GENETIC AND PHENOTYPIC CHARACTERISATION OF FOODBORNE BACTERIA ISOLATED FROM READY-TO-EAT FOODS IN ALICE, SOUTH AFRICA.

BY

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DECLARATION

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture, and the work contained herein is my original work and that this work has not been submitted to any other university in partial or entirely for the award of any degree.

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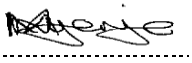
Signature: 

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DEDICATION

To my husband Wilson and lovely daughter Nancy.

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.

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LIST OF ABBREVIATION

AIDS-Acquired immunodeficiency syndrome

ANOVA - Analysis of variance

API-Analytical profile index

ASPIRE-Alice Regeneration Strategy Report

ATCC-American type culture collection

CAC/GL 22R- Codex Alimentarious Commission

CDC- Center for Disease Control and Prevention

CFU-Colony forming units

CLSI- Clinical and Laboratory standard Institute

DNA- Deoxyribose nucleic acid

ESBLs- Extended spectrum β-lactamases

FAO-Food and Agriculture organization

FDA/CFSAN- U.S. Department of Health and Human Services Food and Drug Administration. Center for Food Safety and Applied Nutrition

GAP- Good agriculture practice

HACCP- Hazard Analysis and Critical Control Point

HUS-heamolytic-ureamic syndrome

Ig G- Immunoglobilin G

M.I.C.E- Minimum Inhibition Concentration Evaluator

MPN-Most probable number

NCTC-National type culture collection

NSWFA-New Zealand and South Wales Food Authority

PCR-Polymerase chain reaction

RNA-Ribose nucleic acid

SD- Standard deviation

SEA- Staphylococcal enterotoxins superantigens

SEM-Scanning electron microscope

SPSS- Statistical Package for the Social Sciences

SHV - Sulphydryl variable

TEM -Temoneira

WHO- World Health Organization

General abstract

Foodborne illnesses following the ingestion of contaminated food are a major public health problem worldwide. They include a broad group of illnesses ranging from mild to chronic or life-threatening; caused by either toxins released from the disease-causing microbes, or by the microbes themselves. Antimicrobial susceptibility data shows an alarming increase in the frequency of antimicrobial resistance of foodborne pathogens, a situation which is worrisome as it decreases the effectiveness of drugs employed to reduce the morbidity and mortality associated with serious and life-threatening infections and thus, compromising human health. This study was therefore designed to assess the occurrence and characterization of bacterial foodborne pathogens in various foods sold in Alice, Eastern Cape Province of South Africa in an effort to throw more light on the inherent risk associated with such foods.

The study was conducted during the period of 2011 - 2013. Two university restaurants and eight ready-to-eat food vending sites in Alice Town were selected based on their prominence to the students, workers and rest of the community. Microbiological analysis was conducted on 252 samples which included vegetables, potatoes, rice, pies, beef and chicken stew. The isolates were identified using biochemical tests and confirmation of the two most prevalent organisms (*Listeria ivanovii* and *Enterobacter cloacae*) was done using PCR techniques. The antimicrobial susceptibility profile of *Listeria ivanovii* and *Enterobacter cloacae* strains were identified using the disc diffusion technique; minimum inhibitory concentration was determined by the broth dilution method and M.I.C. Evaluator test strips. The microtiter plate adherence assay was employed to ascertain the ability of these isolates to adhere to a surface whereas the role of cell surface properties in biofilm formation was assessed using the coaggregation and autoaggregation assays. The architecture of the formed biofilms was

examined under the scanning electron microscope. The virulence and resistant genes were also detected and characterised by sequencing the PCR products.

Bacterial growth was present in all the food types tested; organisms isolated included: *Listeria* spp. (22%), *Enterobacter* spp. (18%), *Aeromonas hydrophila* (12%), *Klebsiella oxytoca* (8%), *Proteus mirabilis* (6.3%), *Staphylococcus aureus* (3.2%) and *Pseudomonas luteola* (2.4%). PCR confirmed 30 (97%) isolates as *E. cloacae* complex while 44% (22/50) tested positive for *L. ivanovii*. All the strains of *E. cloacae* (100%) and 96% of *L. ivanovii* isolates (based on phenotypic identification) were resistant to at least four or more of the antibiotics. In this study, *bla*-$_{\text{TEM}}$ was also detected from 48 (96%) of *L. ivanovii* and 30 (100%) of *E. cloacae* strains; further analysis of the *bla*-$_{\text{TEM}}$ demonstrated the occurrence of *bla*-$_{\text{TEM-1.}}$ Of the 56 *bla*-$_{\text{TEM-1}}$ positive isolates sequenced, 7% (4/56) had mutation of either insertion or substitution of a nucleotide.

