward pressure using the smooth side of a spreader, and the films left undisturbed for one minute to solidify. Once solidified, the films were stacked in piles of no more than 15 and incubated clear side up in a metal jar with a moisture pack at 35 ± 1 °C for 48 hours. After the incubation period the films were removed and blue colonies associated with gas were identified as *E. coli*. To confirm that the colonies were *E. coli* they were streaked onto ½ blood ½ MacConkey agar plates (DMP), incubated at 36 ± 1 °C for 24 hours, and a rapid indole test (Appendix B) was performed.

2.4 Disk Diffusion Susceptibility Testing

The resistance profiles of the bacteria were tested using the Kirby-Bauer disk diffusion susceptibility test as described in the National Committee for Clinical Laboratory Standards (NCCLS 2001).

A single colony of the bacterium of interest was suspended in 1 ml of normal saline (DMP) and vortexed to disrupt clumps of growth. The cell suspension was adjusted by adding normal saline, until the turbidity was equivalent to that of a McFarland 0.5 barium sulphate standard. The entire surface of a Mueller-Hinton agar plate (DMP) was inoculated using a sterile applicator swab moistened in the cell suspension. The plate was stored at room temperature for 3-5 minutes to allow the medium to absorb

the moisture from the inoculum. Disks containing known amounts of tetracycline and ofloxacin were then placed on the surface of the Mueller-Hinton plate using sterile forceps. The plate was incubated at 36 ± 1 °C for 24 hours. The antimicrobial agents diffuse into the medium causing a zone of inhibition of growth around the disk corresponding to the susceptibility of the strain to that agent.

The diameter of each zone of inhibition was measured using callipers and the susceptibility profiles of the bacteria were determined by referring to the NCCLS criteria (NCCLS 2001) (Table 2.2).

Table 2.2: Zone diameter interpretive standards for Enterobacteriaceae (NCCLS).

Bacterial Species	Antimicrobial Agent	Disk Content	Zone Diameter (mm)		
			Resistant	Intermediate	Sensitive
<i>E. coli</i> and <i>Salmonella</i>	Tetracycline	30 µg	14	15-18	19
<i>E. coli</i> and <i>Salmonella</i>	Ofloxacin	5 µg	12	13-15	16

2.5 Detection of Bacterial Pathogens using Polymerase Chain

Reaction

Polymerase chain reaction (PCR) is a molecular technique that allows for the rapid exponential amplification of a particular region of DNA. In this technique the template DNA is denatured by subjecting it to a high temperature and allowed to

anneal to oligonucleotide sequences that flank the target region. The annealed oligonucleotide sequences then act as primers for the synthesis of DNA which is catalyzed by a thermostable DNA polymerase. The newly formed strands can themselves act as templates for the same primers and repeated cycles of denaturation, annealing and elongation will result in the exponential amplification of the target DNA sequence (Wilson & Walker 1994).

PCR was performed to confirm that the bacteria were correctly identified using the microbiological culture techniques. If no bacteria were isolated from the poultry samples, PCR was performed to assess whether bacteria were present, but the culture techniques were not sensitive enough to isolate them.

2.5.1 Generation of inhibition control constructs

Inhibition of PCR when using food samples can occur due to the presence of inhibitors in the food items. To test for this occurrence inhibition control constructs can be generated and included in the amplification reactions.

Inhibition control constructs were generated to test for inhibition in the *Salmonella* amplification reactions. In order to generate these constructs, primers were designed using the primers already designed for *Salmonella* PCR (Carli et al. 2001) with the addition of a pGEM[®]-5Zf(t) vector sequence (Promega, CA, USA) at the 3' end of each primer.

The primers were as follows:

F 5' to 3': gTgAAATTATCgCCACgTTCgggCAACgTCgTgTAgATAACTACgAT

R 5' to 3': TCATCgCACCGTCAAaggAACCgCCAACTTACTTCTgACAACg

The underlined regions represent the pGEM vector sequence nucleotides 1411-1432 and 1781-1801 of the forward and reverse primers, respectively.

PCR was then performed using these primers to produce a construct of 438 bp. The construct was designed to be 154 bp larger than the product obtained when using the *Salmonella* primers without the vector sequence so that it would be easy to differentiate between the two products separated by electrophoresis through agarose gels.

2.5.2 DNA extraction

The High Pure PCR Template kit (Roche, Penzberg, Germany) was used to extract DNA from the enrichment broths according to the manufacturer's protocol. Five hundred µl of selenite cystine broth was used for *Salmonella* spp. (after 24 hrs of incubation section 2.3.3) and 500 µl of sterile buffered peptone water for *E. coli* spp. (after 24 hrs of incubation section 2.3.4). The sample was centrifuged at 3000 x g for 5 minutes in a benchtop centrifuge and resuspended in 185 µl PBS (DMP). Fifteen µl of lysozyme was added to the suspension which was incubated for 15 minutes at 37 °C. 200 µl of binding buffer and 40 µl of proteinase K were added to the sample and incubated at 72 °C for 10 minutes. Isopropanol was added, the sample mixed

thoroughly, pipetted into the upper reservoir of a combined filter tube-collection tube assembly, and centrifuged for 1 minute at 8000 x g. The flowthrough and collection tube were discarded, 500 µl of inhibitor removal buffer added to the upper reservoir, and the process repeated. The flowthrough and collection tube were discarded, 500 µl of wash buffer was added and the procedure repeated twice. The flowthrough was discarded, and the sample centrifuged at 14000 x g for 10 seconds. 200 µl of elution buffer was added, the sample centrifuged at 8000 x g for 1 minute, and the eluted DNA stored at -15 to -25 °C.

2.5.3 PCR

PCR reactions for all bacteria were performed in total volumes of 25 µl in 0.5 ml thin-walled tubes, using an iCycler thermal cycler (Bio-Rad, CA, USA). The products were separated alongside a mass DNA ladder (Fermentas, MD, USA) through 2% agarose gels (Whitehead Scientific, South Africa) containing 3.75×10^{-4} µg/µl ethidium bromide by horizontal electrophoresis. The gels were visualized and the images analyzed and captured using a Gel Doc 1000 system (Bio-Rad, CA, USA).

2.5.3.1 *Salmonella* spp.

The primers used for the *Salmonella* spp. were those already designed (Carli et al. 2001): invAF: 5' gTgAAATTATCgCCACgTTCgggCAA, and invAR: 5' TCATCgCACCGTCAAAggAACC.

The expected product size was 284 bp. The reaction mixture consisted of 5 µl of template and final concentrations of: 1.5 mM MgCl₂, 0.2 µM dNTP's, 0.5 µM of each primer and 12.5 U Taq DNA polymerase (2 X Master Mix; Roche, Germany). The cycling parameters were: 2 minutes at 94 °C, followed by 35 cycles of 94 °C for 30 seconds, 64 °C for 30 seconds, 72 °C for 45 seconds, and a final cycle of 72 °C for 5 minutes.

A laboratory control strain of the pathogen was used as a positive control. A blank (no DNA) and a positive control were used for each run. Spiking of all samples with 1 µl of the required inhibition control construct was done to test for inhibition.

2.5.3.2 *E. coli*

Multiplex PCR was performed using four primer sets for the detection of *E. coli* O157: H7 genes simultaneously. The primers were those designed by Wang et al (2002). This assay was designed to detect *hlyA*, the gene encoding the virulence factor; *rfbE*, which encodes the *E. coli* somatic antigen O157; *fliC_{H7}*, the gene encoding the *E. coli* structural flagella antigen H7; and *E16S*, the *E. coli* ribosomal gene.

The primer sequences were as follows:

HlyA-a: 5' to 3': AGCTGCAAGTGCGGGTCTG

HlyA-b: 5' to 3': TACGGGTTATGCCTGCAAGTTCAC

RfbE-a: 5' to 3': CTACAGGTGAAGGTCGAATGG

RfbE-b: 5' to 3': ATTCCTCTCTTTCCTCTGCGG

FliC-a: 5' to 3': TACCATCGCAAAAGCAACTCC

FliC-b: 5' to 3': GTCGGCAACGTTAGTGATACC

E16S-a: 5' to 3': CCCCCTGGACGAAGACTGAC

E16S-b: 5' to 3': ACCGCTGGCAACAAAGGATA

The expected product size of the *E. coli* ribosomal gene was 401 bp. The reaction mixture consisted of 5 µl of template and final concentrations of: 1.5 mM MgCl₂, 0.2 µM dNTP's, 1 µM of each primer and 12.5 U Taq DNA polymerase (2 X Master Mix; Roche, Germany). The cycle parameters were as follows: 2 min at 95 °C, followed by 30 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 1 minute, and a final cycle of 72 °C for 7 minutes. A laboratory control strain of the pathogen was used as a positive control. A blank (no DNA) and a positive control were used for each run.

2.6 Macro-Restriction Analysis

Macro-restriction analysis was performed to ensure that the bacteria used in this study were not clonally identical.

Macro-restriction analysis is used to fingerprint bacterial strains. Traditional techniques used to type species are often based on phenotypic techniques; these include cell envelope protein typing, biotyping, antibiograms, and serotyping. These techniques are however not capable of typing all strains, often show little variation within enterococcal species, and are generally not sufficiently discriminatory. For this reason, genotypic methods such as ribotyping, analysis of plasmid profiles, arbitrarily primed PCR, and pulsed-field gel electrophoresis (PFGE) were introduced. Genotypic methods have been shown to be highly discriminatory and often superior to phenotypic techniques (Seifert et al. 1994).

Pulsed-field gel electrophoresis is a technique used to separate large DNA fragments i.e. fragments greater than 60 kb. This method involves electrophoresis in an agarose gel using three electrical fields that are applied at different angles for defined times.

The current is applied first in one direction from one set of electrodes, it shifts to a second set of electrodes for a short period of time (pulse), and then shifts to a third set of electrodes. The electric field is thus applied in pulses that alternate from three sets of electrodes. This causes the DNA to migrate through the gel and the back and forth movement results in a higher level of fragment resolution. The smaller the molecule

is the quicker it can realign itself, and continue moving through the gel; the larger the molecule the longer the time taken. The smaller molecules therefore move through the gel faster than the larger molecules and the fragments separate according to size (Wilson & Walker 1994; Tenover et al. 1997).

2.6.1 Preparation of agarose plugs

Brain heart infusion (BHI) broth (DMP) was inoculated with a single colony of each of the bacteria of interest and incubated at 36 ± 1 °C for 24 hours. The absorbance of each suspension was measured at 600 nm using a standard visible range spectrophotometer and the samples were normalized by using that volume of sample that gave the highest density for analysis. One ml of the sample with the highest absorbance and the appropriate volumes of the remaining samples were aliquoted into 2 ml eppendorf tubes and centrifuged in a bench top centrifuge at $12000 \times g$ for 2 minutes. The supernatants were removed; the pellets resuspended in 500 µl of TE buffer, and warmed to 37 ± 1 °C in a heating block. Five hundred µl of 1.5 % agarose in TE buffer, melted and cooled to ± 50 °C, was added, mixed quickly and pipetted into plug moulds (Bio-Rad, CA, USA). Once set (± 15 minutes) the blocks were pressed out into 15 ml centrifuge tubes containing 6 ml of lysis buffer. These were incubated for 5 hours at 36 ± 1 °C with gentle shaking, after 5 hours the lysis buffer was replaced with fresh lysis buffer and the plugs were incubated overnight at 50 ± 1 °C. The plugs were transferred to 2 ml eppendorf tubes containing storage buffer and stored at 2-8 °C.

2.6.2 Digestion of DNA with restriction endonucleases

One third of each agarose plug was transferred to 2 ml Eppendorf tubes. The plugs were washed twice in 2 ml of storage buffer containing 1 mM Phenylmethanesulfonyl fluoride (PMSF) for 40 minutes at room temperature, followed by three washes of 20 minutes each with 2 ml 10 mM Tris buffer, pH 8. The excess liquid was then removed, 70 µl of the digestion mixture consisting of 30 U *Xba* I (Fermentas, MD, USA) was added to each tube according to the manufacturer's directions and the tubes were incubated at the recommended time and temperature for the specific organism (Table 2.3). Pulsed field gel electrophoresis was performed immediately after digestion.

2.6.3 Pulsed-field gel electrophoresis

A 1 % pulsed-field certified agarose gel (Bio-Rad, CA, USA) was prepared using 0.5 x TBE buffer and left to set for one hour. Two litres of 0.5 x TBE buffer was poured into the electrophoresis chamber of a CHEF DRII system (Bio-Rad) and allowed to cool to 14 °C for at least one hour prior to the digest being completed. A lambda marker (Amersham Biosciences, United Kingdom) and the restricted agarose plugs were inserted into the gel wells and fixed in place using 1 % molten agarose (Whitehead Scientific, South Africa). Once the agarose had set, the gel was placed in the chamber and electrophoresed at the recommended current-switching parameters for optimal separation of the DNA fragments (Table 2.3). The gel was removed from the chamber, stained for 30 minutes in TBE containing 0.5 µg/ml ethidium bromide,

and destained for 20 minutes in distilled water. The gels were visualized and the images analyzed and captured using a Gel Doc 1000 system (Bio-Rad).

The banding patterns were then interpreted using the Tenover criteria (Tenover et al. 1995) for interpreting pulsed field-gel electrophoresis (PFGE) patterns (Table 2.4).

Table 2.3: Optimized conditions for restriction digest.

	Organism	
	<i>E. coli</i>	<i>Salmonella</i>
Enzyme	<i>Xba</i> I	<i>Xba</i> I
Digestion time (hours)	3	5
Digestion temperature	37 °C	37 °C
Voltage	6 V/cm	6 V/cm
Run time (hours)	20	20
Initial switch time (sec)	5	2.2
Final switch time (sec)	50	63.8

Table 2.4: Tenover criteria for interpreting PFGE patterns.

Category	Typical # of fragment differences
Indistinguishable	0
Closely related	2-3
Possibly related	4-6
Different	≥ 7

2.7 Ethidium Bromide Accumulation Assays

To determine whether the bacteria had active efflux pumps, ethidium bromide accumulation was assayed as previously described (Baranova & Neyfakh 1997) with slight modifications.

The bacteria were grown in BHI broth (DMP) at 36 ± 1 °C for 24 hours, normalized to an optical density of 0.2 at A_{600} using a standard visible range spectrophotometer, harvested by centrifugation, resuspended in 3 ml phosphate buffered saline (PBS), pH 7.2, and placed in a fluorimeter cuvette. All spectrophotometric readings were performed using a LS-3B fluorescence spectrometer (Perkin-Elmer, MA, USA). Background fluorescence was recorded and subtracted. Ethidium bromide was added to the cell suspension at a final concentration of 2 µg/ml and the change in fluorescence recorded at excitation and emission wavelengths of 530 and 600 nm, respectively. Fluorescence of the cell suspension was used as an indicator of the amount of ethidium bromide taken up by the cells since fluorescence is dramatically enhanced by binding to intracellular components. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, MO, USA), an efflux pump inhibitor, was then added to the cell suspension 5 minutes after the addition of the ethidium bromide and the change in the fluorescence intensity recorded over a period of 5 minutes. A sharp increase in fluorescence is indicative of the inactivation of the efflux pumps, due to increased accumulation of ethidium bromide. These experiments were performed on one occasion.

2.8 Measurement of Chemical Accumulation

To determine if the functional components in household detergents could be extruded by the efflux pumps, chemical uptake was assayed as previously described (Chapman & Georgopapadakou 1988) with slight modifications.

The bacteria were grown to the late logarithmic phase at 36 ± 1 °C in Luria Bertani (LB) broth and the absorbance values at 600 nm recorded using a standard visible range spectrophotometer. The bacterial cells were harvested by centrifugation in a benchtop centrifuge at $5700 \times g$ for 5 minutes, washed once in 15mM PBS, and resuspended in 10 ml of the same buffer to a calculated A_{600} of 10.0. The bacterial suspension was placed in a shaker at 37 °C and left for 10 min to equilibrate. The chemical of interest, sodium salicylate (Sigma Aldrich, MO, USA), triclosan (kind gift from Ciba Geigy, Manchester, UK), or *ortho*-phenylphenol (Sigma Aldrich), was then added to the required final concentration (Table 2.5). After addition of the chemical, 0.5-ml samples were removed at different time intervals. At 7 min after addition of the chemical, the efflux pump inhibitor, CCCP was added to one-half of the reaction mixture (5 ml) to a final concentration of 200 μ M and the other half of the reaction mixture (5 ml) was used as a control (no CCCP). Each of the collected samples at different time points was immediately diluted in 1 ml of ice-cold PBS to stop the reaction and centrifuged at $5700 \times g$ for 5 minutes. The pellets were washed once with 1 ml of ice-cold PBS to remove any agent not associated with the bacteria, resuspended in 1 ml of PBS and boiled for 5 minutes to lyse the cells. The samples

were then centrifuged at 5,700 x g for 10 minutes and the absorbance of the supernatant was measured using a 3 series UV spectrophotometer (BioMate, NY, USA) at the appropriate wavelengths (Table 2.5). The absorbance readings are a measurement of the degree to which the agent was internalized by the bacterial cells, and a relative increase suggests that efflux pumps were involved in extruding the chemical.

This procedure was done in duplicate on two independent occasions for each bacterium and the results plotted as graphs of time vs. absorbance.

Table 2.5: Final concentration of chemical added to the cell suspension and wavelengths at which absorbance readings were recorded.

Chemical	Final Concentration	Wavelength
Sodium Salicylate	14 mM	296 nm
<i>Ortho</i> -Phenylphenol	8 mM	251 nm
Triclosan	6 mM	382 nm

These concentrations are slightly higher than the concentrations recommended by the manufacturer; this was to ensure that once the antimicrobials were added to the cell suspension they would be diluted in the PBS to the concentrations recommended by the manufacturers.

2.9 Chemical Disinfection Procedure

An in-use disinfectant test was established to see what effect, if any, the commonly used household disinfectants had on the gastrointestinal pathogens in the domestic setting. This procedure aims to mimic the situation where bacteria from the kitchen grow in a wet, warm, soiled environment, as occurs daily in many households.

2.9.1 Preparation of contaminated dishcloths

A single colony of the bacterium of interest was inoculated into BHI broth and incubated at 36 ± 1 °C for 24 hours. 10 ml of the cell suspension was added to 50 ml of a 10 % sterilized milk solution (Oxoid) in a 500 ml beaker. One perforated 100 % viscose fibre dishcloth (purchased from a supermarket and autoclaved prior to use) was added to each beaker and squeezed with gloved hands to distribute the test suspension throughout the cloth. The cloth was then transferred to a sterile plastic bag and left to incubate at room temperature for 2 hours.

2.9.2 Disinfection of dishcloths

The dishcloths were aseptically divided into 5 equal portions (approx. 25 cm²). One portion was transferred to quarter-strength Ringer solution to determine the initial bacterial count. One portion was rinsed under running tap water and transferred to quarter-strength Ringer solution to see the effect of merely rinsing the dishcloths (0 % detergent concentration). The remaining cloth portions were immersed in the

commonly used household disinfectant products (containing sodium salicylate, triclosan, or 2-phenylphenol). The products were used at the concentration recommended by the manufacturer, as well as concentrations 25 % and 50 % of the recommended concentration. After 2 minutes, the cloth portions were rinsed in running tap water for 30 seconds and one cloth portion from each disinfectant treatment was transferred to 10 ml of quarter-strength Ringer solution for determination of total counts.

2.9.3 Estimation of total counts in cloth portions

Cloth portions were transferred to 10 ml of quarter-strength Ringer solution in 250 ml conical flasks. The flasks were shaken on a mechanical shaker for 10 minutes to release the organisms from the cloths and serial dilutions of the rinse fluid were prepared in quarter-strength Ringer solution. These dilutions were then plated on nutrient agar plates, and incubated at 36 ± 1 °C for 24 hours. The colony forming units (cfus) were determined and expressed as % reduction in bacterial numbers relative to the cfu from the untreated aliquot.

The chemical disinfection procedure was repeated in duplicate on two different occasions for each bacterium.

2.9.4 Additional dishcloth tests

2.9.4.1 Effect of tap water, saline, and distilled water on the bacterial cells

The running tap water used during the 0 % rinsing step above was hypotonic in comparison to the bacterial cells. This could have resulted in lysis of the bacterial cells, resulting in skewed data. In order to ascertain whether this had occurred, the procedure was repeated as above except that the cloth portions were rinsed in distilled water (most hypotonic), tap water (hypotonic), and normal saline (isotonic). These experiments were done in duplicate on 2 different occasions and the cfu/ml were calculated and the results compared.

2.9.4.2 Suspension tests

Suspension tests were performed to determine if any interactions between the cloth fabric and the ingredients in the disinfectants was taking place, which would result in an altered disinfectant efficacy.

The bacteria were grown overnight in BHI broth, split into three equal portions of 3 ml each, added to a 10 % milk solution and left to incubate for 2 hours at room temperature. The suspensions were centrifuged for 5 minutes at 3000 x g and the supernatant discarded. One pellet was resuspended in 10 ml of tap water, serially diluted and plated on nutrient agar to determine the initial counts. The remaining pellets were resuspended in 5 ml of distilled water and 25 % disinfectant solution

respectively. After 2 minutes the suspensions were centrifuged at 3000 x g for 5 minutes, resuspended in 10 ml tap water, serially diluted in tap water, and plated on nutrient agar to determine the cfu/ml. These experiments were performed in duplicate on 2 different occasions and the cfu/ml were calculated and the results compared.

2.10 Statistical Analysis

Statistical analysis of results was performed using Stata 8 (Statacorp, Lakeway Drive, College Station, Texas, USA).

Regression models for time-series data were used in the prediction of readings to analyze the results from the chemical accumulation assays, as well as reproducibility of assays. A similar model using clustered-series data was used in the prediction of readings to analyze the results from the chemical disinfection procedure, as well as reproducibility of the procedure. Results were considered significant at the 5 % level.

CHAPTER 3

3 RESULTS

3.1 Bacterial Strains

A total of 18 bacterial strains were examined in this study. Of these 18 strains, eight were *Salmonella* and ten were *E. coli*.

The *Salmonella* species (Figures 3.1 and 3.2) were isolated from poultry samples and humans with infectious intestinal disease using standard microbiological culture techniques, as well as obtained from laboratory culture collections compiled from previous studies.

The *E. coli* strains (Figure 3.3) were isolated from poultry samples and humans with infectious intestinal disease using microbiological culture techniques, as well as obtained from the Onderstepoort Veterinary Institute, University of Pretoria. The source of each bacterial isolate is shown in Table 3.1.

Table 3.1: Source of each bacterial isolate examined in this study.

Source	Bacterial isolates
Poultry samples	<i>Salmonella</i> #s 1-4 & <i>E. coli</i> #s 1-5
Humans with infectious intestinal disease	<i>Salmonella</i> #s 7 & 8 & <i>E. coli</i> #s 8 & 9
Onderstepoort Veterinary Institute	<i>E. coli</i> #s 6 & 7
Laboratory culture collection	<i>Salmonella</i> #s 5 & 6

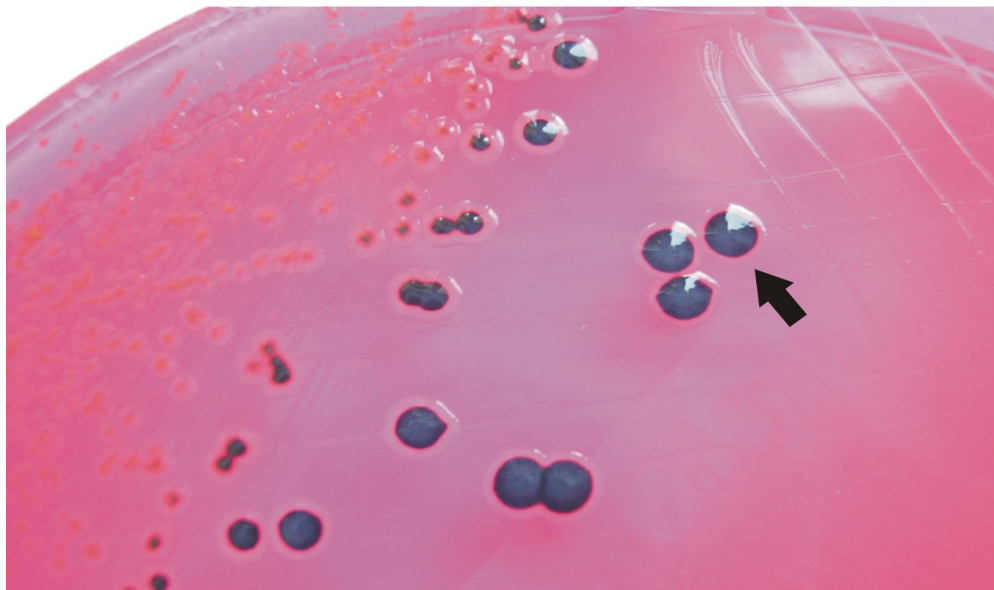


Figure 3.1: Typical growth of salmonellae on XLD agar. Salmonellae grow as translucent red colonies with or without a black centre.

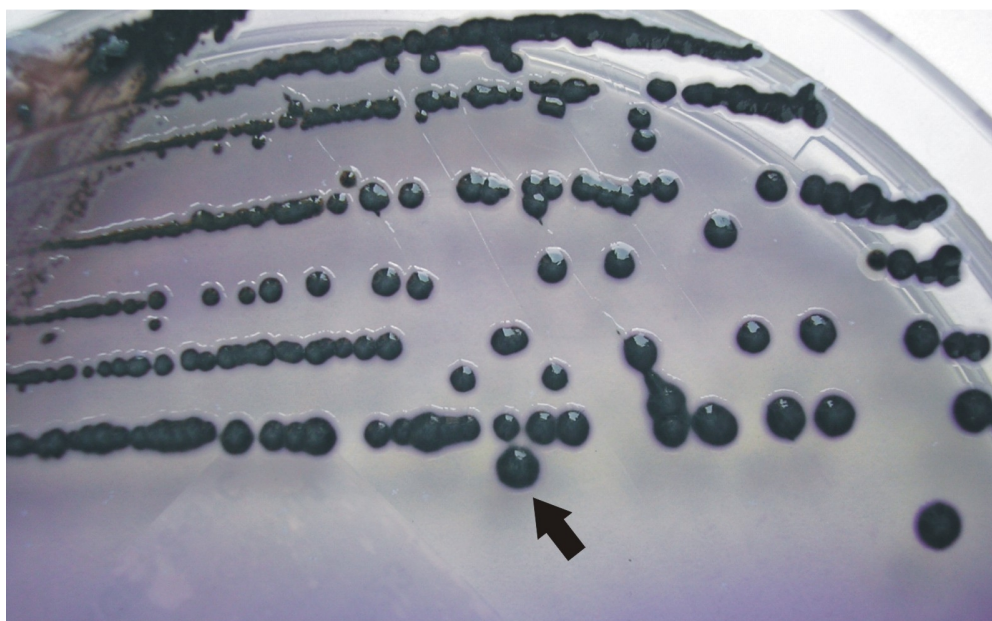


Figure 3.2: Typical growth of salmonellae on MLCB agar. Salmonellae grow as large purple-black colonies due to the production of hydrogen sulphide.

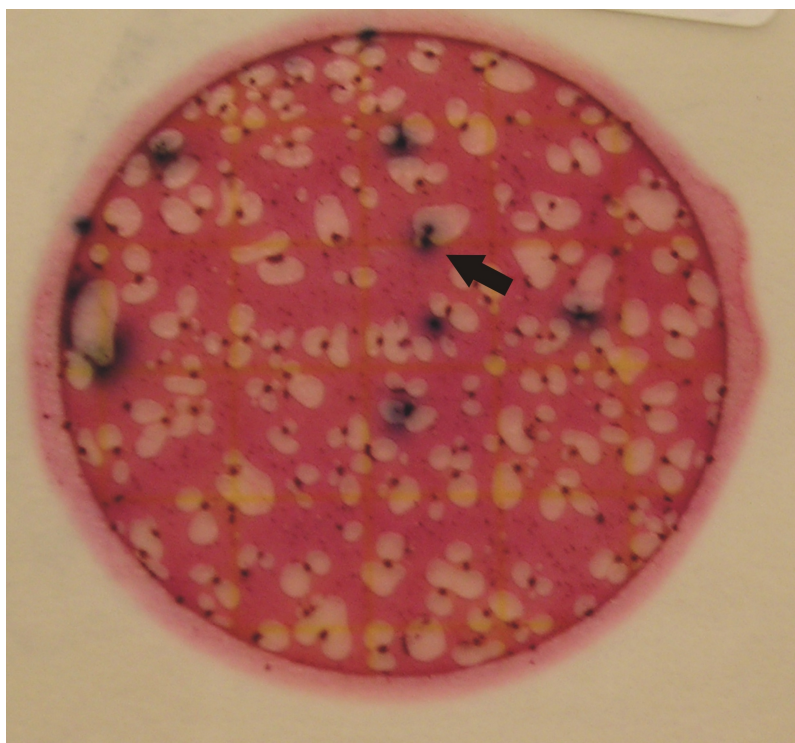


Figure 3.3: Growth of *E. coli* on a 3M Petrifilm™. *E. coli* appear as blue colonies with gas bubbles.

3.2 Disk Diffusion Susceptibility Testing

Disk diffusion susceptibility tests were performed on all the bacterial strains that had survived intermediate concentrations of tetracycline and ofloxacin to determine if they were fully resistant or sensitive. Susceptibility testing was also done using the susceptible strains which were isolated by omitting the antibiotics from the selection broth and plating out onto suitable media.

After multiple screening, eight *Salmonella* strains were selected, four of which were found to be sensitive to both tetracycline and ofloxacin, and four were resistant to only tetracycline (Table 3.2). The four sensitive isolates were used in the ethidium bromide accumulation assays as well as the chemical accumulation assays as controls against which the resistant isolates could be compared.

Of the many *E. coli* strains examined, ten were selected for the study. Four strains were found to be sensitive to tetracycline and ofloxacin and six strains were resistant to both tetracycline and ofloxacin (Table 3.3). The sensitive isolates were used in the ethidium bromide accumulation assays as well as the chemical accumulation assays as controls against which the resistant isolates could be compared.

Table 3.2: Zone diameters (including disk) of the eight *Salmonella* strains used in the study. A zone size of ≥ 14 and ≥ 12 is considered as resistant for tetracycline and ofloxacin, respectively (NCCLS 2001).

Bacterial species	Zone diameter (mm)		Result (R/S)	
	Tetracycline (30 µg)	Ofloxacin (5 µg)	Tetracycline (30 µg)	Ofloxacin (5 µg)
<i>Salmonella</i> # 1	19	29	S	S
<i>Salmonella</i> # 2	20	28	S	S
<i>Salmonella</i> # 3	21	28	S	S
<i>Salmonella</i> # 4	21	30	S	S
<i>Salmonella</i> # 5	6	30	R	S
<i>Salmonella</i> # 6	8	29	R	S
<i>Salmonella</i> # 7	13	39	R	S
<i>Salmonella</i> # 8	8	46	R	S

Table 3.3: Zone diameters (including disk) of the ten *E. coli* strains used in the study. A zone size of ≥ 14 and ≥ 12 is considered as resistant for tetracycline and ofloxacin, respectively (NCCLS 2001).

Bacterial species	Zone diameter (mm)		Result (R/S)	
	Tetracycline (30 µg)	Ofloxacin (5 µg)	Tetracycline (30 µg)	Ofloxacin (5 µg)
<i>E. coli</i> # 1	28	36	S	S
<i>E. coli</i> # 2	26	33	S	S
<i>E. coli</i> # 3	29	42	S	S
<i>E. coli</i> # 4	28	38	S	S
<i>E. coli</i> # 5	6	11	R	R
<i>E. coli</i> # 6	6	6	R	R
<i>E. coli</i> # 7	6	7	R	R
<i>E. coli</i> # 8	6	8	R	R
<i>E. coli</i> # 9	14	6	R	R
<i>E. coli</i> # 10	6	6	R	R

3.3 Polymerase Chain Reaction

PCR was performed to confirm that the bacteria used in the study were correctly identified using microbiological culture techniques. If no bacteria were isolated from the poultry samples, PCR was performed to investigate the possibility that the culture techniques were not sensitive enough to isolate bacteria.

Amplification of a 284 bp product in the salmonellae and a 401 bp product in the *E. coli* PCR reactions confirmed that the bacteria isolated were as expected from cultural methods. None of the *E. coli* samples were 0157 positive but one sample did encode the *hlyA* gene that produces the H7 flagellar antigen. Figures 3.4 and 3.5 show the presence of the amplified products after agarose gel electrophoresis.

PCR amplification reactions were also carried out on the enrichment broths from which no bacteria were isolated using standard microbiological culture techniques. In these PCR reactions no amplification products were observed after agarose gel electrophoresis, indicating that if any *Salmonella* or *E. coli* were present they were below the detectable limits. The PCR detection limits were determined to be 10^{-6} and at least 10^{-8} cfu/ml for *Salmonella* and *E. coli*, respectively. In the *Salmonella* PCR the inhibitor control constructs were not amplified, indicating that inhibition could have occurred. If this was the case then *Salmonella* could have been present but were not amplified.

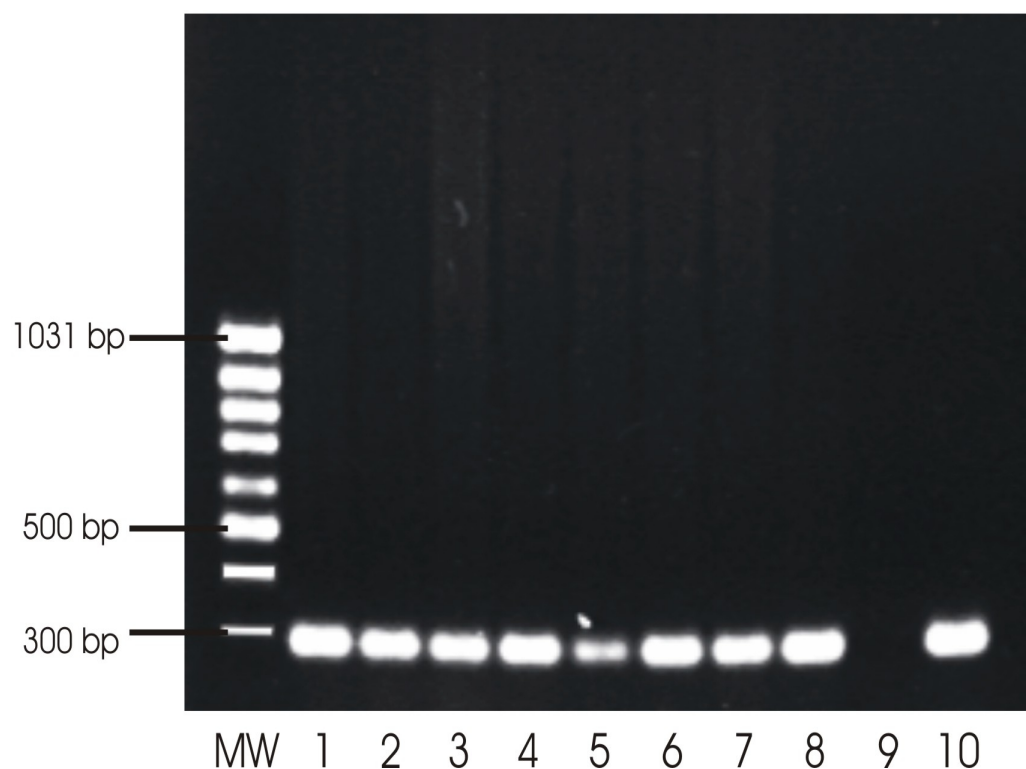


Figure 3.4: PCR amplification profiles from the *Salmonella* species used in the study. Molecular masses are indicated on the left of the gel.

Lane MW, Molecular weight marker

Lane 1, *Salmonella* # 1

Lane 2, *Salmonella* # 2

Lane 3, *Salmonella* # 3

Lane 4, *Salmonella* # 4

Lane 5, *Salmonella* # 5

Lane 6, *Salmonella* # 6

Lane 7, *Salmonella* # 7

Lane 8, *Salmonella* # 8

Lane 9, negative control (no DNA)

Lane 10, positive control (284 bp)

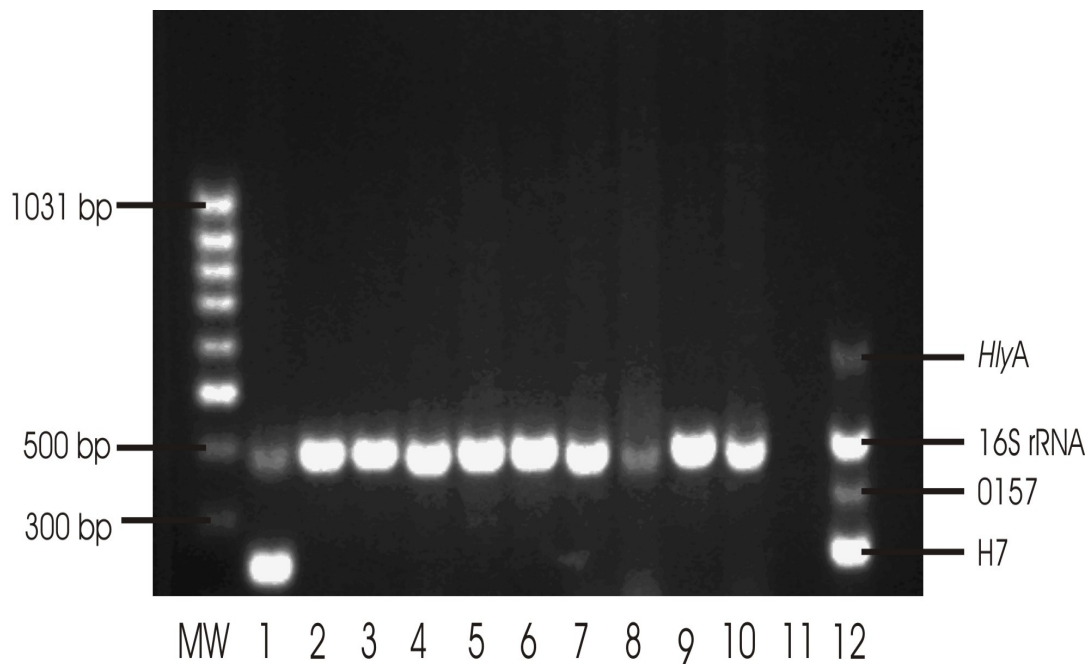


Figure 3.5: Multiplex PCR amplification profiles from the *E. coli* strains used in the study. Molecular masses are indicated on the left of the gel.

Lane MW: Molecular weight marker

Lane 1, *E. coli* # 1

Lane 2, *E. coli* # 2

Lane 3, *E. coli* # 3

Lane 4, *E. coli* # 4

Lane 5, *E. coli* # 5

Lane 6, *E. coli* # 6

Lane 7, *E. coli* # 7

Lane 8, *E. coli* # 8

Lane 9, *E. coli* # 9

Lane 10, *E. coli* # 10

Lane 11, negative control (no DNA)

Lane 12, positive control of *hlyA* (569 bp), 16S rRNA (401 bp), *rfbE*_{O157} (327 bp), and *fliC*_{H7} (247 bp)

3.4 Macro-Restriction Analysis

Macro-restriction analysis was performed on the isolated bacterial strains in order to ascertain whether or not the bacteria had similar genetic profiles. This was to ensure that the isolates were not identical, and that the experiments were not being performed on the same bacterial clone resulting in duplication of results.

As seen in Figure 3.6 dissimilar banding patterns (7 or more band differences) were observed in all eight of the *Salmonella* species examined, indicating that they were all different strains.

A difference of only one band was seen between *E. coli* isolates # 6 and 7, indicating that the strains are related. *E. coli* isolate # 7 was therefore excluded from the study. Similar banding patterns (i.e. a four band difference) were observed in *E. coli* #s 1 and 9, 3 and 4, and 6 and 9, indicating that they were possibly the same strain. The remaining *E. coli* species had dissimilar banding patterns (7 or more band differences) indicating that they were different strains (Figure 3.7).

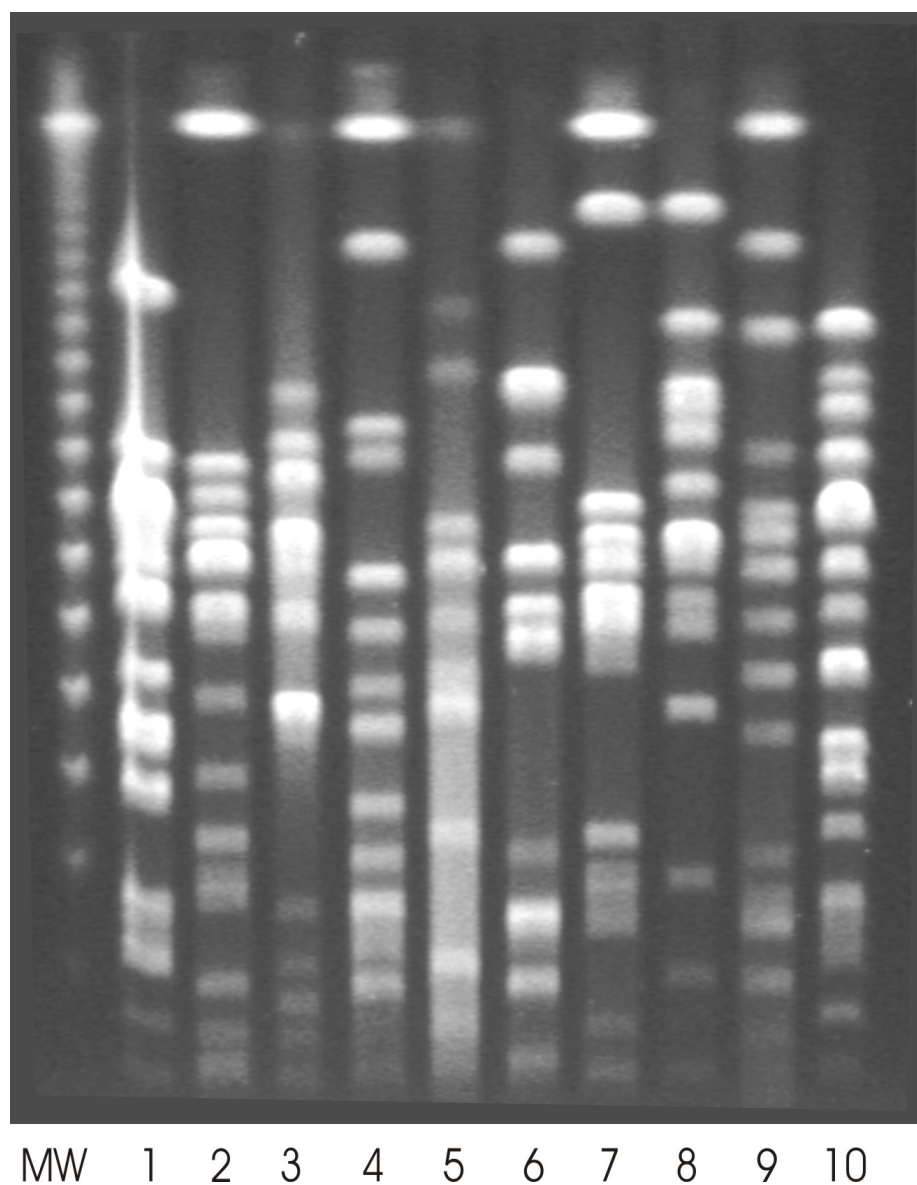


Figure 3.6: Macro-restriction analysis profiles from the *Salmonella* species used in the study. Lane MW, Lambda marker; lane 1, Unrelated *Salmonella* strain; lane 2, *Salmonella* # 1; lane 3, *Salmonella* # 2; lane 4, *Salmonella* # 3; lane 5, *Salmonella* # 4; lane 6, *Salmonella* # 5; lane 7, *Salmonella* # 6; lane 8, *Salmonella* # 7; lane 9, *Salmonella* # 8; lane 10, unrelated *Salmonella* strain.

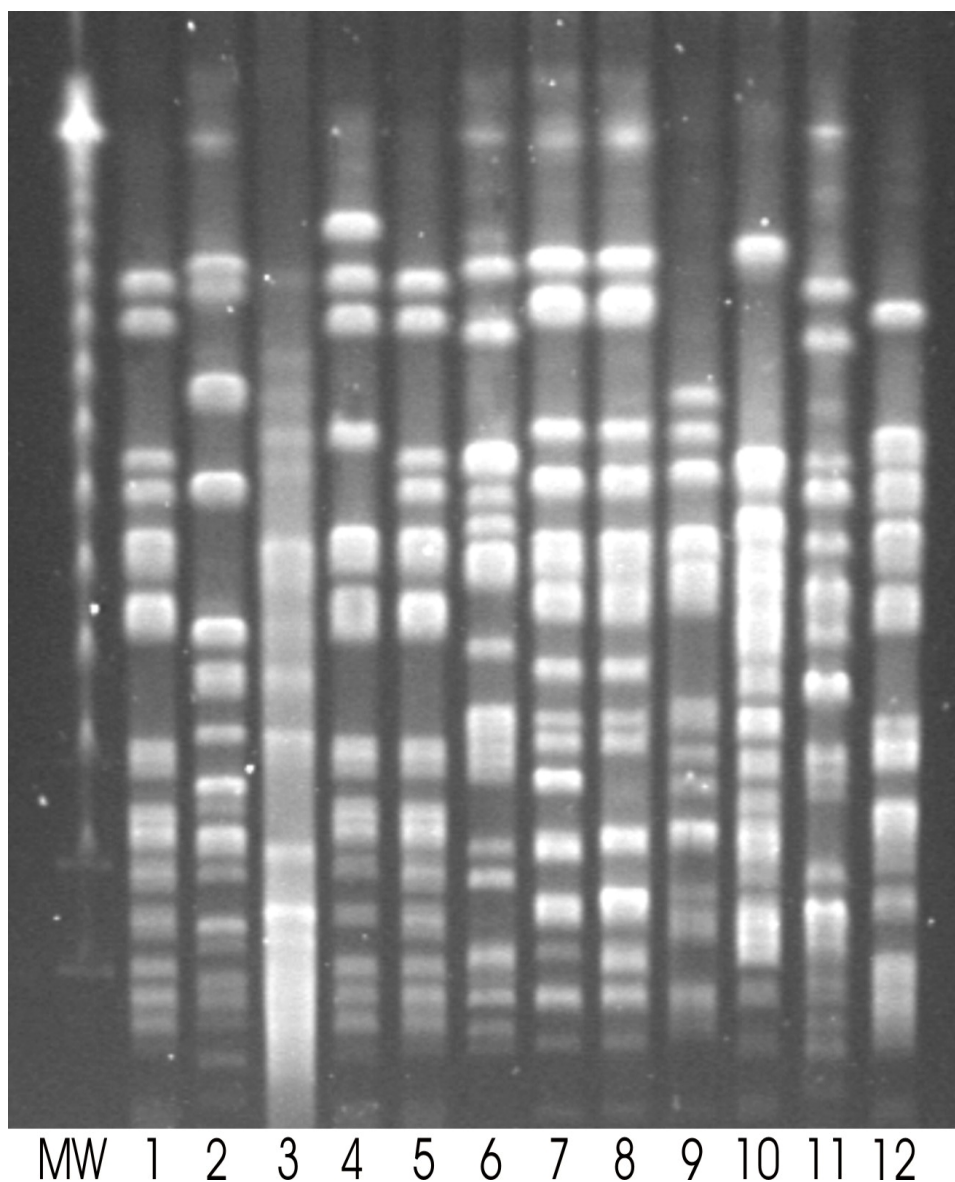


Figure 3.7: Macro-restriction analysis profiles from the *E. coli* species used in the study. Lane MW, Lambda marker; lane 1, unrelated *E. coli* strain; lane 2, *E. coli* # 1; lane 3, *E. coli* # 2; lane 4, *E. coli* # 3; lane 5, *E. coli* # 4; lane 6, *E. coli* # 5; lane 7, *E. coli* # 6; lane 8, *E. coli* # 7; lane 9, *E. coli* # 8; lane 10, *E. coli* # 9; lane 11, *E. coli* # 10; lane 12, unrelated *E. coli* strain.

3.5 Ethidium Bromide Accumulation Assays

All the bacterial isolates were analyzed using an ethidium bromide accumulation assay to determine if they showed active efflux pump activity.

The CCCP added to the cell suspension five minutes after ethidium bromide addition was dissolved in the organic solvent dimethyl sulfoxide (DMSO). Therefore to ensure that any effects observed in the ethidium bromide accumulation assays after CCCP addition could be attributed to the CCCP alone; the accumulation assays were performed using only DMSO. No changes in fluorescence values were observed after the addition of DMSO in any of the accumulation assays. A representative sample is shown in Figure 3.8.

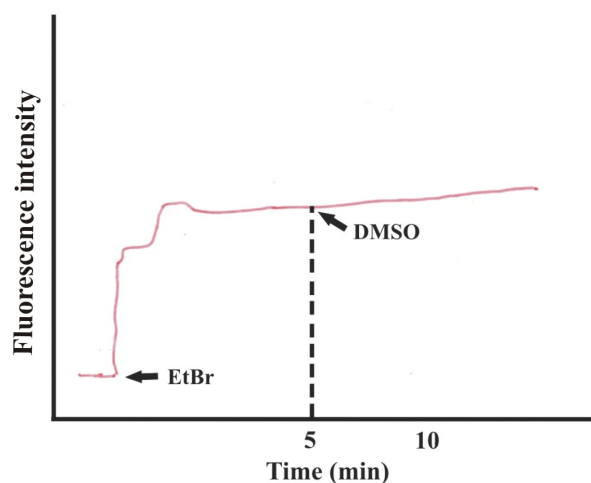


Figure 3.8: Accumulation of ethidium bromide by tetracycline and ofloxacin resistant *Salmonella* # 5. On addition of ethidium bromide, fluorescence intensity (y-axis) increased substantially, and then leveled off. Addition of DMSO had no effect on fluorescence intensity.

3.5.1 Sensitive *Salmonella* and *E. coli* strains

In each instance the fluorescence intensity was seen to increase substantially on addition of ethidium bromide. This was indicative of the fact that the cells were taking up the ethidium bromide, as binding of ethidium bromide to intracellular components results in increased fluorescence (Tables 3.4 and 3.5 and Figures 3.9 and 3.10). By 5 minutes, after the addition of ethidium bromide, a plateau in the fluorescence values was reached.

On addition of CCCP, the fluorescence intensity remained the same or only increased slightly (Tables 3.4 and 3.5 and Figures 3.9 & 3.10). These results indicate that no more, or very little ethidium bromide was entering the cells. This effect could be due to three causes: the absence of efflux pumps, the presence of inactive efflux pumps that could not be induced upon addition of ethidium bromide, or the presence of inactive efflux pumps that could only be marginally induced upon addition of ethidium bromide.

Table 3.4: Spectrofluorimetric values obtained from the ethidium bromide accumulation assays for the sensitive *Salmonella* strains used in the study. The fluorescence values were recorded once the plateau in fluorescence was reached and five minutes after the addition of CCCP.

Bacterium	Fluorescence	
	EtBr addition	CCCP addition
<i>Salmonella</i> # 1	21.5	25.2
<i>Salmonella</i> # 2	23.4	25.7
<i>Salmonella</i> # 3	16.4	18.9
<i>Salmonella</i> # 4	34.6	36.2

Table 3.5: Spectrofluorimetric values obtained from the ethidium bromide accumulation assays for the sensitive *E. coli* strains used in the study. The fluorescence values were recorded once the plateau in fluorescence was reached and five minutes after the addition of CCCP.