Two virulence genes (*ucaA* and *hlyA*) were detected in *E. cloacae* isolates and none in *L. ivanovii* using PCR. Sequence analysis of the *hsp60* gene reported the presence of two sub-species for *E. cloacae*; *E. cloacae* cluster III (75%) and *E. cloacae* cluster IV (25%); while analysis of the *iap60* gene demonstrated that 55.8% (19/34) were *L. ivanovii*, 44% (15/34) *L. seeligeri* and 14.7% (5/34) *L. welshemeri*.

A total of 90% *L. ivanovii* and 88% *E. cloacae* strains demonstrated the ability to form biofilms; the coaggregation index ranged from 12 to 77% while the autoaggregation index varied from 11 to 55% for *L. ivanovii* and 27% to 98% for *E. cloacae*.

The findings of this study indicate that most of the ready-to-eat food samples examined did not meet bacteriological quality standards, thus posing potential risks to consumers. This should draw the attention of the relevant authorities to certify that hygienic standards are improved to curtain foodborne infections. Furthermore, the presence of multi-resistant strains

is of major concern as these foods could serve as important vehicles transmitting multi-resistant bacteria and genes to humans. In addition the ability of these pathogens to form biofilms may lead to adherence of these organisms to kitchen utensils and other environments leading to cross-contamination of food processed in these areas and increase resistance of organisms to antimicrobial agents.

CHAPTER ONE

General Introduction

General introduction

1.1 Background

Foodborne illnesses are a major public health problem and important cause of reduced economic growth worldwide. They result from the ingestion of contaminated foods and food products. Foodborne diseases include a broad group of illnesses with varying degrees of severity, ranging from mild indisposition to chronic or life-threatening illness caused by either toxins released from the disease-causing microbes, or by the microbes themselves (Tauxe *et al*., 2010; Farzan *et al*., 2011). The most common clinical presentation takes the form of gastrointestinal symptoms which are generally mild and self-limiting. However, in susceptible individuals such as the elderly, pregnant women, very sick or weak, the diseases can lead to chronic, life-threatening symptoms including neurological, gynaecological or immunological disorders as well as multi-organ failure, cancer and death (Allerberger and Wagner, 2010; WHO, 2011).

Worldwide, diarrheal diseases alone, a considerable proportion of which is foodborne, kills approximately 2.2 million people annually (Saba and Gonzalez-Zorn, 2012). Although most of these diarrheal associated deaths occur in poor countries, foodborne diseases are not limited to developing countries. According to Tauxe *et al*. (2010), foodborne diseases result in 76 million illnesses and 5000 deaths each year in the United States, whereas in the United Kingdom, foodborne diseases are estimated at 1.7 million cases yearly (Adak *et al*., 2005). It is contemplated that the growing international trade, migration and travel, accelerate the spread of dangerous pathogens and contaminants in food; thus, increasing our universal vulnerability (Tauxe *et al*., 2010). For instance, the Latin American cholera epidemic, which was thought to have begun with contaminated water and seafood in Peru, rapidly spread across Latin America resulting in approximately 400,000 reported cases and more than 4,000

deaths in several countries (PAHO, 1995). Similarly, the 2011 *Escherichia coli* O104:H4 (STEC O104:H4) outbreak which was first reported in Germany spread to other European countries and North America (Wu *et al*., 2011). It is also believed that the global spread of *Salmonella* serotype Enteritidis (now the most important *Salmonella* serotype in most countries) result from exports of food or food animals from developed countries (Tauxe, 1999).

Despite important developments in reducing foodborne diseases through better farm practices and food regulations such as the Hazard Analysis and Critical Control Point (HACCP) and Good Agricultural Practices (GAP) system, foodborne illnesses still remains a challange globally (Tauxe *et al*., 2010). This could be in-part that new pathogens are emerging while established pathogens are acquiring new characteristics and appear in unexpected food vehicles. In addition, consumer tastes and requirements are changing; hence, new food production, preparation and distribution techniques have developed to reflect this development (Quested *et al*., 2010; Tauxe *et al*., 2010).

In recent decades, public health promotion of healthier lifestyles has led to increased demand for fresh, tasty and wholesome food products that requires less handling and preparation. Food processing involve multiple stages of handling compared to unprocessed foods; thus, increasing the chances of contamination and consequently greater risk of foodborne illness. Furthermore, social and economic changes as well as increased travel and tourism have all contributed to an increased demand for street foods or ready-to-eat foods (Nyachuba *et al*., 2010).

The Food and Agriculture Organisation (FAO) defines street foods or fast foods as ready-to-eat foods and beverages prepared and/or sold by vendors especially in streets and other public places for immediate consumption without further processing or preparation. These foods are well appreciated by consumers, mostly by urban workers because of their taste, low cost and ready availability for immediate consumption. It includes fast foods, snacks, beverages, salads and sliced fruits (Simopoulos, 2000). However, food poisoning caused by contaminated food sold or served in public places has been well reported (Mensah *et al*., 2002; Schmid *et al*., 2008; Farzan *et al*., 2011).

The contamination of street foods is mainly due to unhygienic water, improper washing of utensils, improper handling and packaging, low quality of raw materials used, unhealthy preparation and serving areas as well as improper storage practices. In addition, several intrinsic and extrinsic factors such as, pH, moisture content, water availability, temperature and gases (CO_2, O_2) play a role in creating a favourable environment for microbial growth and multiplication (Rath and Patra, 2012).

Although most foodborne illnesses are generally self-limiting, antimicrobial therapy is prerequisite in severe cases, extraintestinal disease, or in immunocompromised subjects. Drugs such as fluoroquinolones or third-generation cephalosporins, ampicillin, gentamicin and sulfamethoxazole/trimethoprim are frequently used; however, drug resistance has been the hitch in the success of managing these severe foodborne illnesses (Mølbak, 2005; Conter, 2009; Ruiz-Bolivar *et al*., 2011). Antimicrobial resistance is an inevitable consequence of antimicrobial use in human medicine, agriculture and animal husbandry where it affords selective pressure for the emergence and spread of resistance genes in non-resistant strains (Davies and Davies, 2010). Selective pressure is a situation when the antibiotic used will not only kill susceptible bacteria, but also "enrich" resistant bacteria as they lack competitors for

space, resources, hosts, etc. Thus, those resistant organisms can often thrive and multiply, passing on their resistant genes to the next generation (Canton, 2009).

The development of antimicrobial resistance through the food chain has been well acknowledged (White *et al*., 2002; Knezevic and Petrovic, 2008; Hunter *et al*., 2010). During food production and manufacturing, a variety of antimicrobials are applied to improve the efficiency of the system, and ensure food quality and safety of the products; which have all reported to have an impact on the incidences of antibiotic resistance in humans (Marshall and Levy, 2011). Antimicrobial resistant bacteria and resistance genes can be spread to humans via food by different routes and mechanisms: for instance, through resistant zoonotic bacteria, like *Salmonella* and *Campylobacter* which may originate from various sources, *e.g*. animals, the environment and humans; or through resistant non-zoonotic human pathogenic bacteria *e.g*. the species of *Shigella* and *Vibrio.* Resistant genes can also be spread by resistant commensal bacteria carrying transferable antimicrobial resistance genes that can be passed on to human pathogenic bacteria (Teuxe *et al*., 2010; Enitan *et al*., 2011). These resistant commensal bacteria may originate from various sources, including animals, the environment and humans. In addition, food handlers can contaminate food during preparation, for example, methicillin-resistant *Staphylococcus aureus* (MRSA) and resistant *Shigella* species (Kozlov *et al*., 2010; Enitan *et al*., 2011).