Bacterium	Fluorescence	
	EtBr addition	CCCP addition
<i>E. coli</i> # 1	44.0	47.5
<i>E. coli</i> # 2	121.5	124.1
<i>E. coli</i> # 3	19.9	22.3
<i>E. coli</i> # 4	20.6	23.3

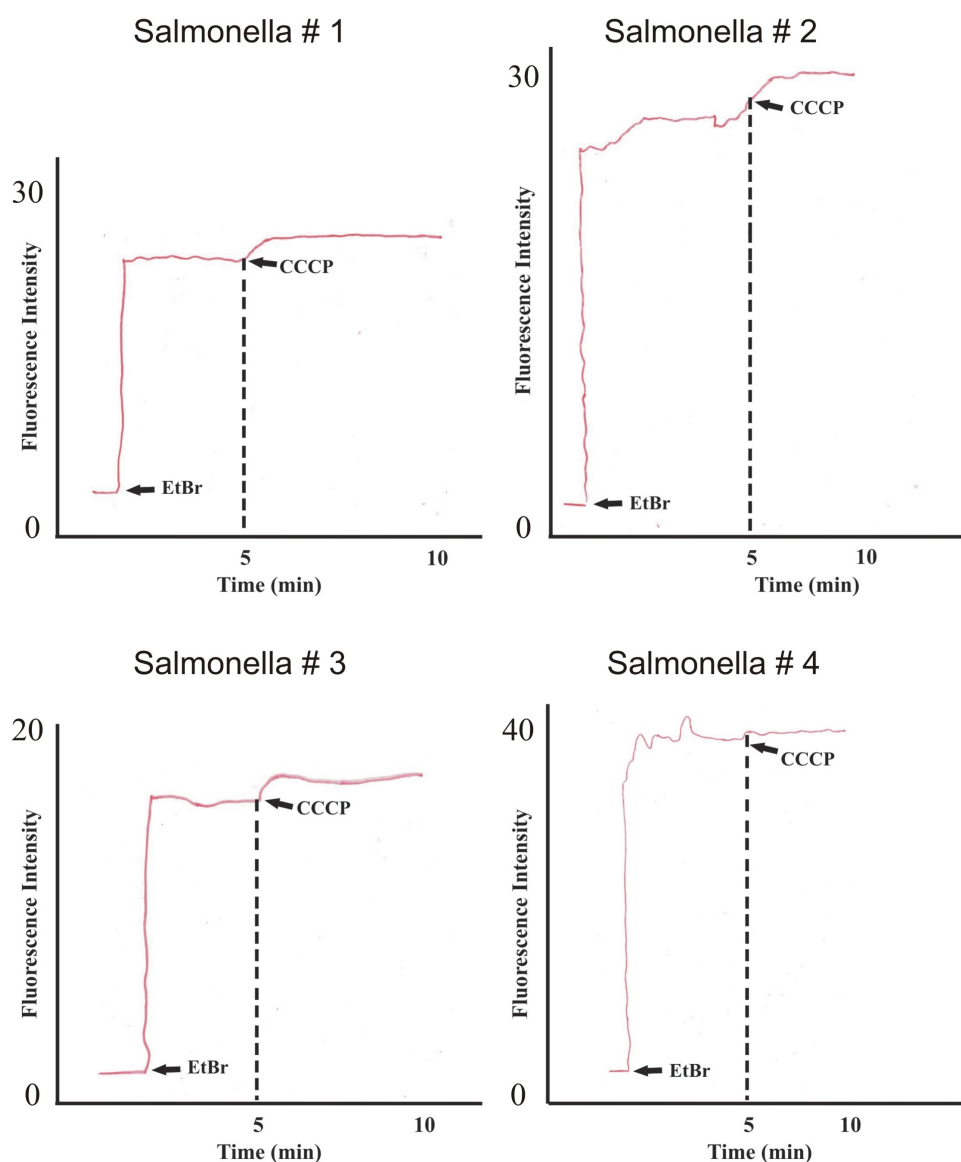


Figure 3.9: Accumulation of ethidium bromide by the four tetracycline and ofloxacin sensitive *Salmonella* #s 1, 2, 3, and 4. On addition of ethidium bromide, fluorescence intensity (y-axis) increased substantially, and then leveled off. Addition of the efflux pump inhibitor, CCCP, had little or no effect on fluorescence intensity indicating that no efflux pumps were inhibited.

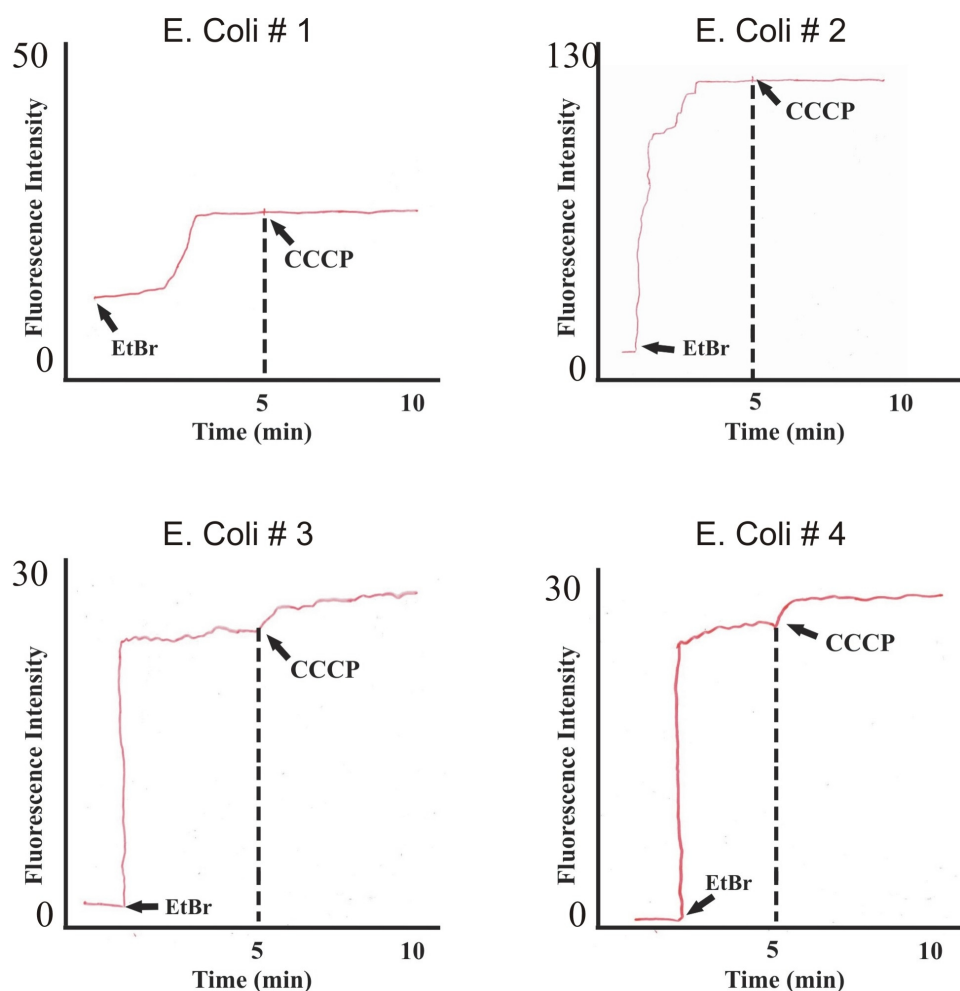


Figure 3.10: Accumulation of ethidium bromide by the four tetracycline and ofloxacin sensitive *E. coli* #s 1, 2, 3, and 4. On addition of ethidium bromide, fluorescence intensity (y-axis) increased, and then leveled off. Addition of the efflux pump inhibitor, CCCP, had little or no effect on fluorescence intensity indicating that no efflux pumps were inhibited.

3.5.2 Resistant *Salmonella* and *E. coli* strains

In each bacterium tested the fluorescence intensity was seen to increase substantially on addition of ethidium bromide, as binding of ethidium bromide to intracellular components resulted in fluorescence (Tables 3.6 & 3.7 and Figures 3.11 & 3.12). Five minutes after the addition of ethidium bromide the fluorescence levels remained constant as a state of equilibrium had been established.

Addition of CCCP resulted in a rapid and dramatic increase in fluorescence. This was due to inactivation of energy dependent efflux pumps, resulting in greater net accumulation of ethidium bromide (Tables 3.6 & 3.7 and Figures 3.11 & 3.12). These results indicated the presence of efflux pumps that could be activated upon the addition of ethidium bromide and inactivated upon addition of CCCP (proton motive force uncoupler). These effects were observed in all the resistant bacterial strains tested.

Table 3.6: Spectrofluorimetric values obtained from the ethidium bromide accumulation assays for the tetracycline and ofloxacin resistant *Salmonella* spp. used in this study. The fluorescence values were recorded once the plateau in fluorescence was reached and five minutes after the addition of CCCP.

Bacterium	Fluorescence	
	EtBr addition	CCCP addition
<i>Salmonella</i> # 5	14.9	28.4
<i>Salmonella</i> # 6	6.9	19.9
<i>Salmonella</i> # 7	19.5	38.0
<i>Salmonella</i> # 8	25.5	52.0

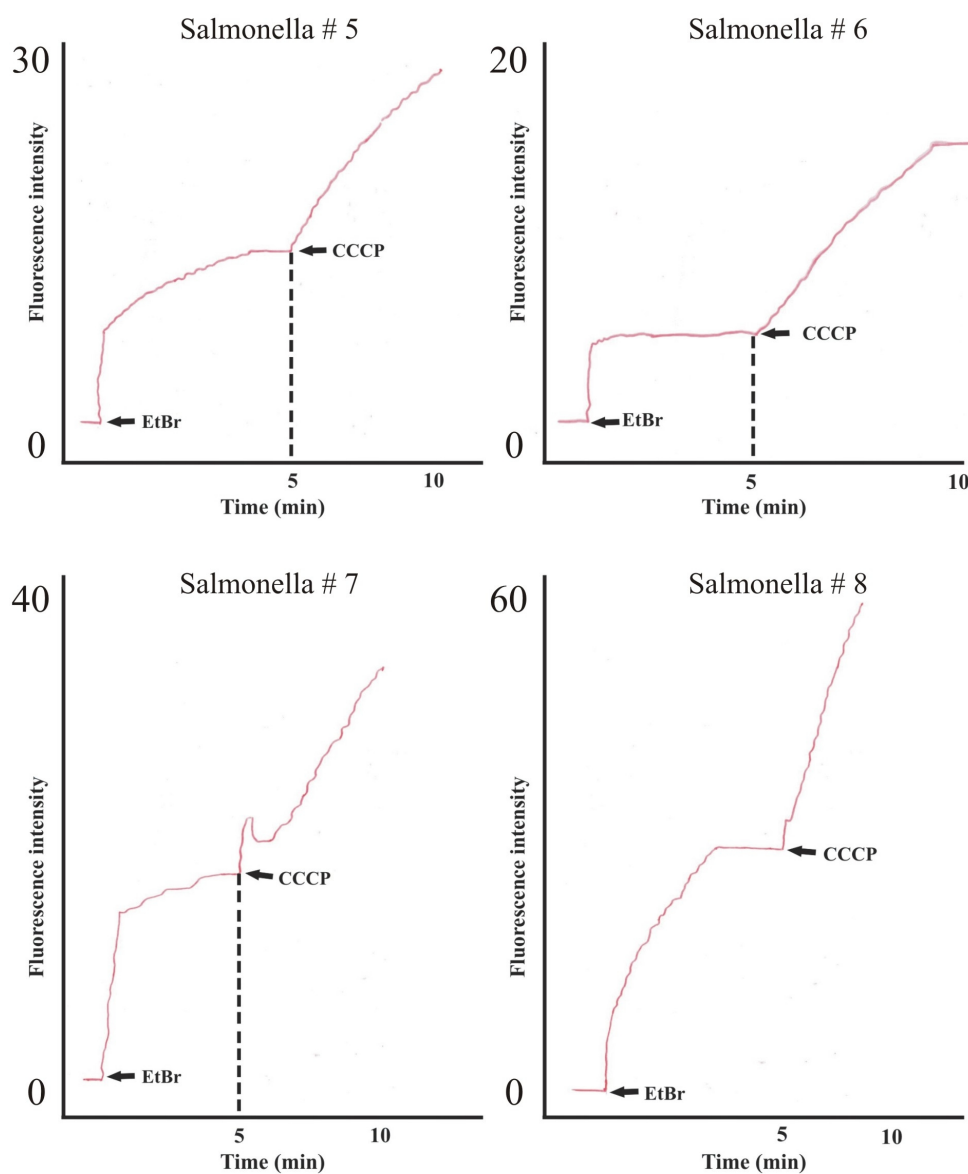
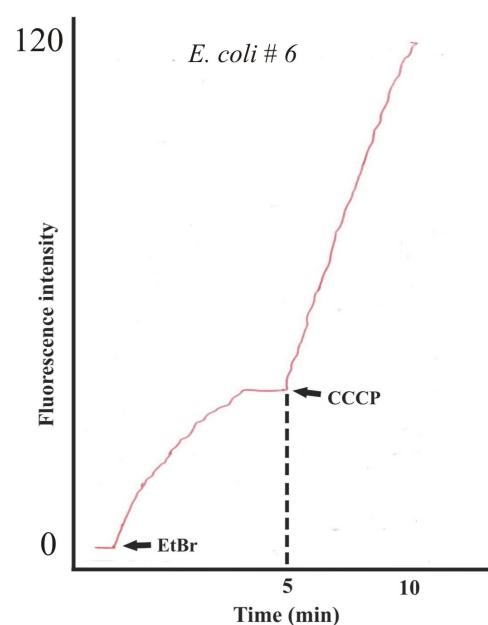
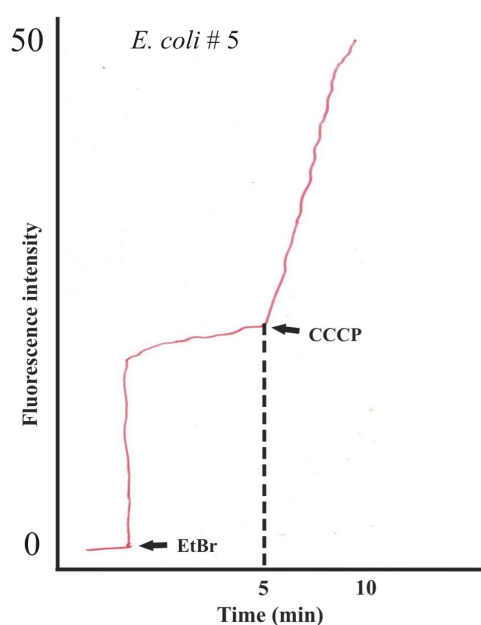


Figure 3.11: Accumulation of ethidium bromide by tetracycline and ofloxacin resistant *Salmonella* #s 5, 6, 7, and 8. On addition of ethidium bromide, fluorescence intensity (y-axis) increased substantially, and then leveled off. Addition of the efflux pump inhibitor, CCCP, resulted in a sharp increase in fluorescence indicating inactivation of efflux pumps.

Table 3.7: Spectrofluorimetric values obtained from the ethidium bromide accumulation assays for the tetracycline and ofloxacin resistant *E. coli* species used in this study. The fluorescence values were recorded once the plateau in fluorescence was reached and five minutes after the addition of CCCP.

Bacterium	Fluorescence	
	EtBr addition	CCCP addition
<i>E. coli</i> # 5	41.7	119.8
<i>E. coli</i> # 6	17.2	54.4
<i>E. coli</i> # 8	94.4	171.6
<i>E. coli</i> # 9	2.8	32.3
<i>E. coli</i> # 10	12.2	30.4



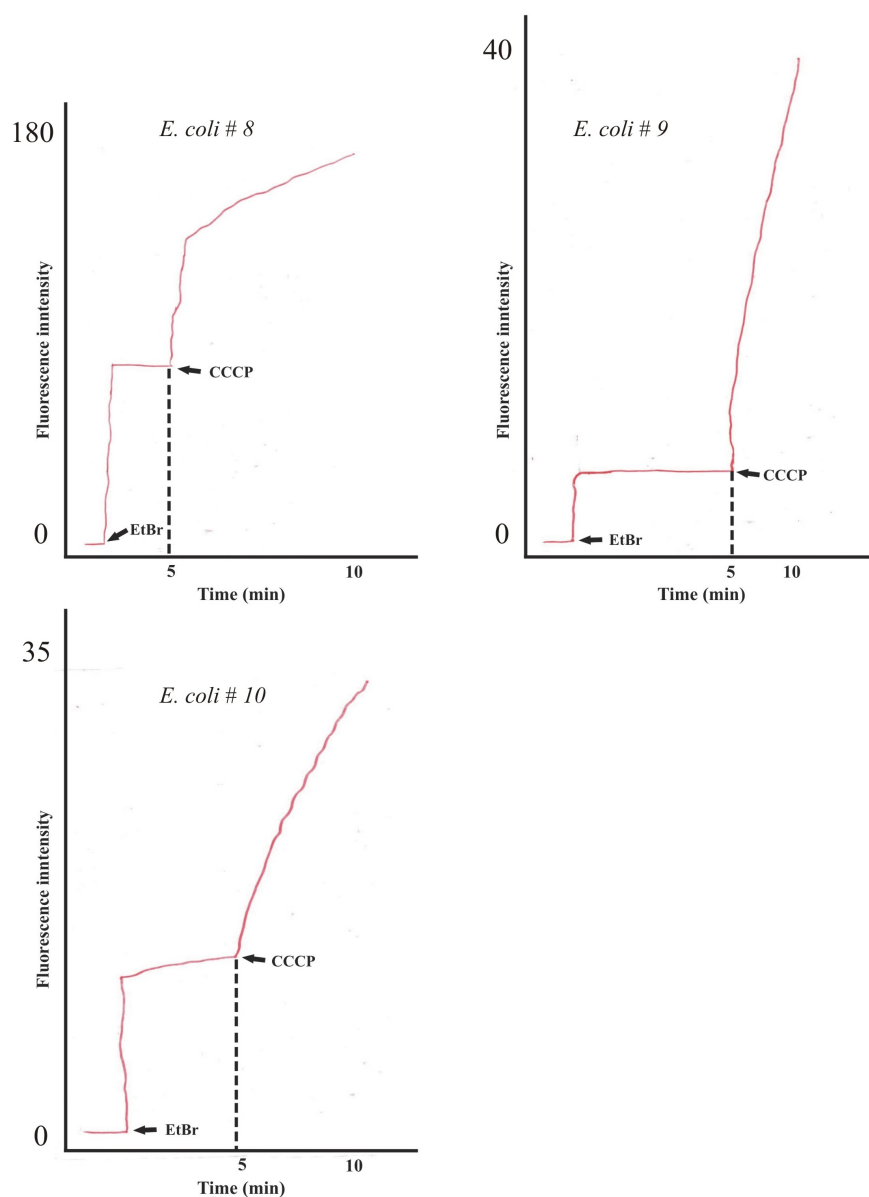


Figure 3.12: Accumulation of ethidium bromide by tetracycline and ofloxacin resistant *E. coli* #s 5, 6, 8, 9 and 10. On addition of ethidium bromide fluorescence intensity (y-axis) increased, and then leveled off. Addition of the efflux pump inhibitor, CCCP, resulted in a sharp increase in fluorescence indicating inactivation of efflux pumps.

The ethidium bromide accumulation assays also needed to be reproducible. In order to ensure this, the assay was performed in duplicate on separate occasions on a representative strain from each genus. It was observed that when done in duplicate the results showed the same trends, indicating that the technique was reproducible. A representative sample is shown in Figure 3.13 A & B.

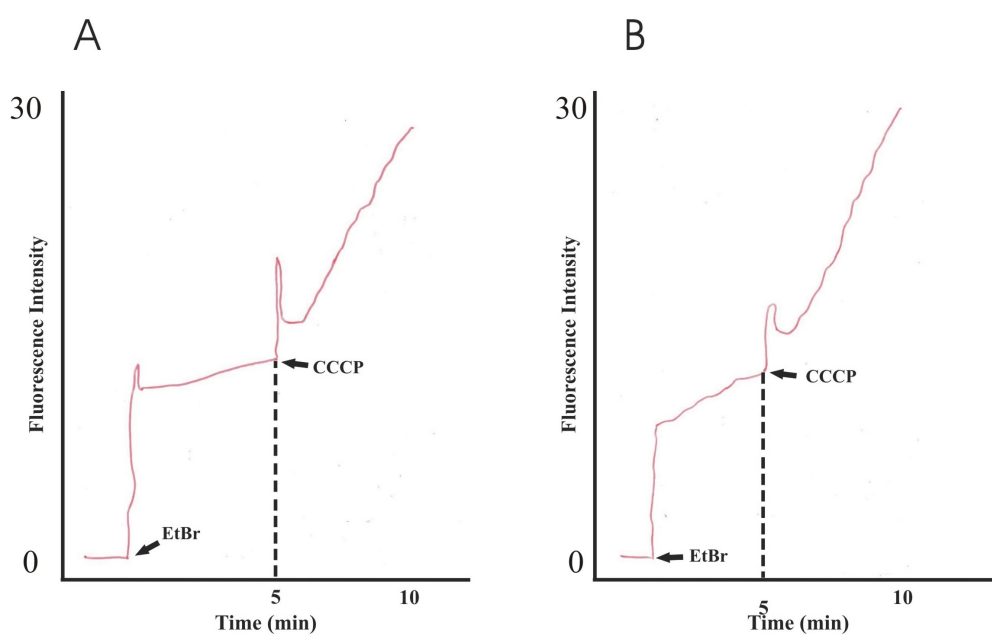


Figure 3.13 (A & B): Accumulation of ethidium bromide by tetracycline and ofloxacin resistant *Salmonella* # 7. On addition of ethidium bromide fluorescence intensity (y-axis) increased, and then leveled off. Addition of the efflux pump inhibitor, CCCP, resulted in a sharp increase in fluorescence indicating inactivation of efflux pumps.

3.6 Measurement of Chemical Accumulation

To determine if the active ingredients in three commonly used household chemical detergents were being extruded by efflux pumps, chemical accumulation assays were performed. The active ingredients were sodium salicylate, triclosan, and *ortho*-phenylphenol.

After a steady state was reached between entry and exit of the active agent, efflux pumps were disrupted in one aliquot using CCCP. After washing the bacteria, the cells were lysed and the intracellular concentration of the active agent assessed. An increase in the absorbance readings in both the absence and presence of CCCP indicated accumulation of the chemical in the bacterial cells. This effect would be observed in bacteria lacking an active efflux process. An increase in absorbance levels after CCCP addition, whilst the absorbance levels in the absence of CCCP remained constant, was indicative of an active efflux process.

3.6.1 Sensitive *Salmonella* and *E. coli* strains

In all the sensitive bacterial strains there was an increase in the absorbance readings in both in both the presence and absence of CCCP, indicating that the chemicals were accumulating in the bacterial cells. This was observed with all three of the active ingredients tested. These results indicate the absence of efflux of the active ingredients in the sensitive bacterial cells. Representative Figures 3.14- 3.17 are shown below and the remaining Figures C1-C6 are shown in Appendix C.

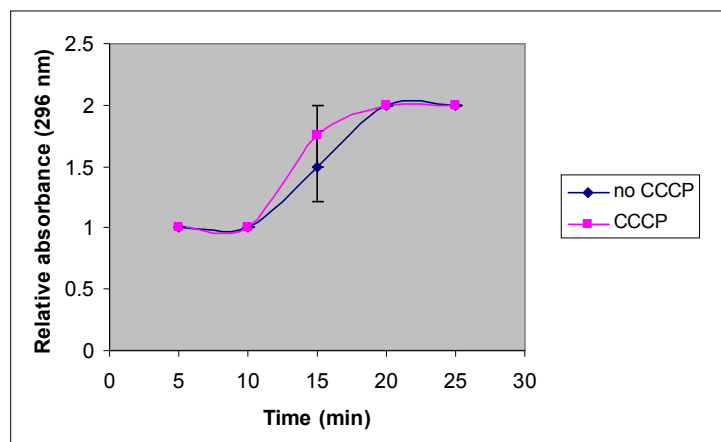


Figure 3.14: Graph of accumulation of sodium salicylate by tetracycline and ofloxacin sensitive *Salmonella* # 3. Absorbance on the y axis is a measure of the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

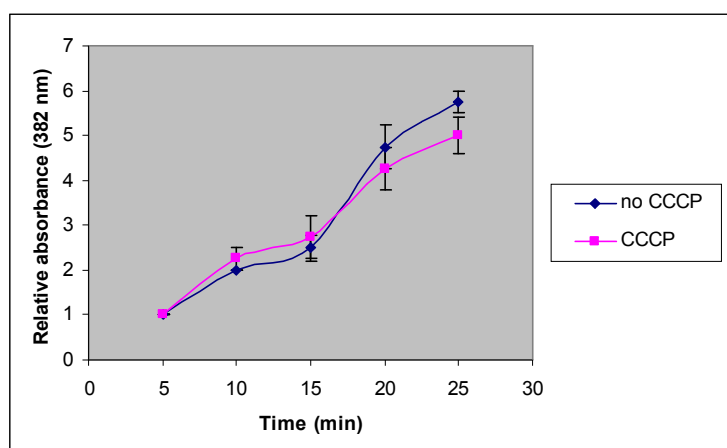


Figure 3.15: Graph of accumulation of triclosan by tetracycline and ofloxacin sensitive *Salmonella* # 2. Absorbance on the y axis is a measure of the levels of accumulated triclosan in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

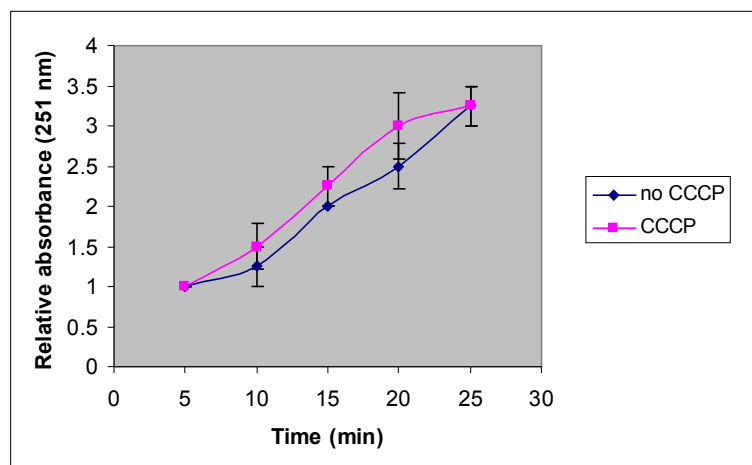


Figure 3.16: Graph of accumulation of *ortho*-phenylphenol by tetracycline and ofloxacin sensitive *E. coli* # 4. Absorbance on the y axis is a measure of the levels of accumulated *ortho*-phenylphenol in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

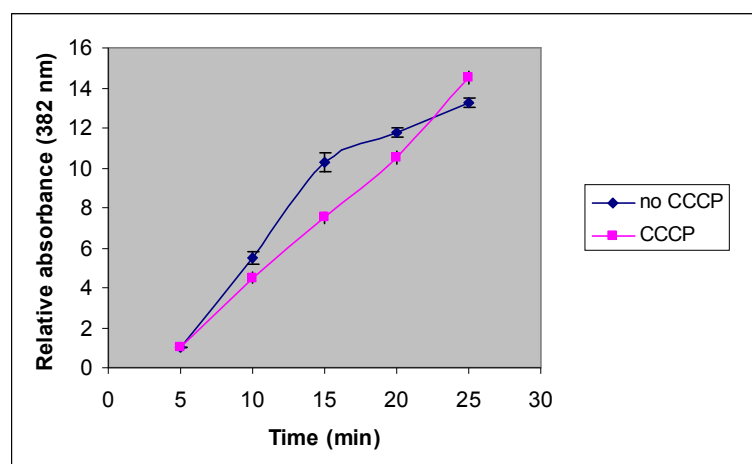


Figure 3.17: Graph of accumulation of triclosan by tetracycline and ofloxacin sensitive *E. coli* # 2. Absorbance on the y axis is a measure of the levels of accumulated triclosan in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

3.6.2 Resistant *Salmonella* and *E. coli* strains

In the resistant strains, absorbance values in the absence of CCCP remained constant. However, upon addition of CCCP, the absorbance values increased as a result of greater cell-associated chemical amounts. Representative Figures 3.18-3.21 are shown below and Figures C7-C12 in Appendix C.

These results showed that the resistant strains had efflux pumps that were extruding the active ingredients found in commonly used household disinfectant products and these efflux pumps were inactivated upon addition of CCCP.

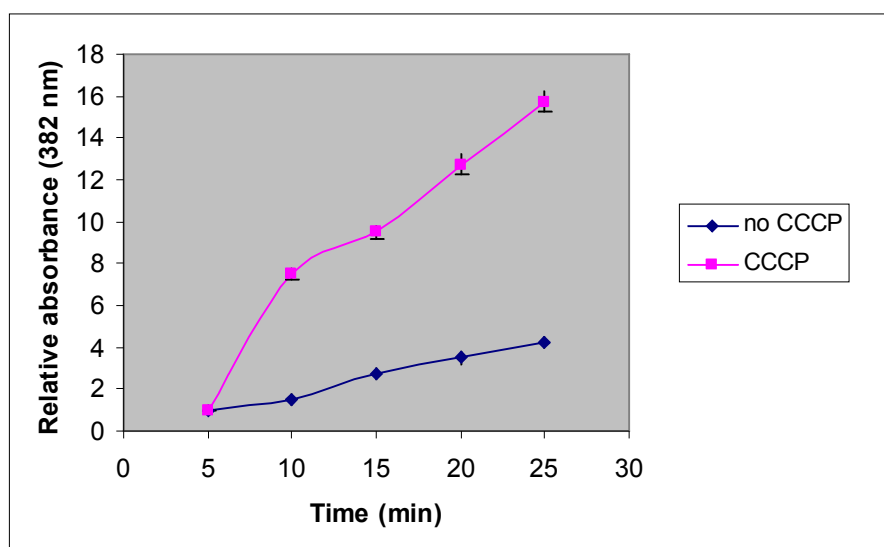


Figure 3.18: Graph of accumulation of triclosan by tetracycline and ofloxacin resistant *Salmonella* # 8. Absorbance on the y axis is a measure of the levels of accumulated triclosan in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

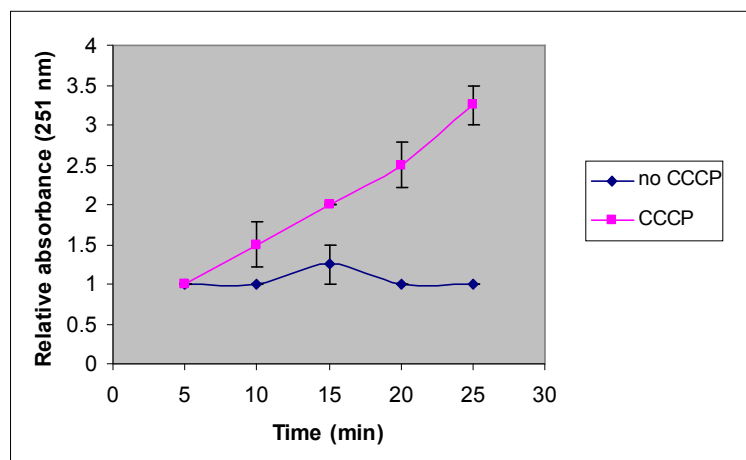


Figure 3.19: Graph of accumulation of *ortho*-phenylphenol by tetracycline and ofloxacin resistant *Salmonella* # 6. Absorbance on the y axis is a measure of the levels of accumulated *ortho*-phenylphenol in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

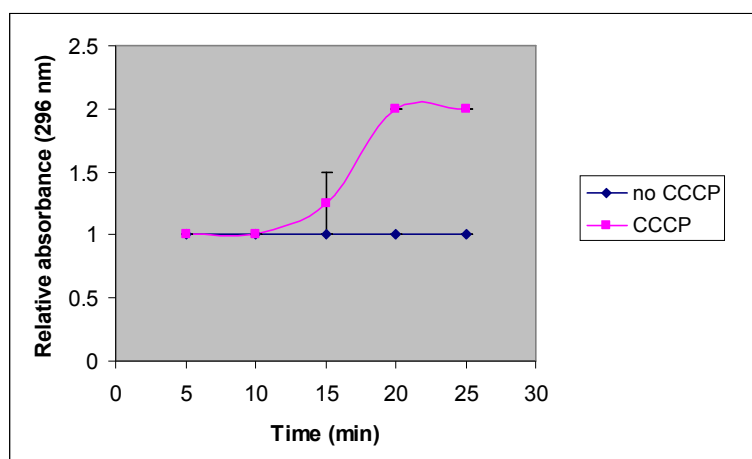


Figure 3.20: Graph of accumulation of sodium salicylate by tetracycline and ofloxacin resistant *E. coli* # 10. Absorbance on the y axis is a measure of the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ error of the mean of 2 independent experiments.

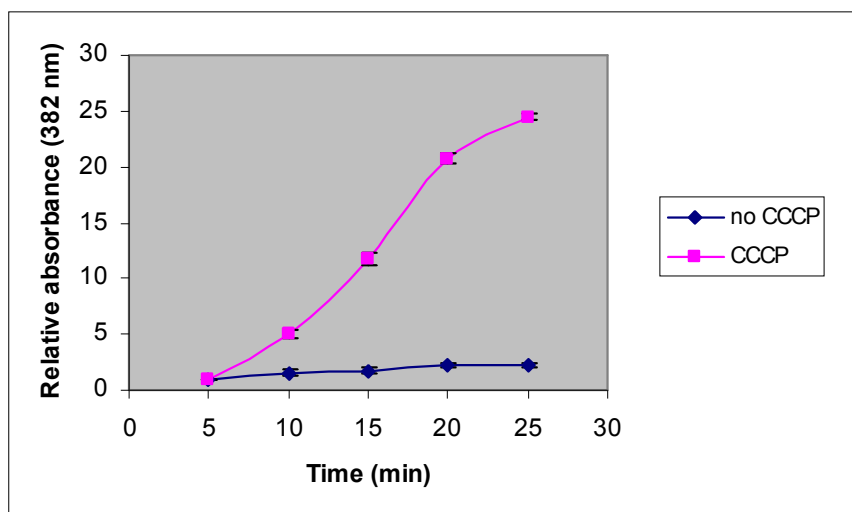


Figure 3.21: Graph of accumulation of triclosan by tetracycline and ofloxacin resistant *E. coli* # 6. Absorbance on the y axis is a measure of the levels of accumulated triclosan in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

3.6.3 Statistical analysis

Statistical analysis showed that there was no significant difference in the results from the duplicate experiments (P -value = 0.652), indicating that the experiments were reproducible.

Statistical analysis of the absorbance readings with or without the addition of CCCP in the sensitive bacterial strains showed no significance (P = 0.381), indicating that these bacteria did not have an active efflux mechanism capable of extruding the household antimicrobials. There was however a significant difference between the

absorbance readings with or without the addition of CCCP in the resistant bacterial strains ($P = 0.000$). This indicates that the resistant bacteria have an active efflux process that is capable of extruding the antimicrobial agents present in household disinfectants.

3.7 Disinfection of Dishcloths

An in-use disinfectant test was used to see the effect of various concentrations of three commonly used household disinfectants on the sensitive and resistant bacteria. 100 % represents the concentration of detergent recommended for use by the manufacturer, 50 % is half the recommended concentration, 25 % is a quarter of the recommended concentration, and 0 % represents a water wash.

3.7.1 Sensitive *Salmonella* and *E. coli* strains

After the cleaning cloths were disinfected with all three household products, there was a dramatic reduction in the number of bacteria remaining on the cloths; this can be seen in Figures 3.22- 3.25 and Figures D1-D6 in Appendix D. The greater the concentration of disinfectant used, the greater the reduction in bacterial numbers. There was however not much difference in the number of remaining bacteria at the different disinfectant concentrations. The water wash (0 %) was also seen to eliminate the bacteria almost as efficiently as the 25 % detergent solution.

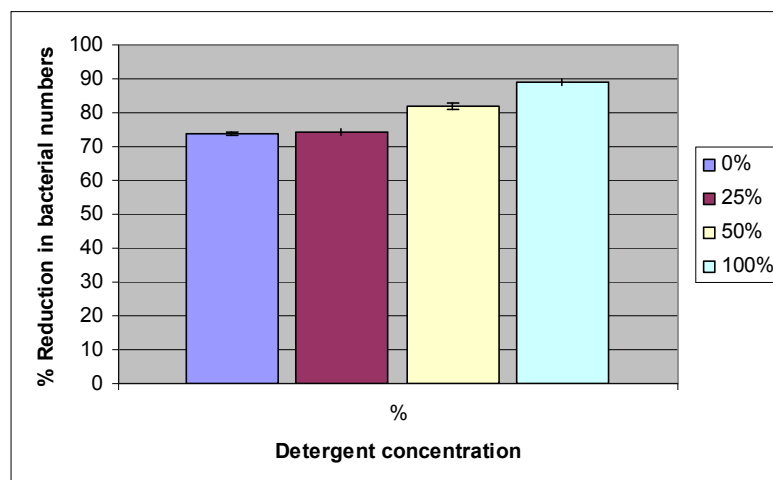


Figure 3.22: Effect of different concentrations of household disinfectant product (containing sodium salicylate) on *Salmonella* # 2 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

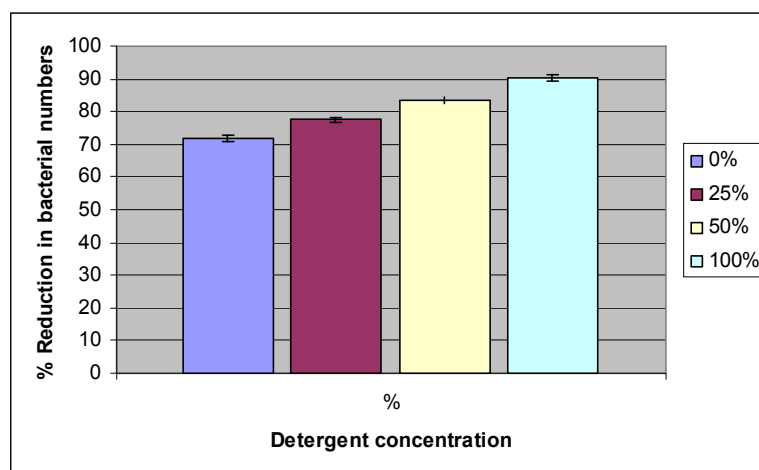


Figure 3.23: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on *Salmonella* # 4 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

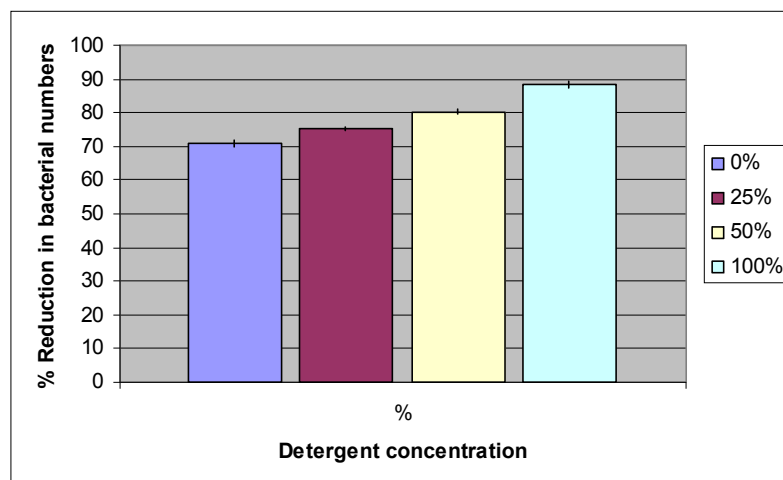


Figure 3.24: Effect of different concentrations of household disinfectant product (containing triclosan) on *E. coli* # 3 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

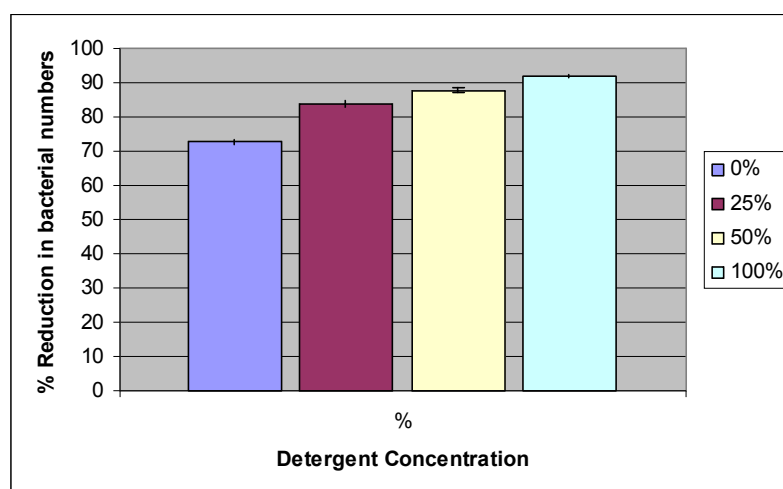


Figure 3.25: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on *E. coli* # 2 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

3.7.2 Resistant *Salmonella* and *E. coli* strains

After the disinfection procedure with all three household products there was a drastic decrease in the number of viable bacteria remaining on the dishcloths. The greater the concentration of disinfectant used, the greater the reduction in bacterial numbers. There was a considerable difference in the number of remaining bacteria at the different concentrations. The water wash (0 %) was also seen to eliminate the bacteria even more efficiently than the 25 % and 50 % detergent solutions. Representative Figures 3.27-3.30 are shown below and Figures D7-D12 in Appendix D.

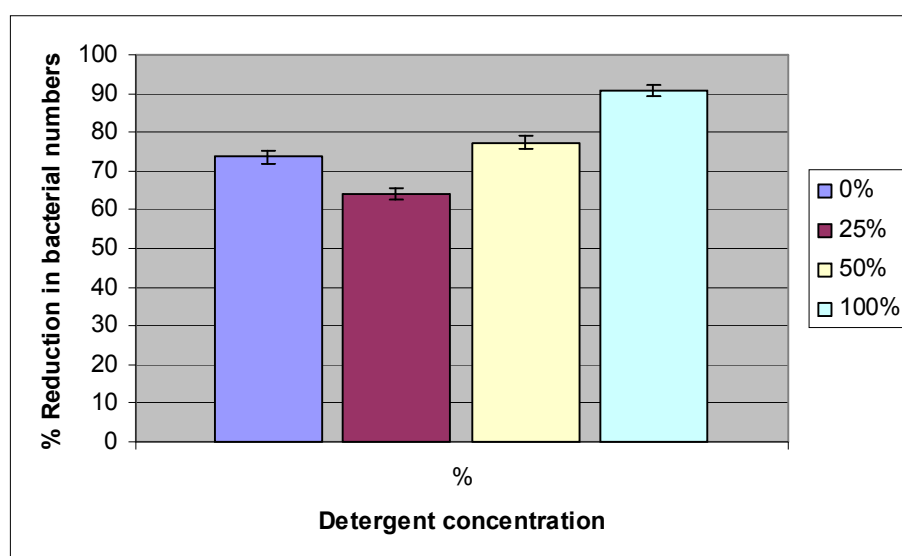


Figure 3.26: Effect of different concentrations of household disinfectant product (containing triclosan) on *Salmonella* # 5 in artificially contaminated dishcloths.

Each error bar represents the standard error of the mean of 2 independent experiments.

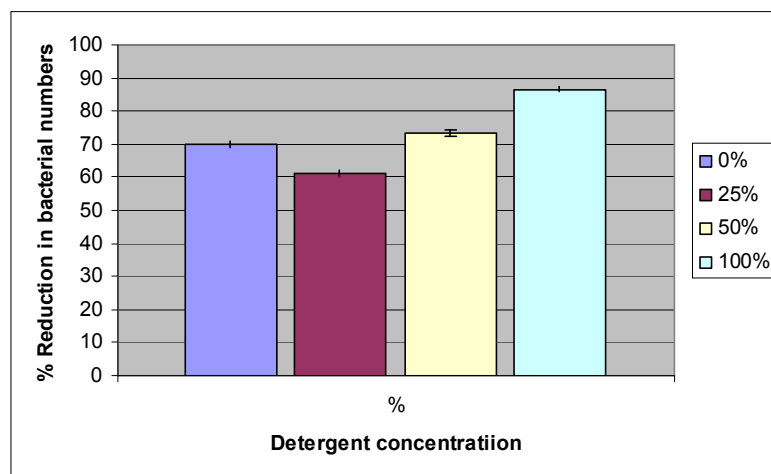


Figure 3.27: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on *Salmonella* # 7 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

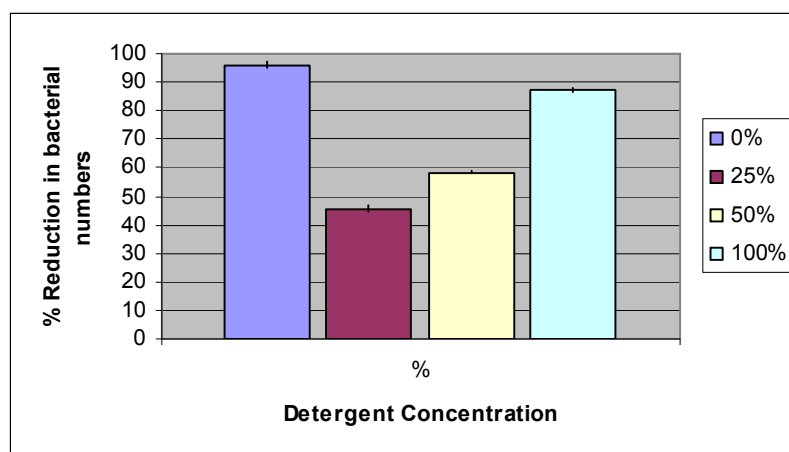


Figure 3.28: Effect of different concentrations of household disinfectant product (containing sodium salicylate) on *E. coli* # 5 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

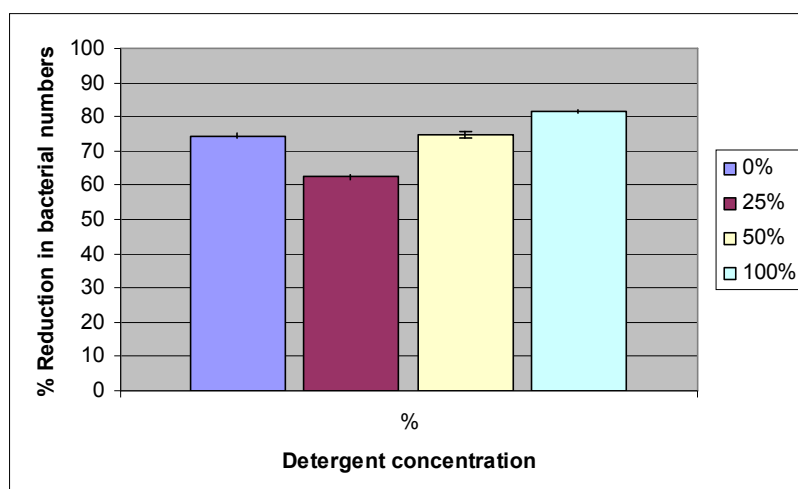


Figure 3.29: Effect of different concentrations of household disinfectant product (containing triclosan) on *E. coli* # 10 in artificially contaminated dishcloths. Each error bar represents the standard error of the mean of 2 independent experiments.

3.7.3 Statistical analysis

Statistical analysis performed on the measurements carried out on different days were not statistically different from each other ($P = 0.955$), indicating reproducibility of the technique.

There was an overall significant difference between the resistant and sensitive strains in terms of susceptibility to the household antimicrobial agents ($P = 0.001$) with the most significant difference being between the sensitive and resistant bacteria at the 25 % disinfection solution ($P = 0.000$). From this it can be concluded that more resistant than sensitive bacteria survive the disinfection procedure, with the highest number of bacteria remaining at the 25 % solution.

3.7.4 Effect of tap water, saline, and distilled water on the bacterial cells

As seen in the results above, the water wash (0 %) eliminated the tetracycline and ofloxacin resistant bacteria more efficiently than the 25 % and 50 % detergent solutions. To establish if this was due to lysis of the cells caused by influx of water, the disinfection procedure was repeated on selected bacteria using distilled water, tap water, and normal saline.

On comparison of the cfu, it was observed that the three different washes yielded very similar results (Table 3.8). These results indicate that lysis of the bacterial cells was due to hypotonicity was not taking place during the rinsing step of the disinfection procedure.

Table 3.8: Number of bacteria (10^{-3} dilution) remaining on the dishcloths after rinsing them with distilled water, tap water, and normal saline.

Bacterial species	Distilled water		Tap water		Normal saline	
<i>Salmonella</i> # 7	69	77	64	80	67	78
<i>E. coli</i> # 9	33	48	38	45	36	42

3.7.5 Suspension tests

The results from the disinfection procedure with the tetracycline and ofloxacin resistant bacteria showed that the water wash eliminated more bacteria than the 25 % and 50 % disinfectant solutions. Suspension tests were performed to determine if this

was a result of an interaction between the cloth portions and any of the ingredients in the disinfectant products.

The results from the suspension tests showed that the 25 % disinfectant solutions eliminated the bacteria more efficiently than the distilled water (Table 3.8). This indicated that an interaction between the cloths and disinfectants was taking place and that the disinfectant solutions were eliminating the bacteria more efficiently than the water wash.

Table 3.9: Initial counts and number of bacteria (10^{-4} dilution) remaining on the dishcloths after the suspension tests.

Bacterial species	Initial count		Distilled water		25 % triclosan	
<i>Salmonella</i> # 6	230	217	146	129	12	10
<i>E. coli</i> # 8	197	183	119	98	9	7

3.8 Trend Summary

	Sensitive <i>Salmonella</i> and <i>E. coli</i>	Resistant <i>Salmonella</i> and <i>E. coli</i>
Ethidium bromide accumulation assay	Fluorescence intensity remained the same or only increased slightly on addition of CCCP, indicating absence of active efflux pumps.	Addition of CCCP resulted in a rapid and dramatic increase in fluorescence, indicating the presence of active efflux pumps.
Chemical accumulation	An increase in absorbance readings in both the presence and absence of CCCP indicated the absence of efflux of the active ingredients in household disinfectants.	Absorbance values in the absence of CCCP remained constant. Absorbance values increased on addition of CCCP indicating efflux pumps capable of extruding the active ingredients in household disinfectants.
Dishcloth Disinfection	Bacteria were eliminated efficiently at disinfectant concentrations of 25, 50, and 100 %	Bacteria were eliminated efficiently at disinfectant concentrations of 50 and 100 %

Even though two different bacterial strains (*Salmonella* and *E. coli*) were examined in this study no differences were observed between the two groups. As such, the organisms were grouped as sensitive or resistant and not according to strain.

In the sensitive and resistant groups, the chemical accumulation and dishcloth disinfection procedures results showed the same trends within the two groups. Representatives from both sensitive and resistant *Salmonella* and *E. coli* were shown in the results section and the remaining results were placed in Appendices C and D.

CHAPTER 4

4 DISCUSSION

The purpose of this study was to determine whether antibiotic resistant enteric bacteria were less susceptible to antimicrobial agents found in commonly used household disinfectant products, due to the action of efflux pumps.

4.1 Selection and Characterization of Microorganisms

Since most cases of infectious intestinal disease occur in the home and are associated with the incorrect storage, preparation, and handling of food items (Gorman et al. 2002), it seemed fitting to use food products as a source of the gastrointestinal pathogens for this study. Chicken products were chosen as many studies have demonstrated that chickens are reservoirs for these potentially pathogenic bacteria: in the United Kingdom 33-41 % of chickens brought into homes are contaminated with *Salmonella* and more than 80 % with *Campylobacter* (Cogan et al. 2002). In a study looking at 99 chicken carcasses in Gauteng, South Africa it was found that 60.6 % of carcasses are contaminated with gastrointestinal pathogens, with a prevalence of 19.2 % for *Salmonella* spp. and 32.3 % for *Campylobacter* spp (van Nierop et al. 2005).

Of the many known gastrointestinal pathogens, *Salmonella* and *E. coli* were chosen because they represent examples of zoonotic pathogens that can exist in both animals

and humans. *Salmonella* spp. can result in diseases in both humans and animals, whereas *E. coli* can exist as normal flora without causing any adverse effects, or can result in disease if they encode virulence factors or if they are found in other sites (Chopra & Roberts 2001).

Tetracycline and ofloxacin were chosen as indicator antibiotics since they have been shown to be associated with the efflux mechanism of resistance (Heisig 1996), with efflux pumps first being identified in tetracycline resistant Gram-negative bacteria (Poole 2000a). *Salmonella* and *E. coli* that were resistant to tetracycline and ofloxacin were chosen to increase the likelihood of isolating bacteria that possessed efflux pumps that could be capable of extruding both antibiotics and household antimicrobials.

Less tetracycline and ofloxacin resistant bacteria were isolated than what was expected from examination of the literature (Manie et al. 1998). This may be a result of *Salmonella* and *E. coli* with the specified resistance profiles not being common in the food items tested. Another possibility was that the microbiological culture techniques used were not sensitive enough to isolate the bacteria or that the bacteria were unculturable due to factors such as cold injury. Since the concentration of antibiotics added to the enrichment broths were low enough to allow for the isolation of bacteria with only intermediate resistance, it is unlikely that the low isolation rates were due to the addition of antibiotics.

To discount the possibility that the microbiological culture techniques used were not sensitive enough, as well as to confirm that the bacteria were correctly identified using microbiological culture techniques, PCR was performed. The amplification reactions, performed on each bacterium isolated using standard microbiological culture techniques, showed that the bacteria were identified correctly. This demonstrates that the correct use of microbiological culture techniques resulted in isolation of the bacteria of interest.

No amplification products were detected when PCR was performed using the enrichment broths from which no bacteria were isolated. This could indicate one of three possibilities:

1. The bacteria of interest were below the detectable limits of the reactions,
2. Inhibition of the reaction occurred due to inhibitors present in the food samples, or
3. Failure of the amplification reactions occurred.

It is unlikely that reaction failure occurred, as the positive controls were amplified in each reaction. In the *Salmonella* PCR, the inhibitor constructs were not amplified, indicating that inhibition was taking place. If this was the case then *Salmonella* could have been present but were just not being amplified. This would indicate that the microbiological culture techniques used were not sensitive enough to isolate the bacteria. No inhibitor constructs were included in the *E. coli* amplification reactions,

thus the negative result could have been due to inhibition of the reactions, or the presence of bacteria below the detection limit in the sample.

Macro-restriction analysis was used to type the bacterial strains. Since discriminatory power and reproducibility are important attributes in any typing system, this method was chosen because its applicability to most of the common bacterial pathogens is well established. This technique is however only reliable if PFGE resolves at least 10 distinct fragments. If fewer fragments are resolved the discriminatory power is unknown (Tenover et al. 1995).

Random genetic events such as point mutations, and insertions and deletions of DNA can alter PFGE patterns. This makes interpretation of the patterns more difficult, but knowledge of genetic events allows one to correctly assign the patterns of the isolates into four categories. These categories are indistinguishable, closely related, possibly related, or unrelated. Isolates are said to be genetically indistinguishable if their restriction patterns have the same number of bands and the corresponding bands are the same size. If there is a two to three band difference isolates are considered to be closely related, whereas four to six band changes results in isolates being considered as possibly related. Seven or more band differences indicates that the strains are unrelated (Tenover et al. 1995).