1.2 Rationale

Recent global developments are increasingly challenging international health security. These developments include the growing industrialization and trade of food production, the rapid urbanization associated with food preparation/consumption outside the home and the emergence of new and antibiotic resistant pathogens (WHO, 2011). Food contamination with pathogens of human reservoir is common when growing areas are contaminated with human sewage. Such threats are further augmented if the food is mishandled during processing and preparations (Nyenje and Ndip, 2013). Food-handlers with poor personal hygiene working in food-serving establishments could be potential sources of foodborne infections (Gashaw *et al*., 2008). Of significance are the unhygienic conditions under which street food vendors operate, and lack of basic food safety training of the street vendors (Mensah *et al*., 2002) makes these foods more vulnerable to bacterial contaminants and deserves immediate attention on their microbial quality (Viswanathan and Kaur, 2001).

The use of antimicrobials as therapeutic agents or as growth promoters in livestock and plant production has adverse public health consequences by creating a reservoir of resistant bacteria and bacteria-borne resistance genes that can be passed on to humans, both directly or indirectly (Andreoletti *et al*., 2008). Resistant strains of *E. coli, Salmonella, Campylobacter, Staphylococcus*, *Shigella* and *Vibrio* species involved in human disease are mostly spread through ingestion of contaminated food. Therefore, control measures applied to the prevention and the spread of pathogenic bacteria via food will also contribute to the prevention and the spread of antimicrobial-resistant pathogenic bacteria (Andreoletti *et al*., 2008).

The biofilm mode of growth is the preferred lifestyle in the microbial world as it enhances growth and survival by providing access to nutrients and protection from predators and antimicrobial compounds (Yildiz and Visick, 2009). Biofilms have been of considerable interest in the context of food hygiene. Of particular significance is the ability of microorganisms to attach and grow on food and food-contact surfaces under favourable conditions. *Salmonella*, *S. aureus*, *E. coli*, *L. monocytogenes* and *Cronobacter sakazakii* are important pathogenic bacteria and represent a serious problem for the industrial environment and food manufactures because of their ability to adhere to different surfaces (polymers, metals, glass) and form biofilm (Nostro *et al*., 2010; Bayoumi *et al*., 2012; Gramile da Silva *et al*., 2012). Cells in the inner most of the biofilm receive less oxygen and fewer nutrients than those at the biofilm surface; hence, these cells may present altered growth and physiological state resulting in increased resistance to antimicrobial agents (Watnick and Kolter, 2000). Therefore biofilm development in food processing environments is a potential persistent source of contamination that may compromise food quality. However, no studies on the microbial quality of the food sold in Alice exist; hence the current study was performed to shed light on the imminent dangers associated with such foods.

1.3 Aim and Objectives

The present study investigated the occurrence and characterization of bacterial foodborne pathogens in various foods sold in the University of Fort Hare and roadside cafeterias of Alice in the Eastern Cape Province of South Africa; the specific objectives were to:

1. Determine the prevalence and distribution of bacterial foodborne pathogens in various foods from the cafeterias of Alice.
2. Elucidate the antibiotic susceptibility profiles of the isolates.
3. Ascertain the distribution of antibiotic resistance genes in the isolates.
4. Characterize the virulence genes in the isolates.
5. Evaluate the ability of the isolates to form biofilm.

1.4 Scope of thesis

This thesis comprises of nine chapters. Chapter one represents a general introduction to the topic of the thesis.

Chapter two, Literature review: It encompasses the major aetiology, pathogenicity and epidemiology of foodborne illnesses. The chapter also outlines the different detection methods and treatment of foodborne illnesses; as well as the effective prevention and control measures routinely employed. The chapter generated two review articles.

Chapter three assesses the microbiological quality of various ready-to-eat foods sold in Alice, South Africa. Bacterial growth was present in all the food types tested, indicating that the food samples did not meet bacteriological quality standards, therefore posing potential risks to consumers. One research paper has been published from this work.

Chapter four confirmed the isolates using molecular techniques, but also detected and characterised some associated-virulence genes of *Listeria* species and *E. cloacae* isolates in an effort to determine the pathogenic potential of these organisms and the risk they pose to

the community who cunsume these foods. Two virulence genes were detected from *E. cloacae* and none from *Listeria* species. One manuscript has been generated from this work.

Chapter five focuses on the antimicrobial susceptibility of *L. ivanovii* and *E. cloacae* that were previously reported as most prevalent. Multiple resistances to at least four or more of the antibiotics was observed and nineteen antibiotypes were obtained based on the antibiotics used in the study. The study also compared the effectiveness of broth microdilution assay and the E-test strip in determining minimum inhibition concentration. Two research articles have been published from this chapter.

Chapter six determines the prevalence of β-lactamase and tetracycline resistance genes among *L. ivanovii* and *E. cloacae* strains, but also characterizes their variants in an effort to establish baseline data on the common variant in the study area. $Bla_{\text{TEM-1}}$ was the only gene present in both *L. ivanovii* and *E. cloacae* strains. One manuscript has been produced.

Chapters seven and eight discusses the ability of *L. ivanovii* and *E. cloacae* isolates to adhere to a surface and form biofilm at different growth conditions. A great number of these isolates were capable of forming single or multispecies biofilms; this is of great concern to the food industry where these organisms may adhere to kitchen utensils and other environments leading to cross-contamination of food processed in these areas. These chapters produced two research papers which are already published.

Chapter nine assembles the general discussion, conclusions and recommendations.

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CHAPTER TWO

Literature Review

Part of this chapter has been published as:

Nyenje, M. E., Ndip, R. N. (2013). The challenges of foodborne pathogens and antimicrobial chemotherapy: A global perspective. *African Journal of Microbiology Research*, **7**: 1158 – 1172.

Literature review

2.1 Introduction

The battle against foodborne diseases is facing new challenges due to the globalization of the food market, climate change and changing patterns of human consumption (Schelin *et al*., 2011). As food is biological in nature, it is capable of supporting the growth of microorganisms and foodborne diseases result from the ingestion of contaminated foods and food products (Guyader and Atmar, 2008). More than 250 different types of viruses, bacteria, parasites, toxins, metals, and prions are associated with foodborne diseases in humans (Schmidt *et al*., 2009). Although viruses are more responsible for more than 50% of all foodborne illnesses; generally hospitalizations and deaths associated with foodborne infections are due to bacterial agents. The infections range from mild gastroenteritis to life-threatening neurologic, hepatic, and renal syndromes caused by either toxin from the "disease-causing" microbe, or by the human body's reaction to the microbe itself (Teplitski *et al*., 2009).

Food poisoning is divided into three types: 'infection', 'intoxication', and 'intermediate' (Schmidt *et al*., 2009). 'Infection' is caused by the oral ingestion of viable microorganisms in adequate amounts to build up infection and the commencement of symptoms is normally delayed, reflecting the time required for an infection to develop. Examples of food-poisoning that cause infection are enteric viruses, *Salmonella*, *Campylobacter* and *Vibrio* species. 'Intoxication' on the other hand, is caused by the ingestion of toxins that have been pre-formed in the food. Therefore, there is no necessity for live organisms to be present and the onset of the symptoms is rapid. Examples are *Bacillus cereus* and *Staphylococcus aureus*. The 'intermediate' food poisoning occurs when live bacteria are ingested and subsequently

produce a toxin in the host, as in the case of *Clostridium perfringens* food poisoning (Teplitski *et al*., 2009).

Foodborne diseases remain the major source of morbidity and mortality in the general population, mainly in susceptible groups, such as the infants, the elderly and the immunocompromised (WHO, 2011). According to the World Health Organization (WHO), up to 1.5 billion cases of diarrhoea and more than three million deaths that occur in children every year are as a result of food and water contamination (WHO, 2007), and in France, it is estimated that these pathogens cause 10,200 - 17,800 hospitalizations yearly (Vaillant *et al*., 2005). The developing world are not spared, in South East Asia, approximately one million children below the age of five years die each year from diarrheal diseases due to contaminated food and water (WHO, 2000). Several devastating foodborne outbreaks have been reported on the African region; in 2004, Kenya experienced an acute aflatoxicosis outbreak which was attributed to maize, whereas in 2007, Angola registered 400 cases of bromide poisoning, associated with the use of sodium bromide as cooking salt (WHO, 2005).

2.2 Aetiology, pathogenicity and epidemiology of foodborne illnesses.

2.2.1 Viruses

Viruses are very small microorganisms, ranging in size from 0.02 to 0.4 micrometres in diameter and cause a wide range of diseases in plants, animals and humans (Koopmans and Duizer, 2004). They are recognized as the most common pathogens transmitted via food; for example in the United States, viruses account for 67% of food related illnesses, compared to 9.7% and 14.2% for *Salmonella* and *Campylobacter*, respectively (Vasickova *et al*., 2005).