As seen with the *Salmonella* PFGE (Figure 3.15) there was a difference of 7 or more bands between isolates indicating that they were unrelated. In the *E. coli* PFGE

(Figure 3.16) a 7 or more band difference was seen in most of the isolates. However, a difference of only one band was observed in isolates 6 and 7, resulting in them being genotypically indistinguishable. A four band difference was observed between isolates 1 & 9, 3 & 4 and 6 & 9; these isolates are therefore possibly related.

Even though each of the *E. coli* isolates was obtained from a different sample collected at different time points, some of the samples were from the same abattoir. It is therefore likely that one chicken acquired an *E. coli* strain and clonal expansion took place, resulting in many, if not all the chickens on one farm having the same strain of *E. coli*. The reason for the four band difference in isolates that are probably related can be explained by point mutations that resulted in loss or creation of restriction sites, or insertion or deletion of DNA fragments that occurred over time.

4.2 Non-Specific Ethidium Bromide Accumulation Assays

The accumulation of ethidium bromide was monitored using non-specific accumulation assays. Ethidium bromide was chosen for these assays as it is a common substrate for bacterial efflux pumps (Baranova & Neyfakh 1997) and has been used in several other studies (Lin et al. 2002; Morita et al. 1998). The results from these assays (Figures 3.10 – 3.12) gave evidence for the existence of an active efflux process in the tetracycline and ofloxacin resistant bacterial strains. In the tetracycline and ofloxacin sensitive strains (Figures 3.8 – 3.9) an active efflux process was not observed, in some instances, however, slight efflux was observed. This can

be explained by the fact that antibiotic resistant as well as susceptible bacteria carry and express the genes coding for certain efflux pumps.

In each assay a plateau in the fluorescence values was observed by five minutes after the addition of the ethidium bromide. This was because the amount of ethidium bromide entering the cells was equal to the amount being extruded from the cells. In the resistant bacteria this plateau could have been due to passive diffusion as a result of the concentration gradient or was caused by efflux pumps extruding the ethidium bromide from the cells. Since the plateau in the resistant bacteria was reached at lower concentrations of ethidium bromide than seen with the sensitive bacteria it is probable that this was a result of efflux of the compound. In the sensitive strains where no active efflux was observed efflux could not have been the case. It is therefore likely that in the sensitive bacteria passive diffusion was taking place

Drastic increases in the fluorescence values were observed in the ethidium bromide accumulation assays upon addition of CCCP. This result was not observed when the experiments were carried out using the solvent, DMSO, alone. These results confirmed the fact that the increase in fluorescence was due to the CCCP. Since CCCP is a proton motive force uncoupler these results indicate that ethidium bromide efflux is an active transport mechanism relying on oxidative phosphorylation.

The fact that active efflux was observed in the tetracycline and ofloxacin resistant, but not the sensitive, bacterial strains could imply that the resistance, or

susceptibility, of these bacteria to the antibiotics was due to the efflux process observed in the ethidium bromide accumulation assays. This was however not investigated and resistance can be attributed to any one of the many different resistance mechanisms present in these bacteria. The only conclusion that could be drawn from the ethidium bromide accumulation studies was that the resistant bacteria had an active efflux process and that the sensitive bacteria lacked, or had an insignificant efflux process. In order to determine if the antibiotic resistance was attributable to an efflux process, time course tetracycline and ofloxacin uptake experiments could have been performed. These experiments were not done in this study, as tetracycline and ofloxacin resistant bacteria were simply selected as indicator organisms, based on association of these antibiotics with efflux resistance mechanisms.

4.3 Specific Antimicrobial Efflux Assays

Time course sodium salicylate, triclosan, and *ortho*-phenylphenol uptake experiments were performed with the tetracycline and ofloxacin resistant as well as sensitive bacteria. In the sensitive bacteria, with all three chemicals, the absorbance values without and with CCCP increased consistently over time with not much difference noted between the two, even after the addition of CCCP.

In the resistant bacteria, the absorbance readings without CCCP remained constant, whilst there was a notable increase in the absorbance readings after the addition of

CCCP. The constant absorbance values in the controls can be explained by the fact that a level of equilibrium had been reached where the amount of chemical entering the cell was equal to the amount being extruded by the efflux process. After the addition of CCCP the efflux pumps were inactivated resulting in accumulation of the chemicals within the cells. This resulted in greater cell-associated chemical resulting in increasing absorbance readings. These results provide evidence of a lack of an antimicrobial specific active efflux process in the tetracycline and ofloxacin sensitive strains and the presence of such a process in the resistant strains. Since CCCP is a proton motive force uncoupler these results also confirm that the efflux process in the resistant bacterial cells is a proton motive force-dependent process.

4.4 In-use Disinfection Studies

Cleaning cloths and sponges play a very important role in domestic hygiene as they aid in the detachment of microbes and soil present on the surface (Humphrey 2001). Nevertheless, the fact that they frequently remain wet for long periods of time, contain residual amounts of soil, and are not changed frequently, they provide ideal conditions for survival and/or growth of microorganisms (Scott & Bloomfield 1990a). Studies have demonstrated that if cloths are not properly disinfected they can transfer bacteria to clean surfaces in the kitchen or people's hands, resulting in diseases (Rusin et al. 1998). In an effort to combat this problem, household disinfectant products were manufactured.

Several brands of household disinfectant products are distinct from regular products due to the addition of one or more antibacterial compounds, and it is claimed that these products reduce or inactivate bacteria more efficiently than the regular products. The efficacy of these products is typically tested using standard suspension tests, where the established requirement for disinfectants is a log reduction equal or greater than 5 ($\geq 99.999\%$ reduction) during 5 minutes of contact time (Kusumaningrum et al. 2002). Even though efficacy testing was not the purpose of this study, one of the objectives was to see if the commonly used household disinfectant products had any effect on the survival of the bacteria in question in a simulated in-use situation, and if so, whether there were any differences between the sensitive and resistant bacterial strains.

Since the outcome of suspension tests might be a poor predictor for the efficacy under practical conditions, especially when bacteria are attached to surfaces and in the presence of food debris, a variety of test procedures have been developed to mimic these conditions (Kusumaningrum et al. 2002). One variant of these procedures is to contaminate a test surface with a standardized inoculum followed by distribution of the disinfectant solution and determination of the survivors after a given exposure time and rinsing technique (Scott & Bloomfield 1990a). An adaptation of this procedure was used in this study.

For the in-use disinfectant procedure, different concentrations of the products were tested, these being the concentration recommended by the manufacturer, as well as

50 % and 25 % of the recommended concentration. The rationale for using the disinfectant at concentrations other than that recommended was that many people do not adhere to the instructions on the products and often dilute household products to extend the period of use. This is of particular concern when the small-print on disinfectant products is that a 100 % (neat) application of the product is required for anti-germicidal activity, but the product needs to be used with water for cleaning.

As expected during the in-use disinfectant testing, the greater the concentration of disinfectant used, the greater the reduction in bacterial numbers. When the disinfectant products were used at concentrations lower than the recommended concentration it resulted in many bacteria remaining in the dishcloths after the disinfection procedure, especially the 25 % disinfection solutions. This effect was more pronounced in the resistant bacteria. Since these bacteria have an active efflux process, are being subjected to low concentrations of antimicrobial agents, and have been demonstrated (Section 3.6.2) to extrude these agents, it is likely that the bacteria could develop resistance to the antimicrobial agents. It is also possible that these bacteria could be selected for in an environment where there are sufficient antimicrobials to eliminate sensitive bacteria, but insufficient to reduce the less susceptible strains.

Another observation was that merely rinsing the cloth portions containing the tetracycline and ofloxacin resistant bacteria in running tap water reduced the bacterial numbers on the cloths more than the 25 % and 50 % disinfectant solutions.

This effect could have been caused by a number of factors. One being that the water resulted in lysis of the bacterial cells. This is because the water was hypotonic in comparison to the cells and would have moved into the cells via the process of osmosis (Solomon et al. 1996). If lysis was taking place, the reduction in bacterial numbers would have been even more when the distilled water was used, as it was the most hypotonic. With the normal saline the reduction should have been the least because it was the least hypotonic (saline is regarded as isotonic with respect to many biological fluids), meaning that osmosis would not have taken place (Solomon et al. 1996). When the results of all three rinses were compared the results were found to be similar (Table 3.7). This indicates that the water wash was not causing lysis of the bacterial cells.

The other possibility examined was that there was some kind of interaction taking place between the cloths and one or more of the ingredients in the disinfectant products. This interaction could have caused the bacteria to be trapped on the cloths, and would not have been rinsed off the cloths during the rinsing step. Since the cloth portions that were rinsed with tap water did not have this interaction, the bacteria were not trapped and were easily rinsed off the cloths. This would account for the greater reduction in bacterial numbers when rinsed with tap water.

To investigate this, suspension tests were carried out. The suspension tests were similar to the disinfection procedure, with the only difference being the absence of dishcloths. When these results were compared, the 25 % disinfectant solution resulted

in a greater reduction in bacterial numbers than the water on its own (Table 3.8). This indicated that the disinfectant eliminated the bacteria more efficiently than the water wash. Thus the results observed in the in-use disinfection procedure may be related to the bacteria being trapped on the cloth portions due to an interaction between the cloths and ingredients in the disinfectant solutions.

4.5 Possible Identities of the Efflux Pumps

Based on the literature it is known that Gram-negative bacteria possess efflux pumps that fall into the MFS, RND, SMR, and MATE families (Yang et al. 2003). In *Salmonella* species, efflux of quinolones has been shown to be attributed to the transporters AcrA and AcrB which belong to the MFS and RND families, respectively (Poole 2000a). The genes involved in the extrusion of tetracyclines are however only found in the MFS (Chopra & Roberts 2001; Poole 2000a). Since the *Salmonella* species used in this study were only resistant to tetracycline it is probable that the efflux activity observed in the ethidium bromide and chemical accumulation assays can be attributed to efflux pumps belonging to the MFS.

In *E. coli*, efflux pumps that extrude quinolones have been shown to belong to three different superfamilies, namely AcrAB, MdfA, and YdhE. AcrAB is a member of the RND family, MdfA belongs to the MFS, and YdhE is found in the MATE family of efflux pumps (Yang et al. 2003). Tetracycline efflux known to be a result of pumps found in the MFS family (Chopra & Roberts 2001; Poole 2000a). The *E. coli* strains

examined in this study were resistant to both tetracycline and ofloxacin. The efflux pumps could therefore belong to the RND, MFS, or MATE families.

The grouping of efflux pumps observed in this study into one or more of the above mentioned superfamilies is however only a postulate based on the literature, and further analyses would be required to conclusively identify them. It is important to note that tetracycline and ofloxacin resistant bacteria were only selected as indicator organisms, based on association of these antibiotics with efflux resistance mechanisms, and it is unknown if the resistance profiles of the bacteria studied could be attributed solely to the efflux process. Changes in antibiotic target or production of hydrolytic enzymes could also have occurred, but this was not investigated.

CHAPTER 5

5 CONCLUSION

The use of subtherapeutic doses of antibiotics during the rearing of livestock can promote bacterial resistance by eliminating the less resilient microorganisms normally present in animals, leaving an environment in which drug-resistant strains can thrive. If in addition to the antibiotic resistance, these strains are also resistant to household antimicrobial agents, they would be able to survive the disinfection procedures that take place in kitchens and could be transferred to humans. If this occurred these bacteria could result in food-borne infections that do not respond to treatment. For this reason it is important to determine if antibiotic resistant organisms from livestock have developed resistance to household antimicrobials.

This study showed that poultry samples available in Gauteng, whether they were from supermarkets or obtained directly from the abattoirs, are contaminated with tetracycline and ofloxacin resistant *Salmonella* and *E. coli* species. These resistant bacteria were observed to have active efflux mechanisms, and these mechanisms were capable of extruding sodium salicylate, triclosan, and *ortho*-phenylphenol.

This however did not lead to biocide resistance in an in-use situation if household disinfection products were used as directed by the manufacturer. If the cleaning agents were diluted, they were less effective resulting in antibiotic as well as biocide

resistant bacteria remaining in the dishcloths. These resistant bacteria could then be potentially transmitted to humans. If the occupants of a household are healthy individuals this would perhaps not be a problem, but since many South Africans are immuno-compromised due to HIV/AIDS, and are receiving home-based medical care, this could be of significant importance.

5.1 Future Work

In order to further this work it could be established whether or not the tetracycline and fluoroquinolone resistance was due to the efflux resistance mechanism. This can be determined by carrying out chemical accumulation assays as described above with the relevant antibiotic instead of the antimicrobial agent. If the resistance is found to be attributed to this resistance mechanism, the family to which the efflux pumps belong could then be determined (i.e. MFS, RND, SMR, MATE, and ATP-binding cassette transporter families). Identification of the family to which the efflux pumps belong can be established using PCR and knockout experiments. In order to determine the effect of the antimicrobial agents on the organisms in the domestic setting, a kitchen surface/cleaning cloth could be seeded with the organisms, disinfected using the products as recommended by the manufacturers, and the number of viable resistant bacteria calculated.

CHAPTER 6

6 APPENDICES

6.1 Appendix A: Media and Reagents

Buffered Peptone Water	g/litre
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5
pH 7.2 ± 0.2	

Selenite Cystine Broth	g/litre
Tryptone	5.0
Lactose	4.0
Disodium phosphate	10.0
L-Cystine	0.01
pH 7.0 ± 0.2	

Rappaport-Vassiliadis Broth	g/litre
Soya peptone	5.0
Sodium chloride	8.0
Potassium dihydrogen phosphate	1.6
Magnesium chloride 6 H ₂ O	40.0
Malachite green	0.04
pH 5.2 ± 0.2	

Novobiocin Stock Solution	
Novobiocin powder	20 mg
Sterile distilled water	10 ml

Xylose Lysine Desoxycholate Agar

	g/litre
Yeast extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5
pH 7.4 ± 0.2	

Mannitol Lysine Crystal Violet Brilliant Green Agar

	g/litre
Yeast extract	5.0
Peptone	10.0
'Lab-Lemoc' powder	2.0
Sodium chloride	4.0
Mannitol	3.0
L-lysine hydrochloride	5.0
Sodium thiosulphate	4.0
Ferric ammonium citrate	1.0
Brilliant green	0.0125
Crystal violet	0.01
Agar	15.0
pH 6.8 ± 0.2	

Singers Broth

Tryptone	15 g
Sodium chloride	5 g
Lactose	10 g
Distilled water	1000 ml
pH 7.3	
Singers indicator	26.5 ml

Blood Agar 5 %

	g/litre
Columbia agar	39

MacConkey Agar	g/litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH 7.4 ± 0.2	
Normal saline	g/litre
Sodium chloride	8.5
Mueller-Hinton Agar	g/litre
Beef, dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
7.4 ± 0.2	
Phosphate Buffered Saline	g/litre
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2
pH 7.2	
2 % Agarose	40 ml
Agarose	0.4 g
1X TAE buffer	40 ml
Ethidium bromide	1.5 µl
Luria Bertani Broth	g/litre
Tryptone	5.0
Yeast extract	10.0
Sodium chloride	5.0
TE buffer	
EDTA	1 mM
Tris-HCl pH 8.0	10 mM

Lysis buffer

EDTA	0.1 M
Tris-HCl pH 8.0	10 mM
Sarcosyl	1 %

Storage buffer

EDTA	10 mM
Tris-HCl pH 8.0	10 mM

PMSF

	400 mM
PMSF	0.7 g
Ethanol	10 ml

1 M Tris (pH 8.0)

Tris	24.2 g
HCl	± 8.4 ml

10 X TBE

	400 ml
Tris	43.2 g
Borate	22 g
0.5 M EDTA pH 8.0	16 ml

Ringer Solution – ¼ strength

	g/litre
Sodium chloride	2.25
Potassium chloride	0.105
Calcium chloride 6 H ₂ O	0.12
Sodium bicarbonate	0.05
pH 7.0	

Nutrient Agar

	g/litre
‘Lab-Lemco’ powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2	

6.2 Appendix B: Microbiological Procedures for the Identification of Unknown Bacterial Isolates

6.2.1 Rapid Indole Test

A piece of filter paper (Whatman no.1) was placed into a sterile Petri dish and saturated with indole reagent. From an overnight culture grown on a ½ blood ½ MacConkey agar plate, well-isolated colonies from the blood agar portion were selected for testing. Using a sterile loop or wooden applicator stick, a portion of the colony was rubbed onto the filter paper, and the colour changes that occurred within 30 seconds were observed.

A positive reaction was indicated by a blue to blue-green colour (within 30 sec), and a negative reaction was a colourless or light pink colour.

6.3 Appendix C: Chemical Accumulation Assay Graphs

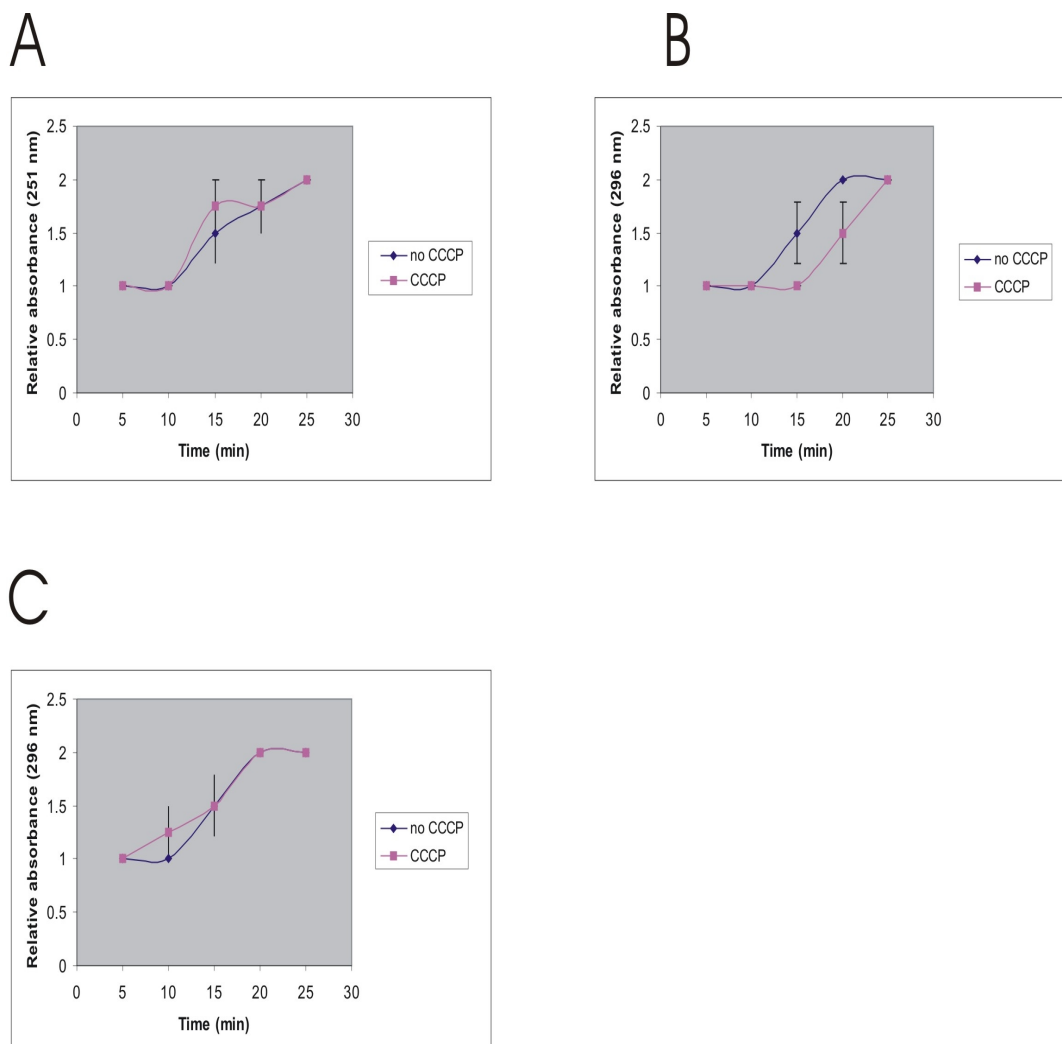


Figure C1: Graph of accumulation of sodium salicylate by tetracycline and ofloxacin sensitive *Salmonella* species. (A) *Salmonella* # 1, (B) *Salmonella* # 2 and (C) *Salmonella* # 4. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

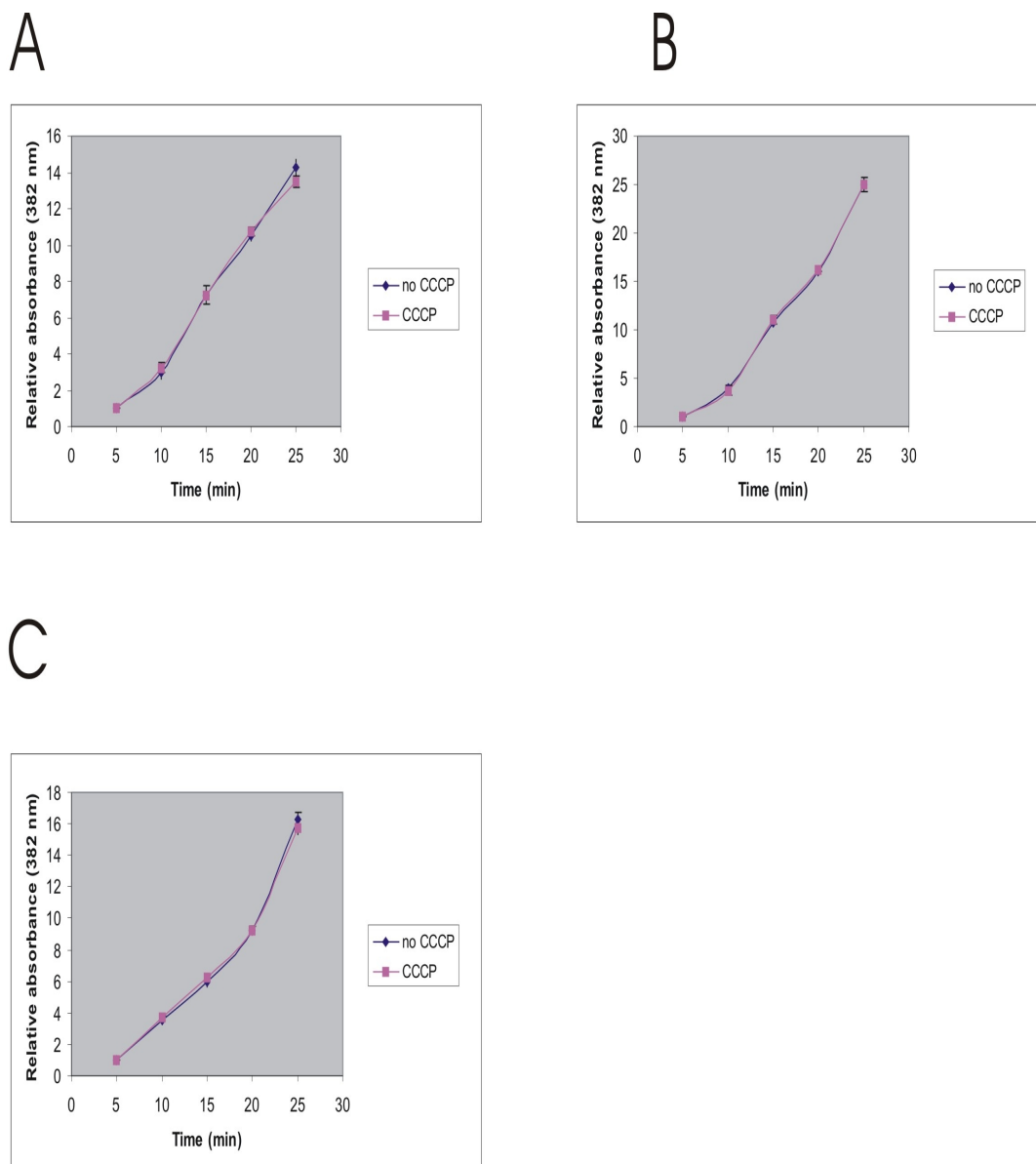


Figure C2: Graph of accumulation of triclosan by tetracycline and ofloxacin sensitive *Salmonella* species. (A) *Salmonella* # 1, (B) *Salmonella* # 3, and (C) *Salmonella* # 4. Absorbance on the y axis represents the levels of accumulated triclosan in the bacterial cells. Each data point represents the mean $\pm$ standard error off the mean of 2 independent experiments.