Viruses are intracellular organisms, which only replicate within living cells of the host; therefore the number of viral particles in food does not increase and sensory features of the contaminated and non-contaminated food will be identical (Koopmans and Duizer, 2004). Foodborne viruses include rotaviruses, noroviruses (NoVs), enteric adenoviruses, hepatitis A virus (HAV), enteroviruses, human astroviruses, aichiviruses, toroviruses, coronaviruses and picobirnaviruses (Chitambar *et al*., 2012). However, overwhelming majority of cases is due to NoVs and HAV (Koopmans and Duizer, 2004).

2.2.1.1 Noroviruses

NoVs are non-enveloped single stranded RNA viruses belonging to the family Caliciviridae. Five genogroups exist, GI to GV, of which GI, GII and GIV are recognized to infect humans; genotype GII.4 is widespread in outbreaks (Koopmans, 2008). NoVs are considered the major cause of epidemic gastroenteritis in all age groups, leading to over 267,000,000 annual infections worldwide (Barrabeig *et al*., 2010). Furthermore, it is estimated that about 900,000 cases of paediatric gastroenteritis in industrialized nations and 1.1 million episodes and 218,000 deaths in developing nations are caused by NoVs (Patel *et al*., 2008).

NoVs are acid resistant; hence they pass through the stomach and replicate in the small intestines where the infected individuals (symptomatic and asymptomatic) shed the virus in both fecal matter and vomitus, and can also be transmitted through contaminated water or food (Loopman *et al*., 2002). Person-to-person transmission is by far the most common route of infection. For example, a surveillance study in New Zealand conducted during 2001- 2007 reported that of the total outbreaks, 19.9% were associated with environmental sources, 17.6% with foodborne infection and 61.0% with person-to-person transmission (Lim *et al*., 2010). Likewise, NoVs outbreaks resulting from contamination by an infected food-handler, water, both directly (example consumption of tainted water) or indirectly (example via washed fruits, by swimming or canoeing in recreational waters) has been documented (Atmar and Estes, 2006).

NoVs infections are mostly manifested as gastroenteritis; characterised by acute onset of nausea (81%), vomiting (54%), diarrhoea (85%) and abdominal cramps (72%). Constitutional symptoms such as fever (51%), rigors, headache, muscle and joint pain are also common (Simmons *et al.*, 2001). In healthy individuals, the symptoms are generally mild and self-limiting however, in vulnerable groups, more serious illnesses have been reported (Teunis *et al*., 2008). Immunity to NoVs infection seems to be short-lived, in the order of several months; after this period, individuals appear to become susceptible to the same strain of virus (Glass *et al*., 2009). Although the pathogenesis is not clearly understood, some studies noted lesion on the small intestinal mucosa and, inflammation, blunting of the villi, shortening of the microvilli and dilation of the endoplasmic reticulum; these conditions leads to abnormal gastric motor function, believed to be the cause of associated nausea and vomiting (Lopman *et al*., 2002; Glass *et al*., 2009).

2.2.1.2 Hepatitis A virus

HAV is a non-enveloped single-stranded RNA virus. It is a member of the Hepatovirus genus, belonging to the Picornaviridae family. There is only one serotype of HAV and six genotypes; genotypes I, II and III cause acute hepatitis in humans and immunity after infection is lifelong (Fiore, 2004). Despite being endemic worldwide, areas with low socio-economic standards have high incidence rates of HAV; exemplified by immunological surveys where almost 90% of children are infected before the age of 10 years, though most infections are asymptomatic (Nainan *et al*., 2006).

The first largest outbreak occurred in China in 1988 where 3 million people were infected after consumption of clams harvested from a sewage-polluted area (Cuthbert, 2001). Other outbreaks associated with oyster, mussels, green onions, lettuce, strawberries in Australia, Brazil, Italy and Spain have been reported (Coelho *et al*., 2003; Wheeler *et al*., 2005). In most of these outbreaks, sewage was the source of pollution.

HAV infections result in a number of symptoms including: fever, anorexia, nausea and abdominal discomfort, followed within a few days by jaundice. HAV infection may also cause liver damage, usually from the host's immune response to the infection of the hepatocytes. In some cases, the liver damage may lead to death. The virus has a low case fatality rate of 0.3% but increases with age and underlying chronic liver diseases (Nainan *et al.*, 2006). The exact pathogenesis of HAV is unclear; however, it is believed that once the virus has been acquired, it enters and replicates in the small intestines (Kumar *et al*., 2010). This primary replication is followed by a viremic stage and transportation to the liver where the virus is further replicated in the hepatocytes (Koopmans, 2008). The virus is secreted into the bile canaliculi from where it passes back into the intestinal tract, and the infected

individuals (asymptomatic and symptomatic) will shed the virus in the feces in high titers. HAV is not cytolytic and hepatic damage is immune-mediated (Pinto *et al*., 2010).

2.2.2 Bacterial agents

Foodborne bacterial agents are the leading cause of severe and fatal foodborne illnesses. Of the many thousands different bacteria species, more than 90% of food-poisoning illness are caused by species of *Staphylococcus, Salmonella, Clostridium, Campylobacter, Listeria, Vibrio, Bacillus,* and entero-pathogenic *E. coli* (Nyenje *et al*., 2012a). For instance in France, in the last decade of the 20th century, *Salmonella* was the most frequent cause of bacterial foodborne illness (5,700 - 10,200 cases), followed by *Campylobacter* (2,600 - 3,500 cases) and *Listeria* (304 cases) (Vaillant *et al*., 2005). In South Africa species of *Listeria, Enterobacter and Aeromonas* were most prevalent bacteria in ready-to-eat foods (Nyenje *et al*., 2012a).

2.2.2.1 Salmonella species

Salmonella is a Gram-negative bacillus (Akoachere *et al*., 2009a); it is a common cause of human bacterial gastroenteritis worldwide, and food animals are important reservoir for non-typhoidal *Salmonella* species (Skov *et al*., 2007). The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori.* Human salmonellosis are mostly caused by *S. enterica* serovar Typhimurium and serovar Enteritidis (Galanis *et al*., 2006), although prevalence of other serovars especially serovar Schwarzengrund in Denmark and the United States has also been reported (Oslen *et al*., 2001; Vugai *et al*., 2004). Mostly, salmonellosis is asymptomatic but in symptomatic cases fever, diarrhoea, abdominal cramps and nausea may be experienced often controlled within a week. However, the organisms may be excreted in the feces for many weeks after symptoms subside (FDA/CFSAN, 2003).

Salmonellosis represents an important public health and economic problem globally. For example in the United States, the organism is responsible for an estimated 1.4 million cases, 16,000 hospitalizations and more than 500 deaths annually at an estimated annual cost of about $2.3 billion and in Denmark, foodborne cases of salmonellosis cost the country about $ 10.4 – $25.5 million in 2001 (Wegener *et al*., 2003; Aarestrup *et al*., 2007). Salmonellosis also affects countries economically, for instance, in 2010; over 550 million eggs were recalled due to possible *Salmonella* contamination, resulting in one of the largest massive recalls. Other products recalled included headcheese, pickles, salami, raw tuna, frozen dinners, alfalfa sprouts, lettuce, tomatoes, and olives (Linscott, 2011).

Salmonella can enter the food supply chain to cause illness in three main ways: food animals harbour the bacteria in their intestines, making meat, poultry, eggs, and milk important vehicles for salmonellosis (Liu *et al*., 2011). It can also be introduced into the environment, through manure and litter subsequently, contaminating farm produce in particular fruits and vegetables which are eaten raw or with minimal cooking. Cross-contamination can also occur in food service environments or homes, often between raw poultry and ready-to-eat products (Mc Entire, 2004). Nevertheless, most studies have documented foods of animal origin as the major vehicles for salmonellosis, which man nurtures through mishandling (Zhao *et al*., 2001; Akoachere *et al*., 2009a). The Center for Disease Control and Prevention (CDC) estimates that 75% of *Salmonella* Enteritidis cases result from the consumption of raw or undercooked eggs; this is because the microorganism is localized inside eggs, making thorough cooking imperative (FDA/CFSAN, 2003). In another study, it was demonstrated that *Salmonella* can colonise the avian reproductive tract, persist in the ovary and oviduct and survive in hen's eggs (Gantois *et al*., 2009). Worthy to note is the fact that there is an increasing trend of salmonellosis outbreaks associated with fresh produce and many such crops are produced in the developing countries where manure is frequently used as a natural

fertilizer and some studies suggest that some *Salmonella* species have now evolved to attach to and colonise vegetables (Klerks *et al*., 2007; Franz and van Bruggen, 2008).