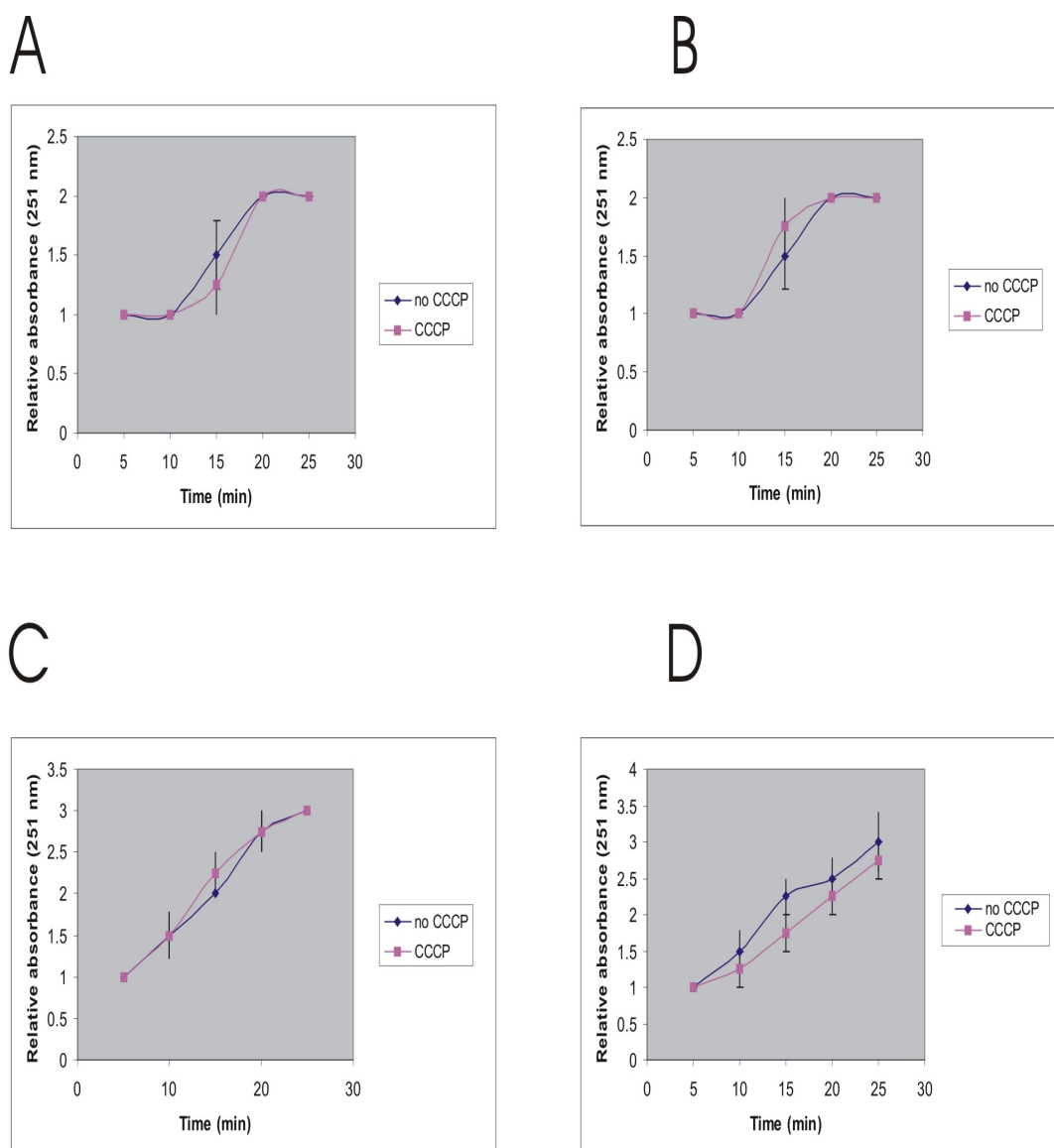


Figure C3: Graph of accumulation of *ortho*-phenylphenol by tetracycline and ofloxacin sensitive *Salmonella* species. (A) *Salmonella* # 1, (B) *Salmonella* # 2, (C) *Salmonella* # 3, and (D) *Salmonella* # 4. Absorbance on the y axis represents the levels of accumulated *ortho*-phenylphenol in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

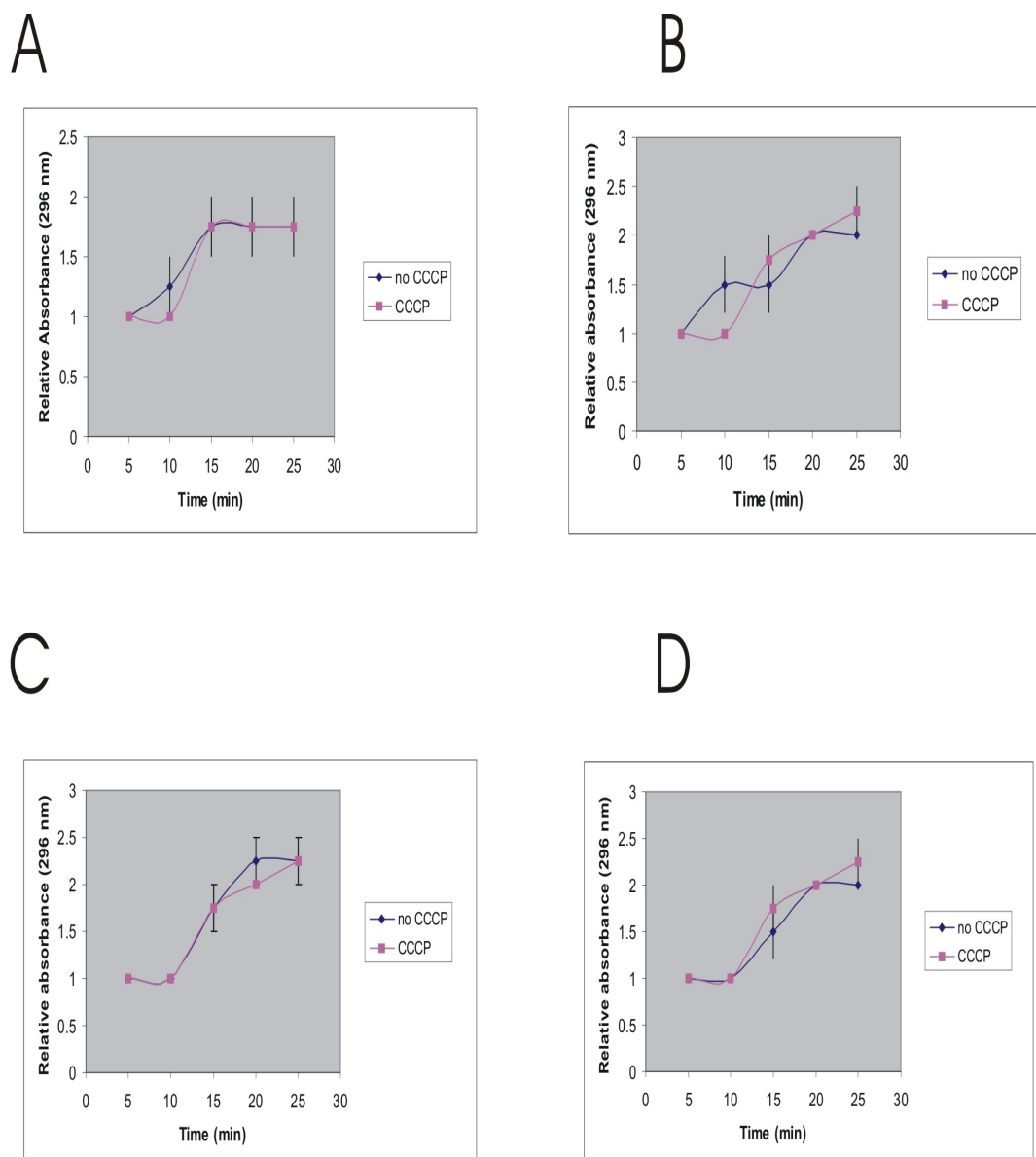


Figure C4: Graph of accumulation of sodium salicylate by tetracycline and ofloxacin sensitive *E. coli* species. (A) *E. coli* # 1, (B) *E. coli* # 2, (C) *E. coli* # 3, and (D) *E. coli* # 4. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

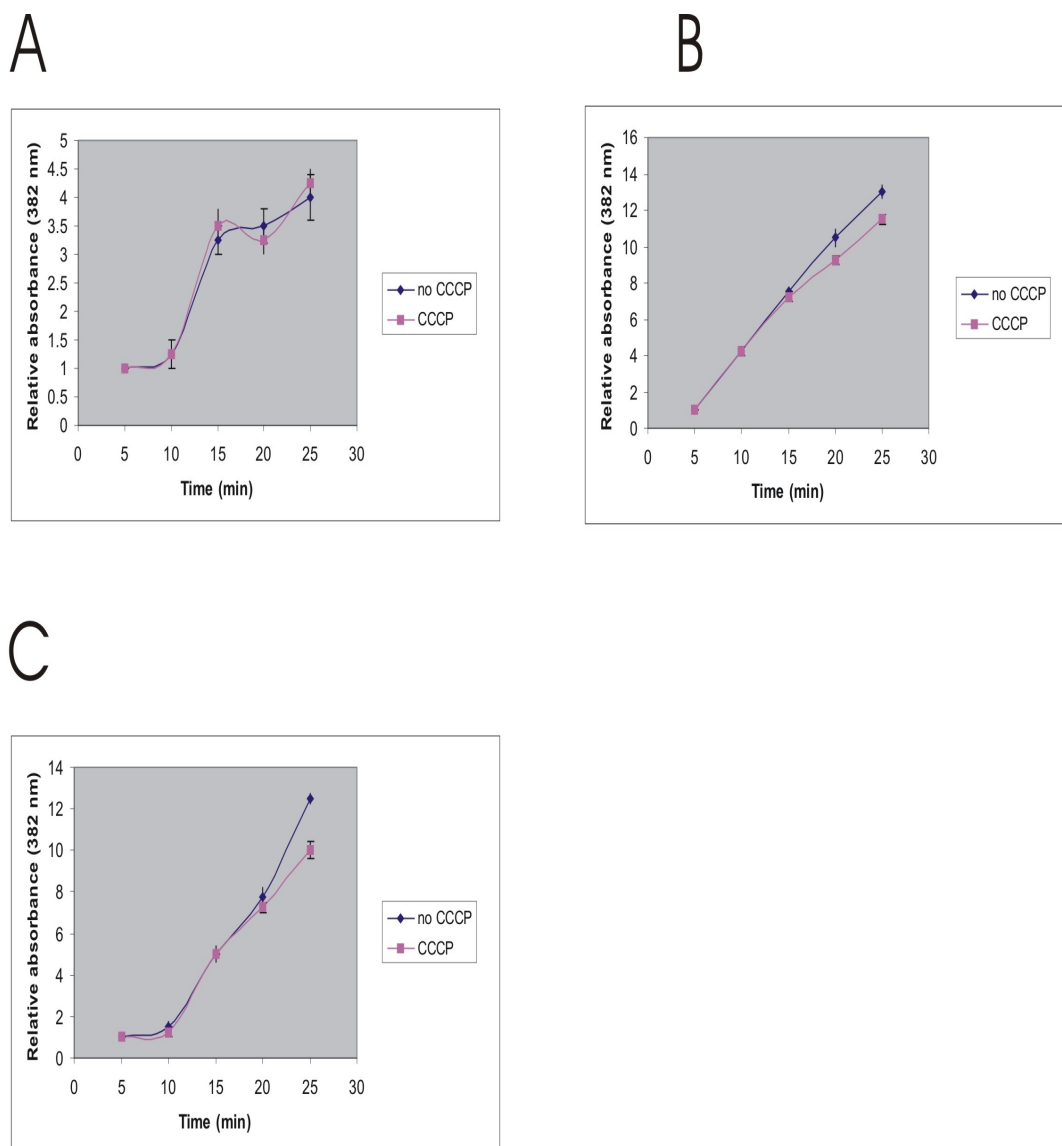


Figure C5: Graph of accumulation of triclosan by tetracycline and ofloxacin sensitive *E. coli* species. (A) *E. coli* # 1, (B) *E. coli* # 3 and (C) *E. coli* # 4. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

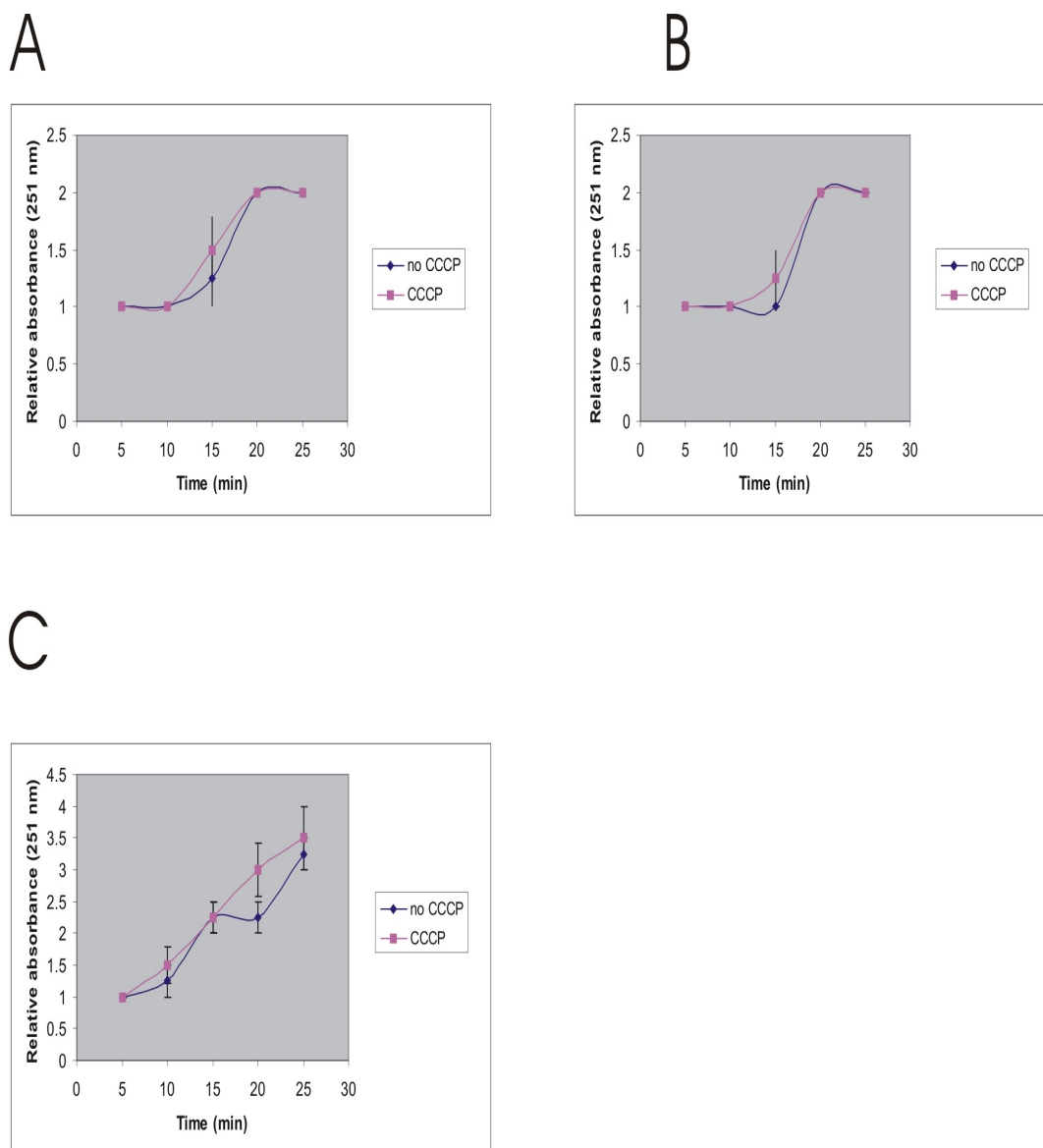


Figure C6: Graph of accumulation of *ortho*-phenylphenol by tetracycline and ofloxacin sensitive *E. coli* species. (A) *E. coli* # 1, (B) *E. coli* # 2 and (C) *E. coli* # 3.

Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

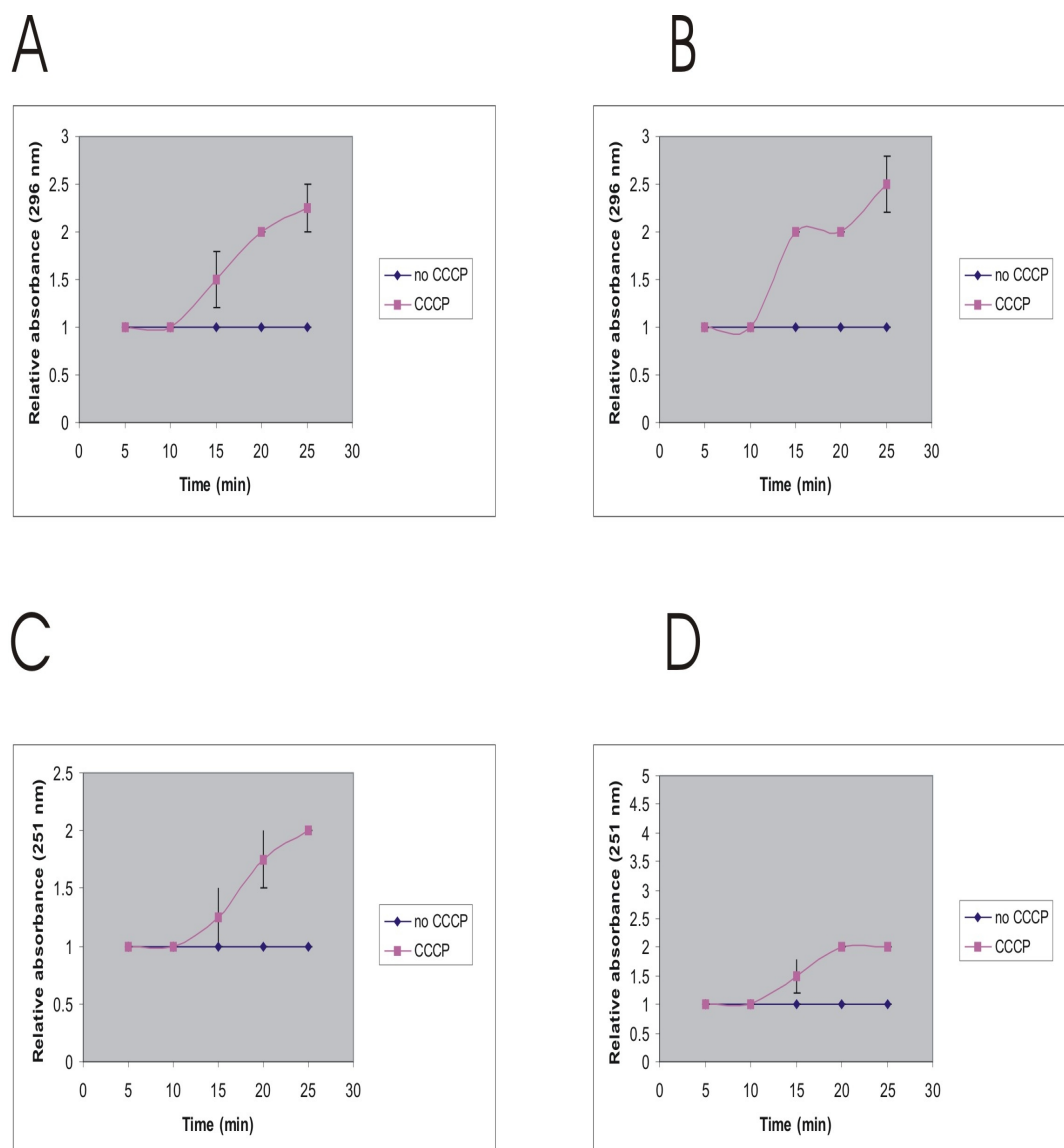


Figure C7: Graph of accumulation of sodium salicylate by tetracycline and ofloxacin resistant *Salmonella* species. (A) *Salmonella* # 5, (B) *Salmonella* # 6, (C) *Salmonella* # 7 and (D) *Salmonella* # 8. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

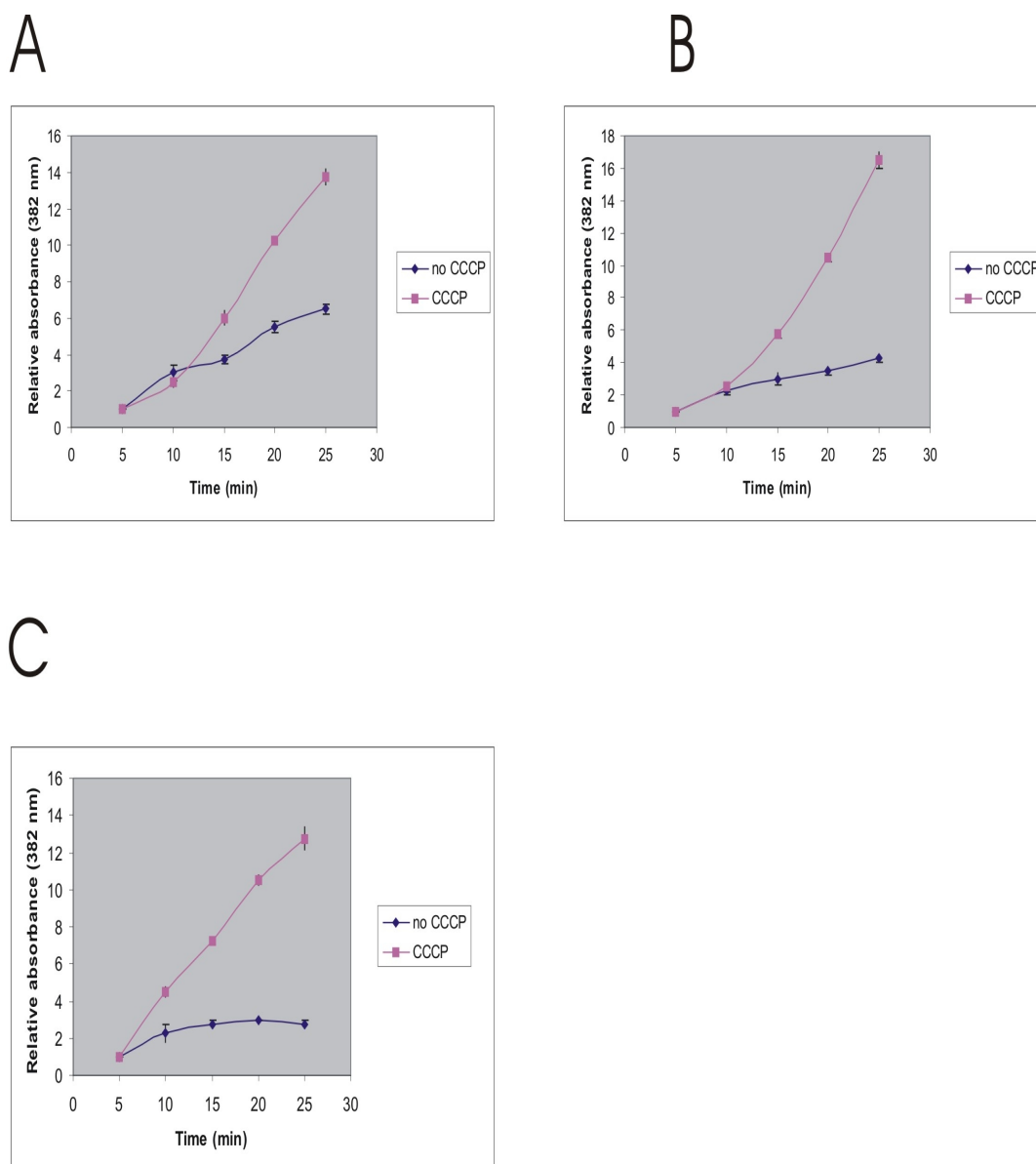


Figure C8: Graph of accumulation of triclosan by tetracycline and ofloxacin resistant *Salmonella* species. (A) *Salmonella* # 5, (B) *Salmonella* # 6, and (C) *Salmonella* # 7. Absorbance on the y axis represents the levels of accumulated triclosan in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

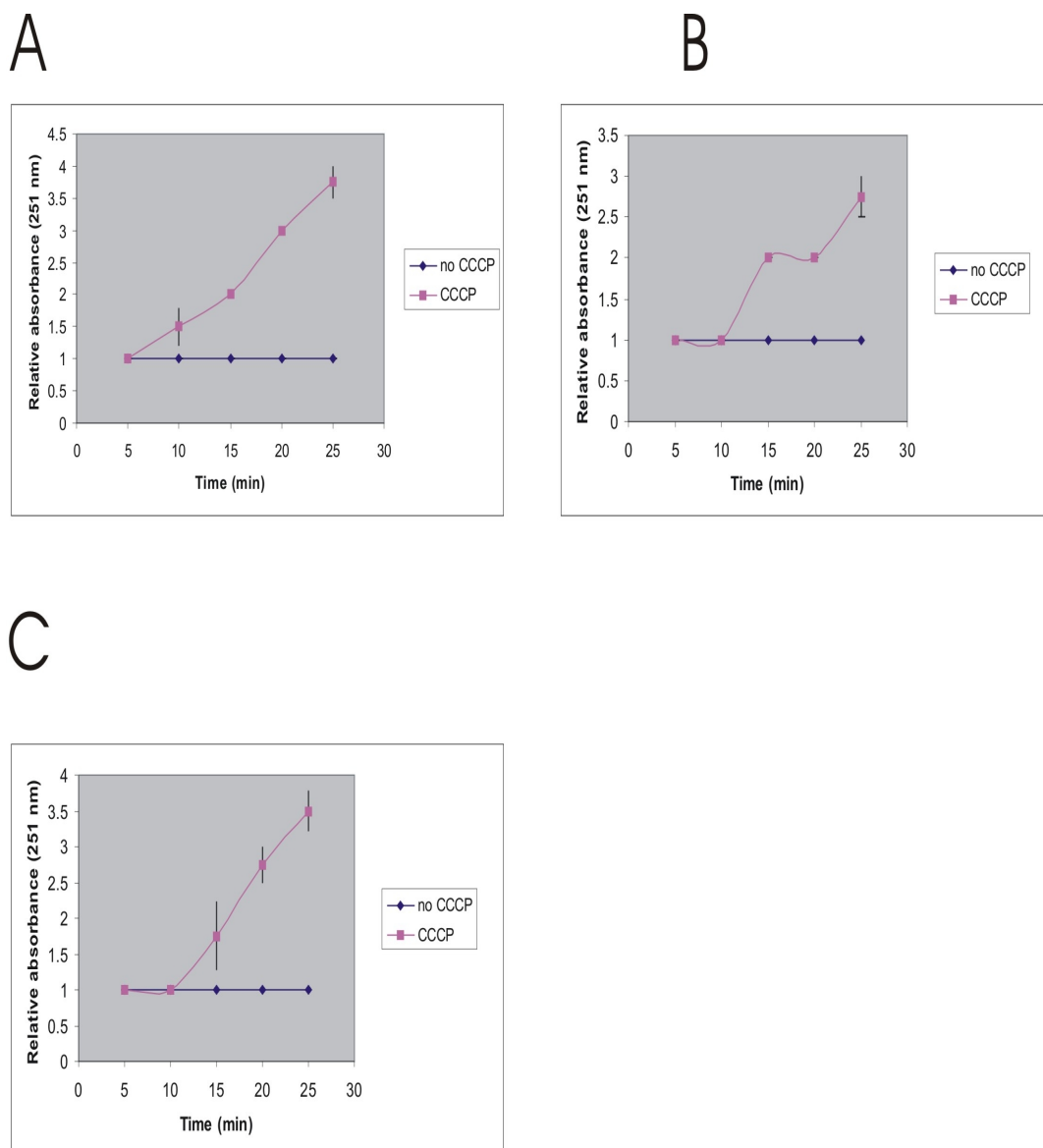


Figure C9: Graph of accumulation of *ortho*-phenylphenol by tetracycline and ofloxacin resistant *Salmonella* species. (A) *Salmonella* # 5, (B) *Salmonella* # 7, and (C) *Salmonella* # 8. Absorbance on the y axis represents the levels of accumulated *ortho*-phenylphenol in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

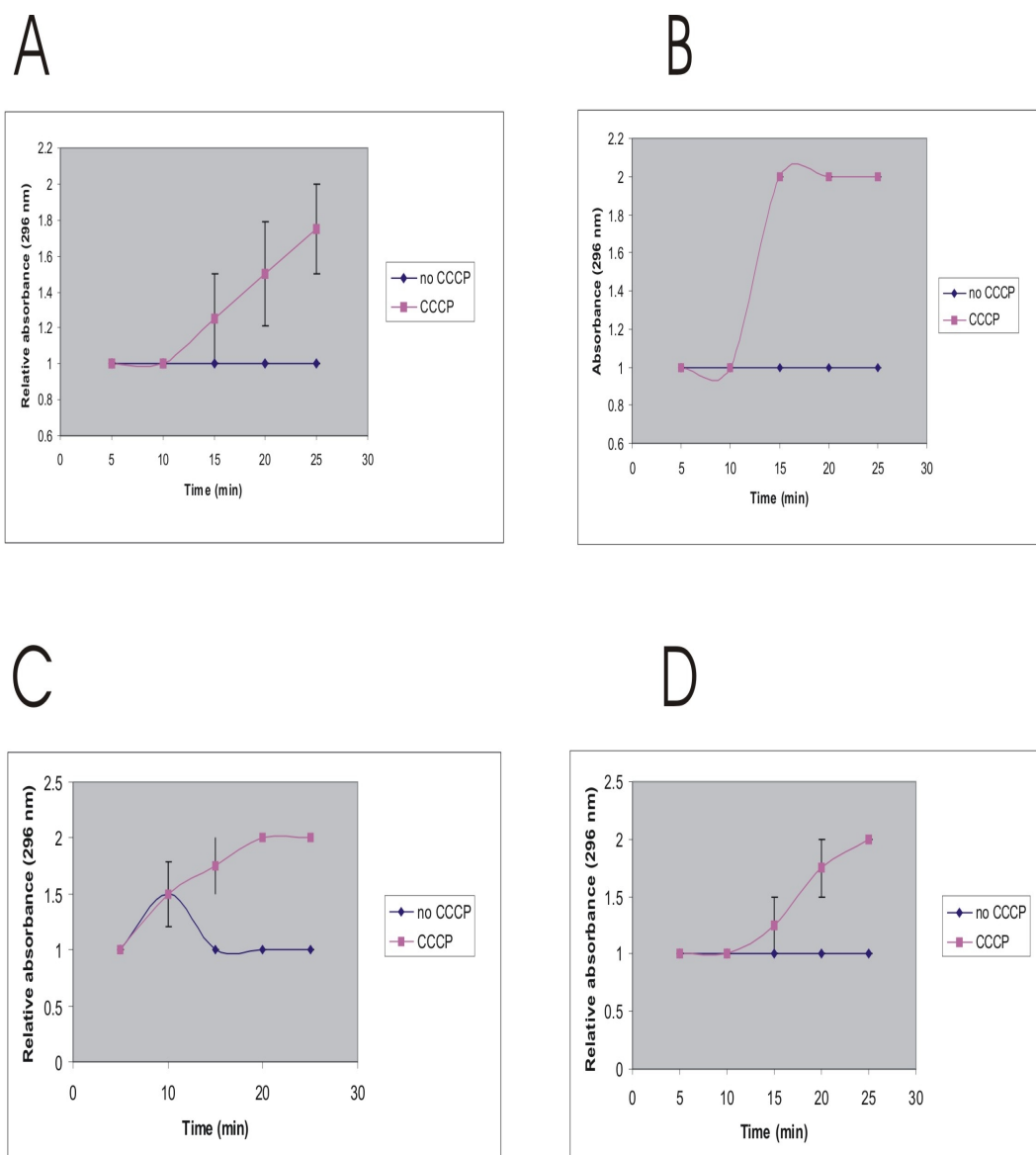


Figure C10: Graph of accumulation of sodium salicylate by tetracycline and ofloxacin resistant *E. coli* species. (A) *E. coli* # 5, (B) *E. coli* # 6, (C) *E. coli* # 8, and (D) *E. coli* # 9. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

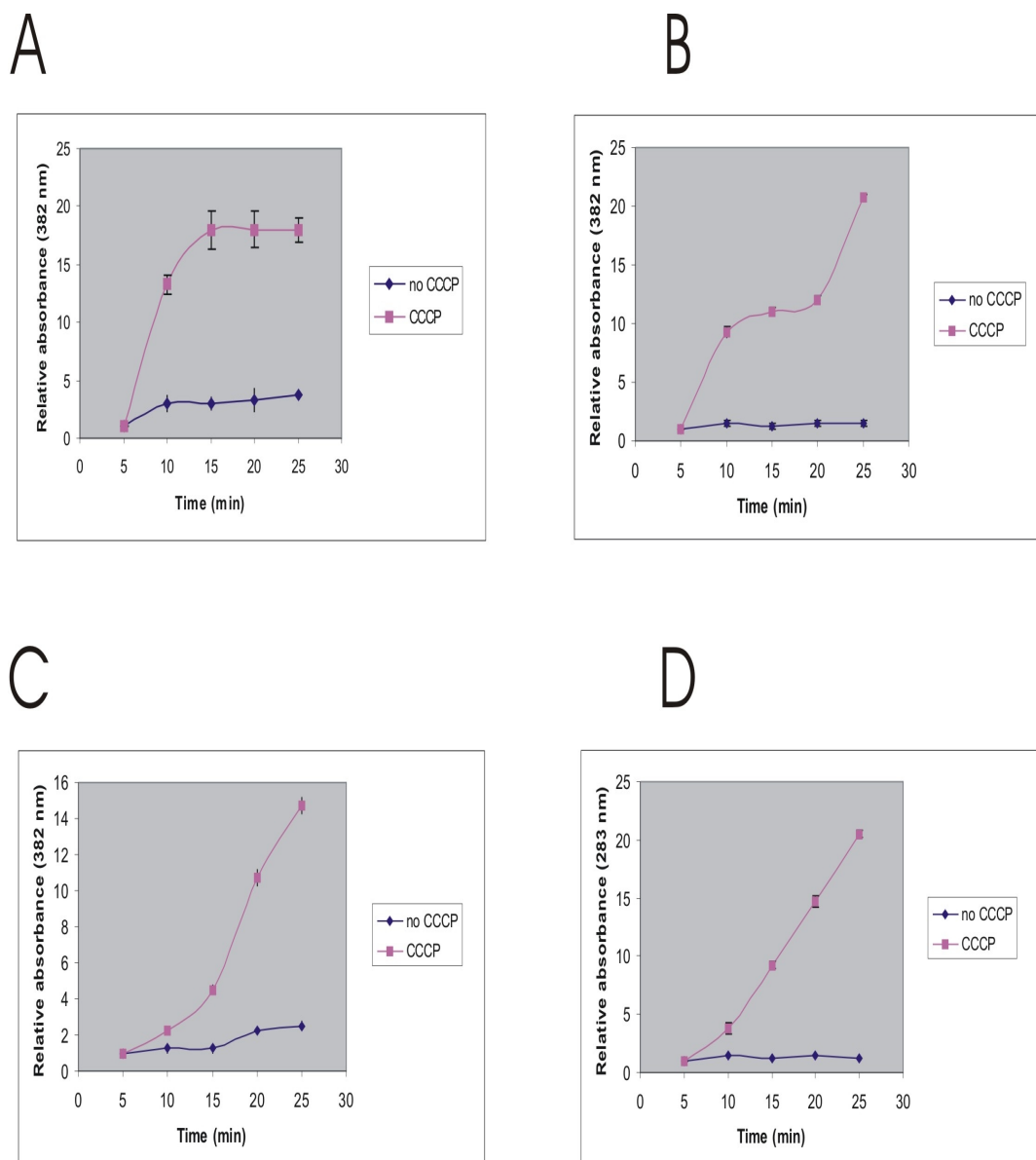


Figure C11: Graph of accumulation of triclosan by tetracycline and ofloxacin resistant *E. coli* species. (A) *E. coli* # 5, (B) *E. coli* # 8, (C) *E. coli* # 9, and (D) *E. coli* # 10. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

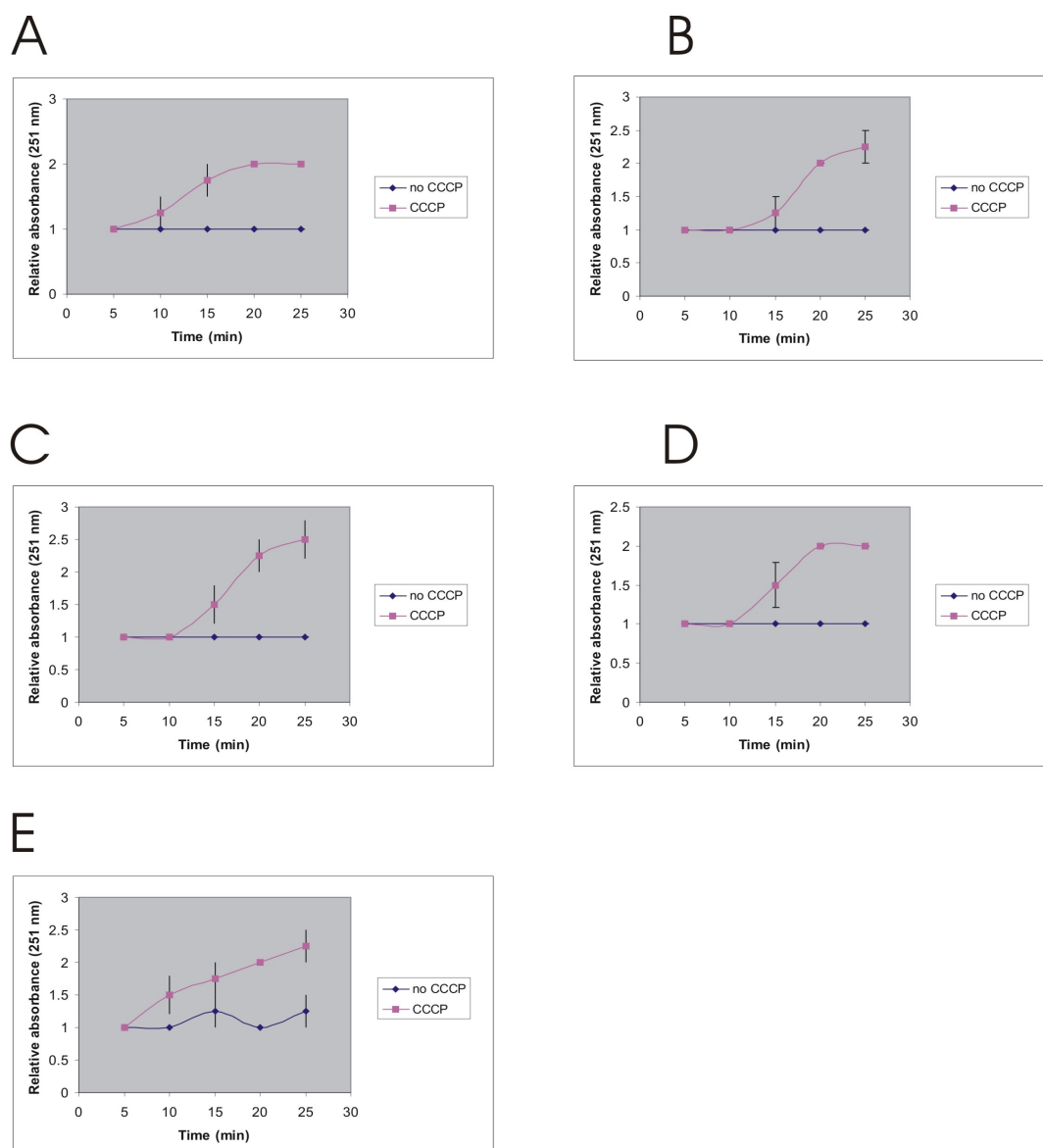


Figure C12: Graph of accumulation of *ortho*-phenylphenol by tetracycline and ofloxacin resistant *E. coli* species. (A) *E. coli* # 5, (B) *E. coli* # 6, (C) *E. coli* # 8, (D) *E. coli* # 9, and (E) *E. coli* # 10. Absorbance on the y axis represents the levels of accumulated sodium salicylate in the bacterial cells. Each data point represents the mean $\pm$ standard error of the mean of 2 independent experiments.

6.4 Appendix D: In-use Disinfectant Testing Graphs

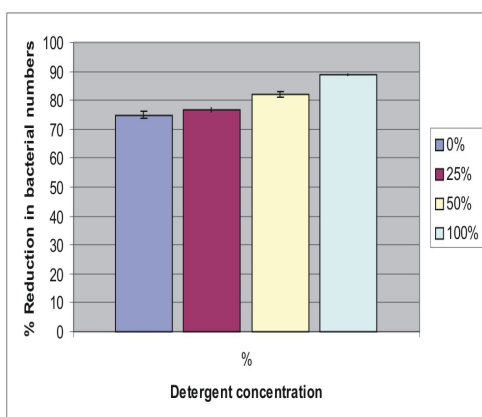
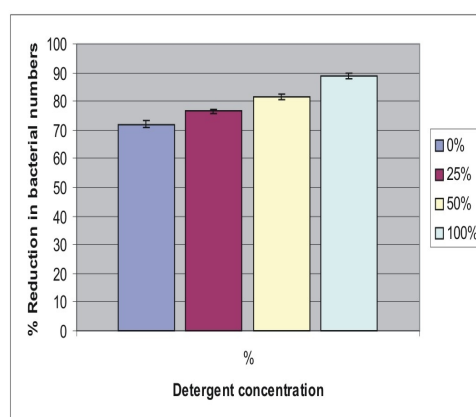
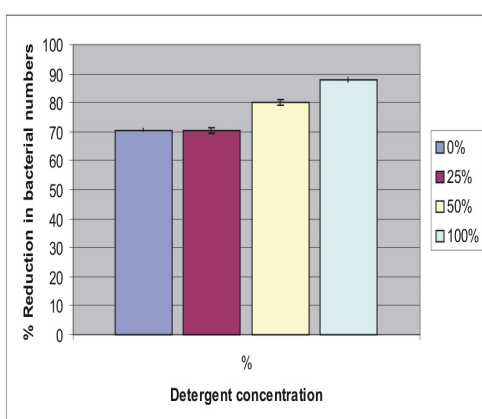
A**B****C**

Figure D1: Effect of different concentrations of household disinfectant product (containing sodium salicylate) on tetracycline and ofloxacin sensitive *Salmonella* species in artificially contaminated dishcloths. (A) *Salmonella* # 1, (B) *Salmonella* # 3, and (C) *Salmonella* # 4. Each error bar represents the standard error of the mean of 2 independent experiments.

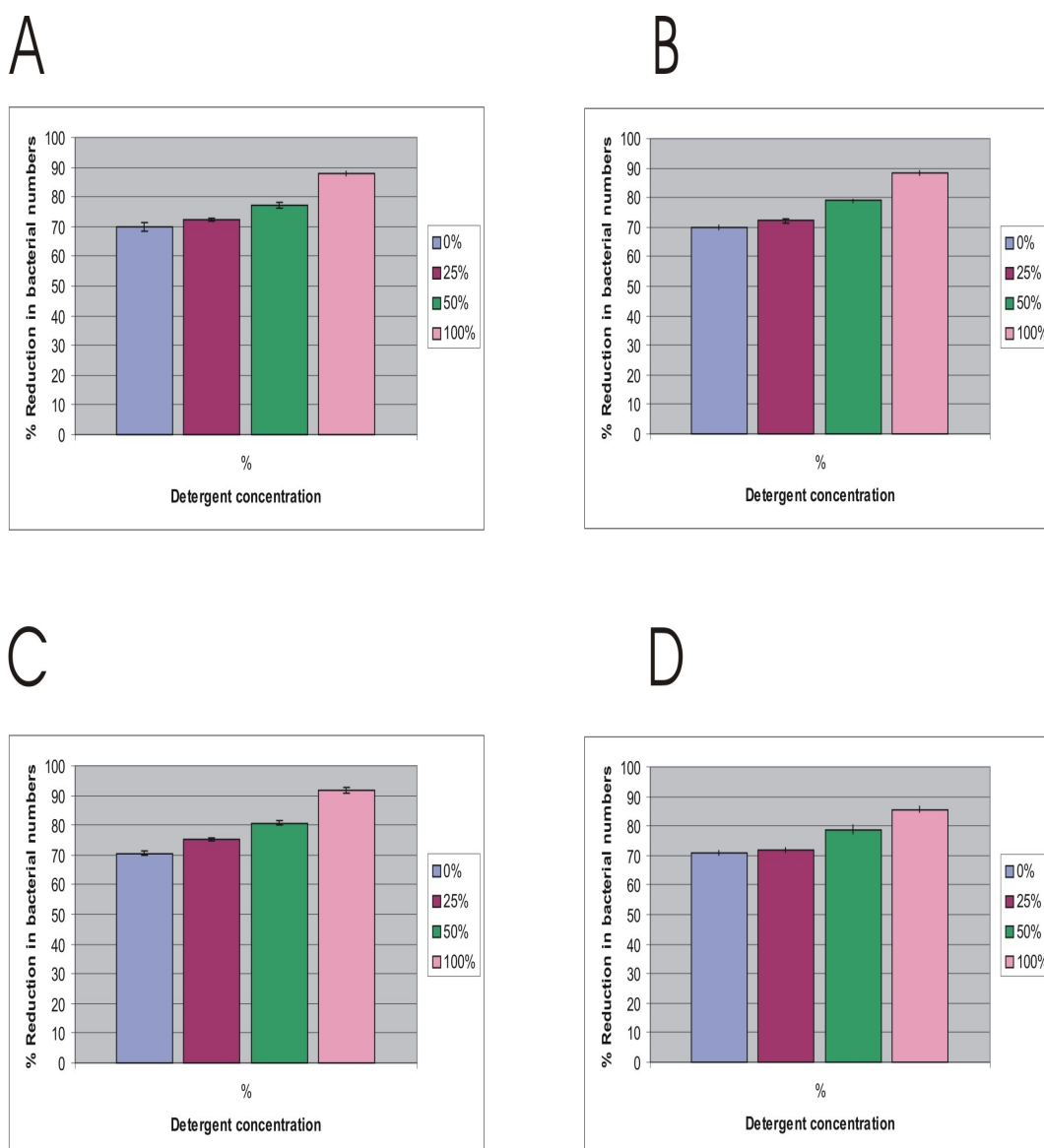


Figure D2: Effect of different concentrations of household disinfectant product (containing triclosan) on tetracycline and ofloxacin sensitive *Salmonella* species in artificially contaminated dishcloths. (A) *Salmonella* # 1, (B) *Salmonella* # 2, (C) *Salmonella* # 3, and (D) *Salmonella* # 4. Each error bar represents the standard error of the mean of 2 independent experiments.

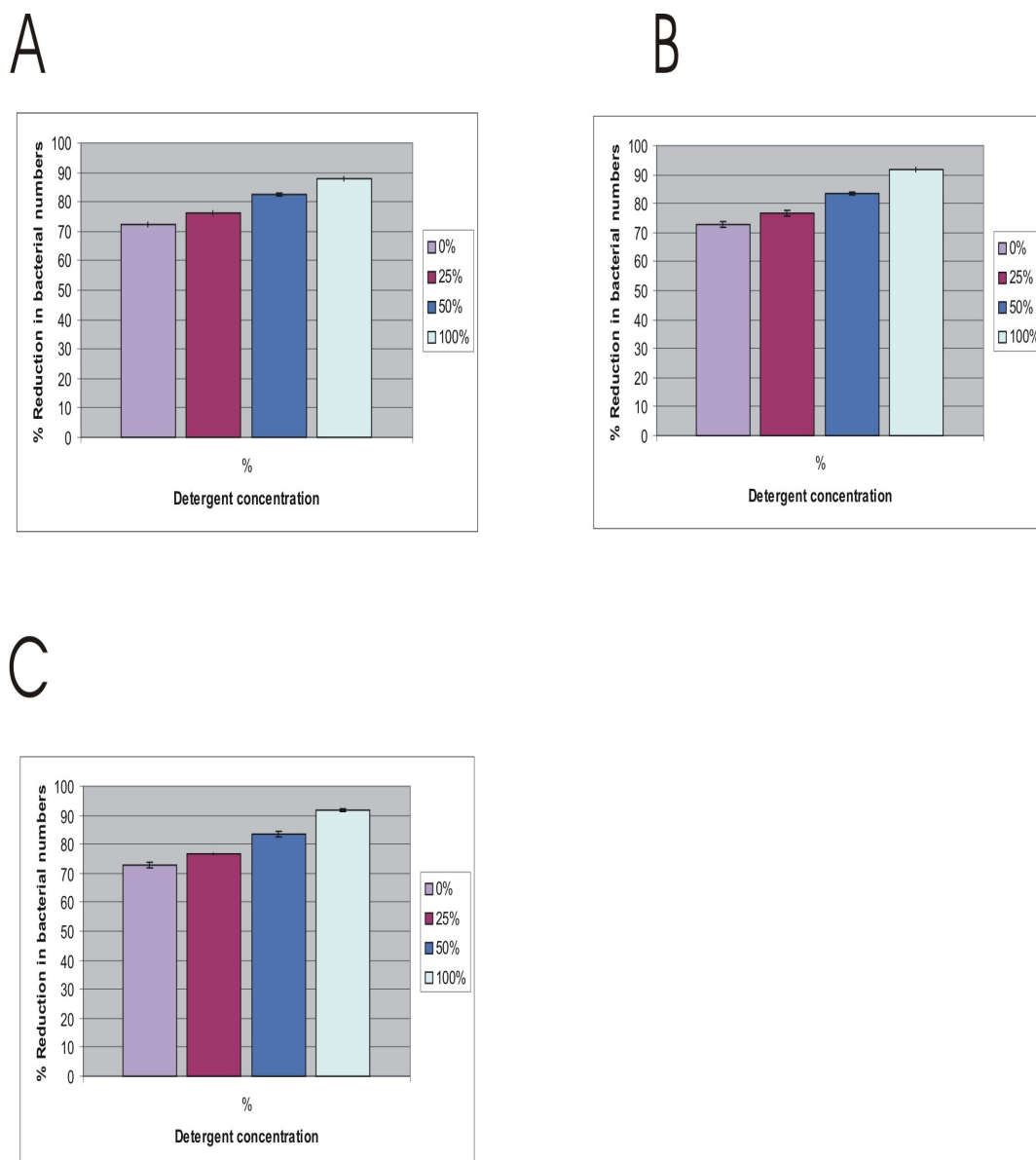


Figure D3: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on tetracycline and ofloxacin sensitive *Salmonella* species in artificially contaminated dishcloths. (A) *Salmonella* # 1, (B) *Salmonella* # 2, and (C) *Salmonella* # 3. Each error bar represents the standard error of the mean of 2 independent experiments.

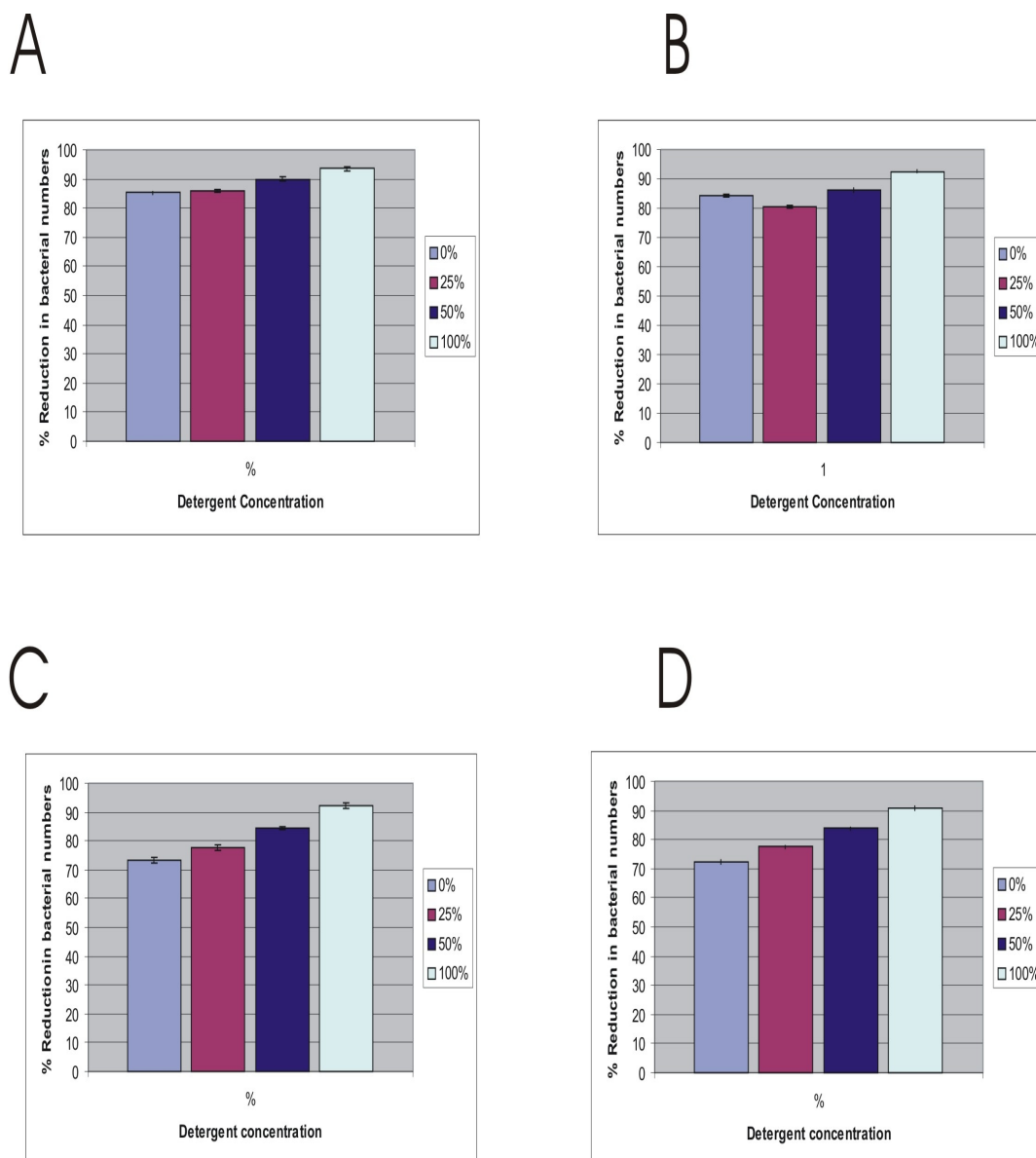
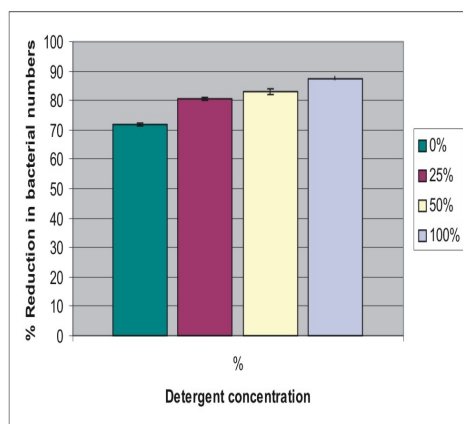
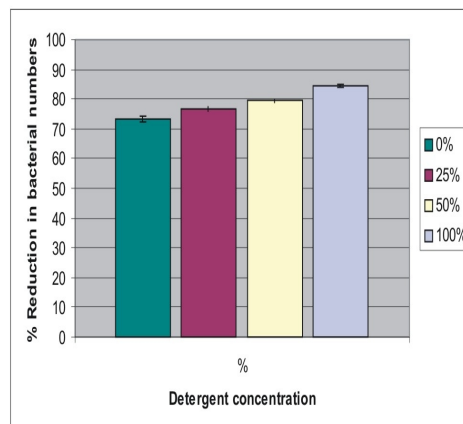


Figure D4: Effect of different concentrations of household disinfectant product (containing sodium salicylate) on tetracycline and ofloxacin sensitive *E. coli* species in artificially contaminated dishcloths. (A) *E. coli* # 1, (B) *E. coli* # 2, (C) *E. coli* # 3, and (D) *E. coli* # 4. Each error bar represents the standard error of the mean of 2 independent experiments.

A



B



C

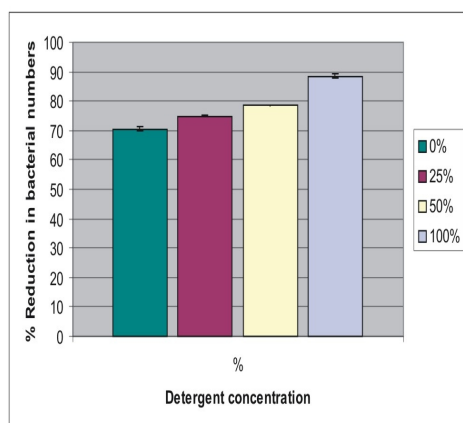
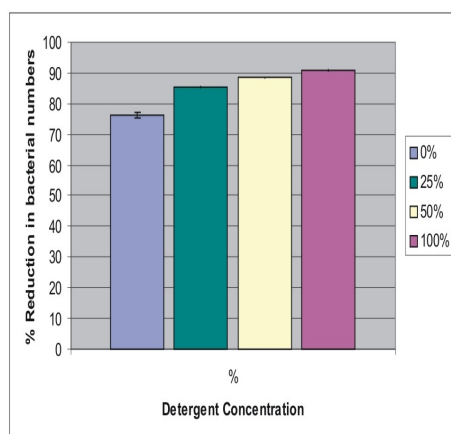
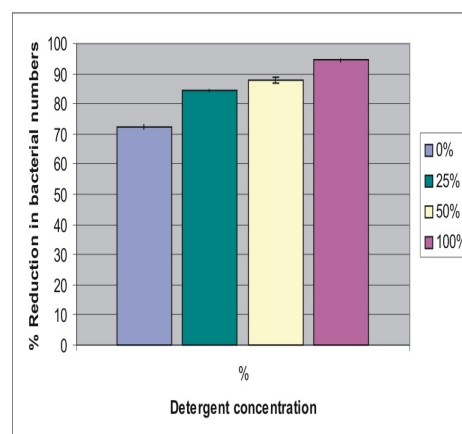


Figure D5: Effect of different concentrations of household disinfectant product (containing triclosan) on tetracycline and ofloxacin sensitive *E. coli* species in artificially contaminated dishcloths. (A) *E. coli* # 1, (B) *E. coli* # 2, and (C) *E. coli* # 4. Each error bar represents the standard error of the mean of 2 independent experiments.

A



B



C

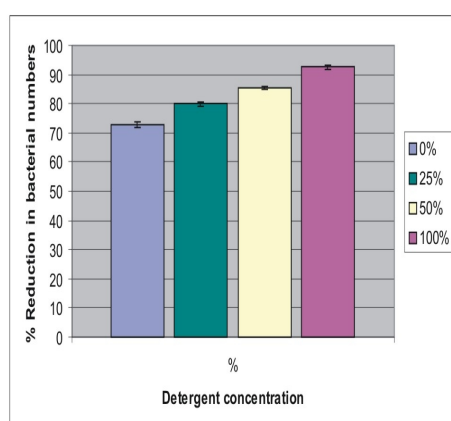


Figure D6: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on tetracycline and ofloxacin sensitive *E. coli* species in artificially contaminated dishcloths. (A) *E. coli* # 1, (B) *E. coli* # 3, and (C) *E. coli* # 4. Each error bar represents the standard error of the mean of 2 independent experiments.

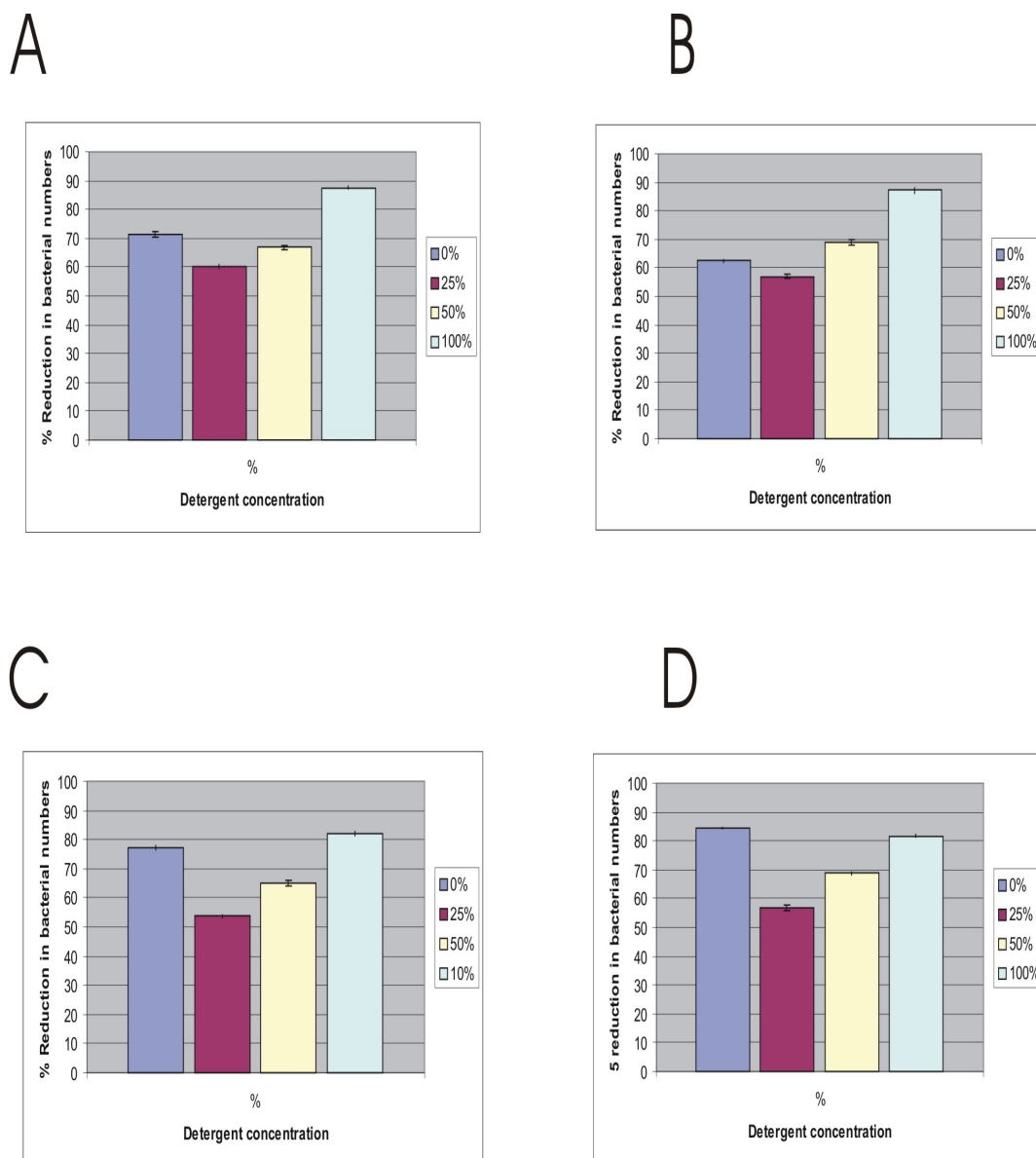
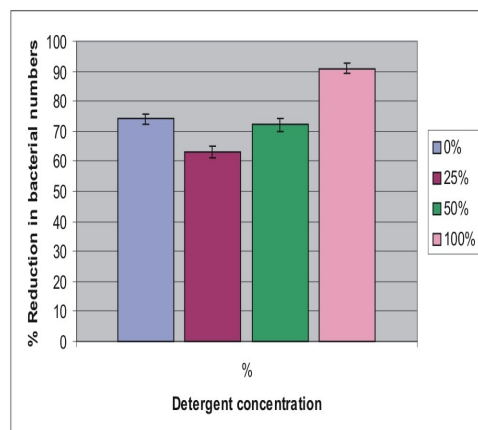
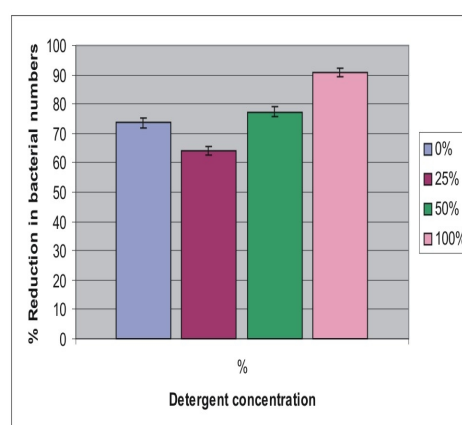


Figure D7: Effect of different concentrations of household disinfectant product (containing sodium salicylate) on tetracycline and ofloxacin resistant *Salmonella* species in artificially contaminated dishcloths. (A) *Salmonella* # 5, (B) *Salmonella* # 6, (C) *Salmonella* # 7, and (D) *Salmonella* # 8. Each error bar represents the standard error of the mean of 2 independent experiments.

A



B



C

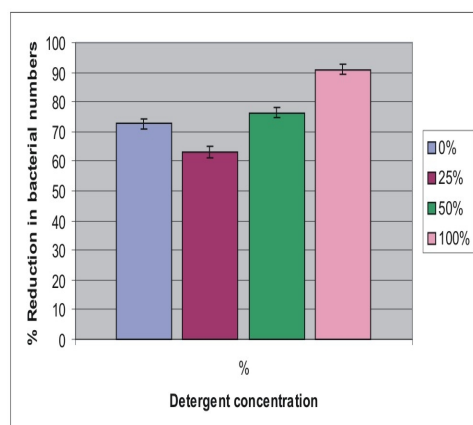


Figure D8: Effect of different concentrations of household disinfectant product (containing triclosan) on tetracycline and ofloxacin resistant *Salmonella* species in artificially contaminated dishcloths. (A) *Salmonella* # 6, (B) *Salmonella* # 7, and (C) *Salmonella* # 8. Each error bar represents the standard error of the mean of 2 independent experiments.

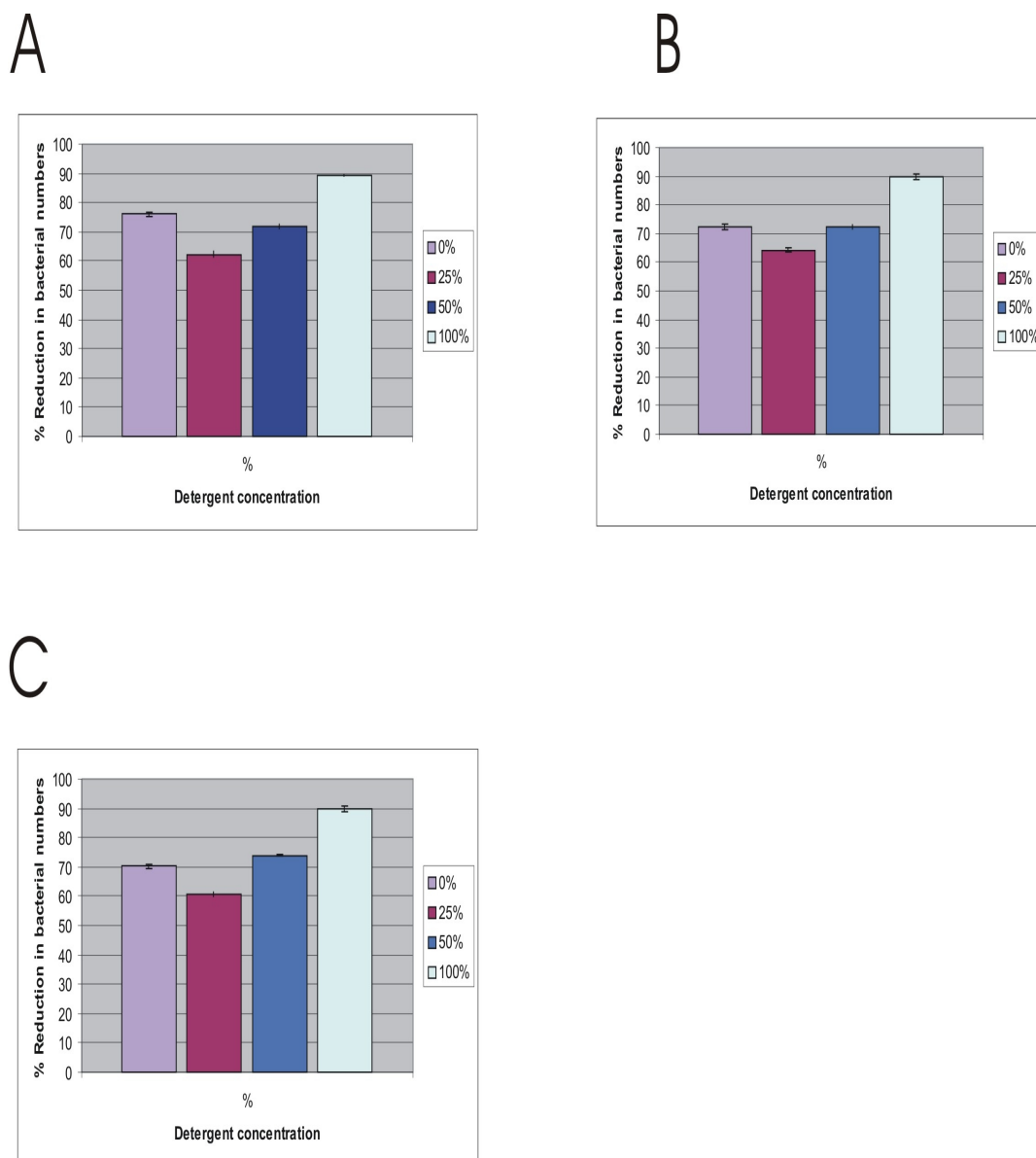


Figure D9: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on tetracycline and ofloxacin resistant *Salmonella* species in artificially contaminated dishcloths. (A) *Salmonella* # 5, (B) *Salmonella* # 6, (C) *Salmonella* # 8. Each error bar represents the standard error of the mean of 2 independent experiments.

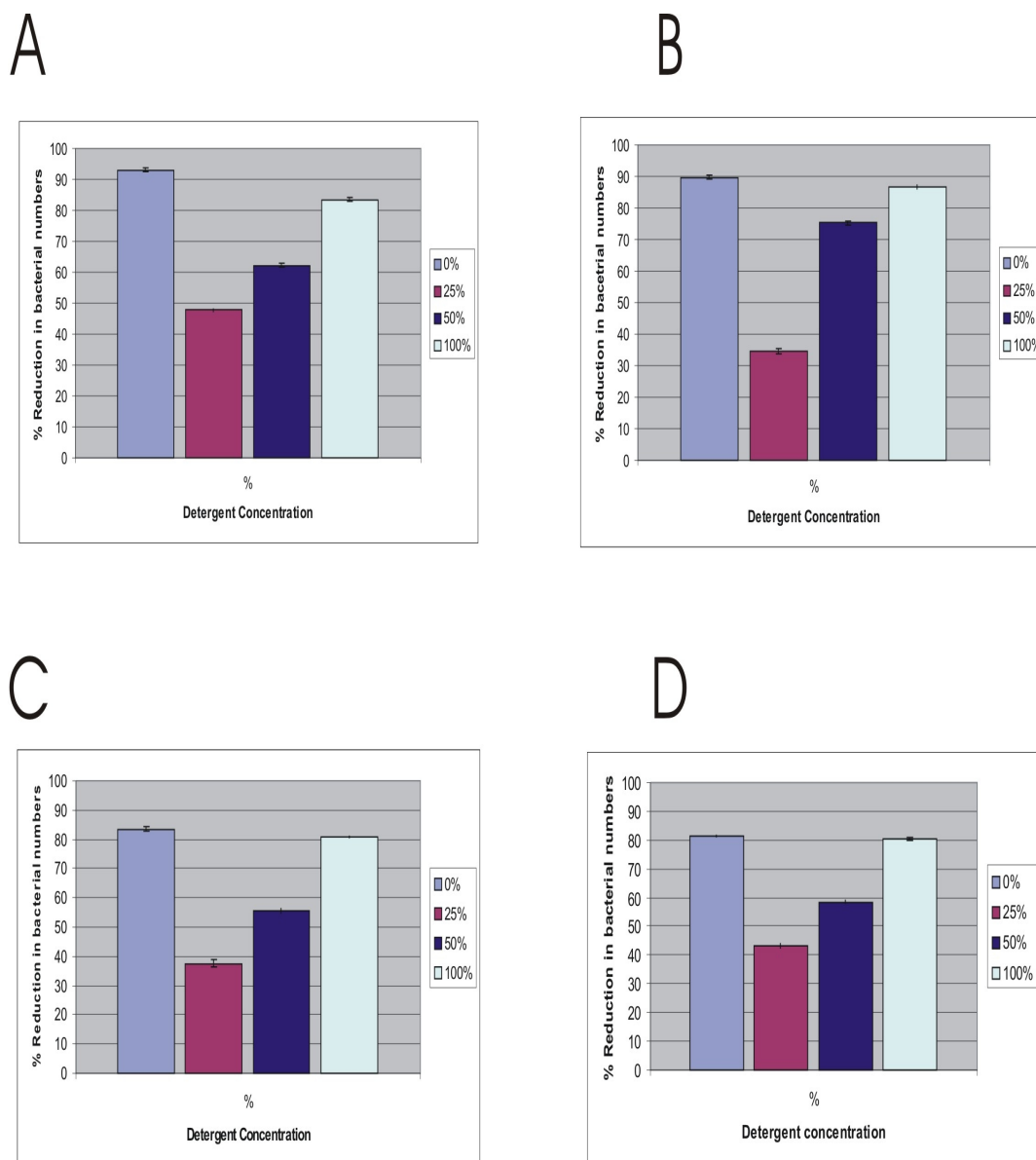


Figure D10: Effect of different concentrations of household disinfectant product (containing sodium salicylate) on tetracycline and ofloxacin resistant *E. coli* species in artificially contaminated dishcloths. (A) *E. coli* # 6, (B) *E. coli* # 8, (C) *E. coli* # 9, and (D) *E. coli* # 10. Each error bar represents the standard error of the mean of 2 independent experiments.

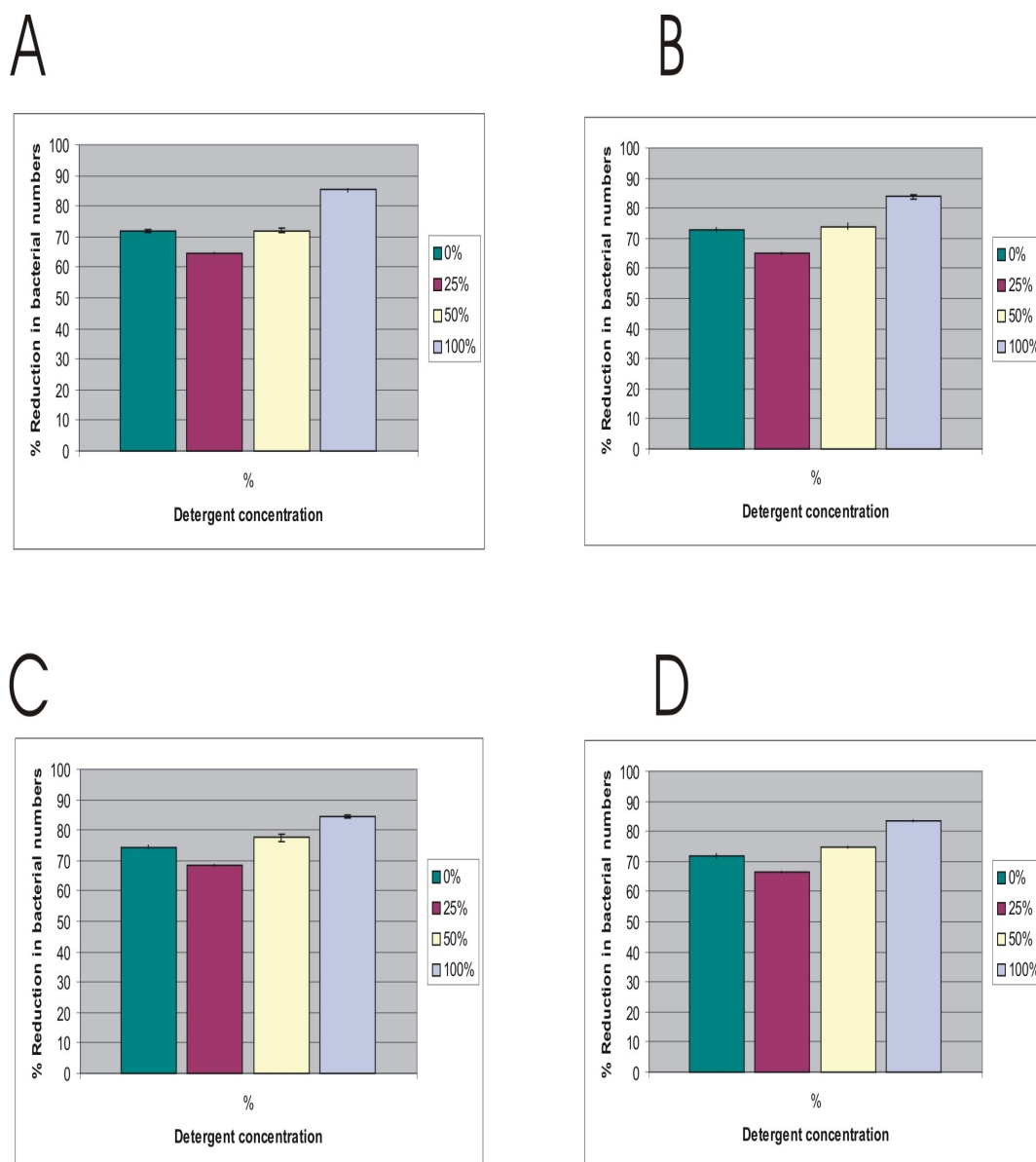


Figure D11: Effect of different concentrations of household disinfectant product (containing triclosan) on tetracycline and ofloxacin resistant *E. coli* species in artificially contaminated dishcloths. (A) *E. coli* # 5, (B) *E. coli* # 6, (C) *E. coli* # 8, and (D) *E. coli* # 9. Each error bar represents the standard error of the mean of 2 independent experiments.

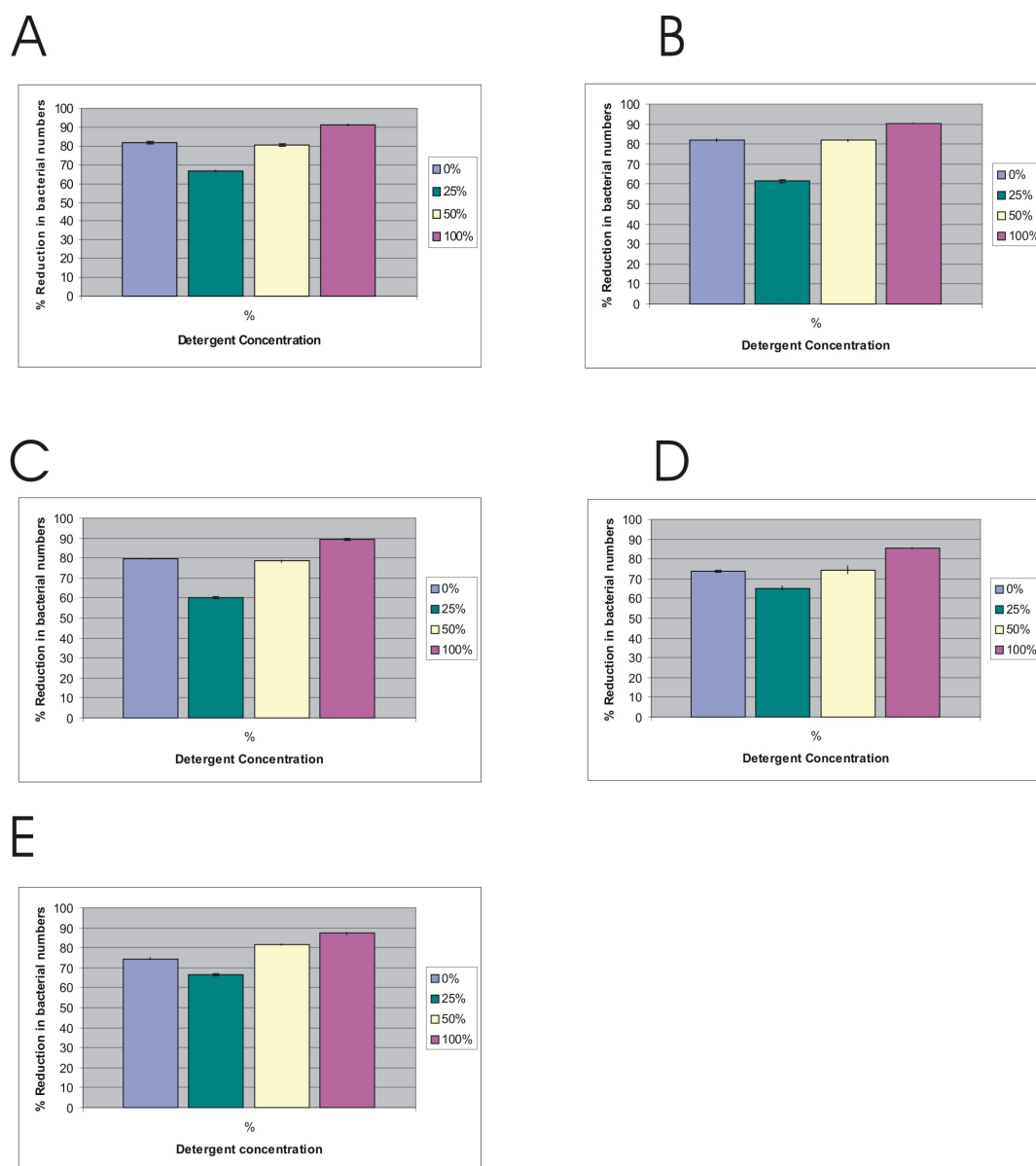


Figure D12: Effect of different concentrations of household disinfectant product (containing *ortho*-phenylphenol) on tetracycline and ofloxacin resistant *E. coli* species in artificially contaminated dishcloths. (A) *E. coli* # 5, (B) *E. coli* # 6, (C) *E. coli* # 8, (D) *E. coli* # 9, and (E) *E. coli* # 10. Each error bar represents the standard error of the mean of 2 independent experiments.

CHAPTER 7

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