2.2.2.2 Staphylococcus aureus

Staphylococci are Gram- and catalase positive, cocci that are ubiquitous in the environment and can be found in the air, dust, sewage, water, environmental surfaces, humans and animals. The species of this organism are classified into two, based on their ability to produce coagulase. Coagulase-positive *Staphylococci* (CPS), in particular *S. aureus* is the pathogenic strain that produces enterotoxin responsible for food poisoning while some strains of Coagulase-negative *Staphylococci* (CNS) are used in the fermentation of meat and milk-based products (Becker *et al*., 2001). Although some studies have reported the existence of certain CNS enterotoxins producing strains (Zell *et al*., 2008; Even *et al*., 2010), the subject has always been controversial because very little information is available about food poisoning caused by CNS (Hennekinne *et al*., 2010).

Staphylococcal food poisoning (SFP) occurs from the ingestion of foods containing preformed staphylococcal enterotoxins (Loir *et al*., 2005). In most cases food-handlers carrying enterotoxin-producing *S. aureus* in their noses or hands are the main source of food contamination due to improper handling and subsequent storage at eminent temperatures which permit growth of *S. aureus* and production of the enterotoxin (Argudin *et al*., 2010).

Various food types have been implicated in SFP and they differ widely from one country to another, probably due to differing food habits (Loir *et al*., 2005). For instance, in the United Kingdom and the United States, meat or meat-based products are the food vehicles mostly involved (Genigeorgis, 1998), while in France, milk-based products are commonly involved than in other countries (De Buyser *et al*., 2001). Salted food products, such as ham, have also been implicated in Japan (Qi and Miller, 2000).

Outbreaks of SFP have been reported worldwide; in Brazil, 42 cases of SFP occurred following a meal at a restaurant (Carmo *et al*., 2003); while in 2009, six food poisoning outbreaks caused by Staphylococcal enterotoxin type E were reported in France and the source of the outbreak was soft cheese made from unpasteurised milk (Ostyn *et al*., 2010). Two *S. aureus* outbreaks from restaurants in the United States in 2003 and 2005 were traced to carrots, green peppers, and leeks (Loir *et al*., 2005).

Staphylococcal enterotoxins (SEs) are superantigenic toxins (SAgs) that cause food poisoning and toxic shock syndrome in humans throughout the world (Balaban and Rasooly, 2000). SAgs belong to the broad family of pyrogenic toxin staphylococcal enterotoxins superantigens (SEA), the toxins that induce emesis (Schelin *et al*., 2011). SEAs are able to evade antigen recognition by interacting with major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells (APC), and with T-cell receptors (TCR) on specific T-cell subsets (Thomas *et al*., 2007). This interaction leads to activation of a large number of T-cells followed by proliferation and massive release of chemokines and proinflammatory cytokines that may led to adverse effects such as lethal toxic shock syndrome (Balaban and Rasooly, 2000). It is suggested that SEA triggers emesis by stimulating the vagus nerve in the abdominal viscera, which transmits the signal to the vomiting centre in the brain (Hu *et al*., 2007). In addition, SEs are able to penetrate the gut lining and activate immune system which respond by releasing inflammatory mediators including histamine, leukotrienes, and neuroenteric peptide that causes vomiting (Shupp *et al*., 2002).

Methicillin resistant *Staphylococcus aureus* (MRSA) strains are now reportedly been isolated in livestock and various foods especially meat and milk, posing a threat over a potential spread of MRSA to consumers via the food chain (Voss *et al*., 2005; Weese *et al*., 2010).

Contamination of meat with MRSA is mostly cross contamination from the colonized body sites of the animal to the carcass, through the environment of processing facilities or by people involved in the handling of carcasses or meat (Weese *et al*., 2010). MRSA bears *mecA* gene which alters penicillin binding proteins (PBP) that has low affinity for all β-lactam antimicrobials (penicillins, cephalosporins and carbapenems) (Weese *et al*., 2010). Hence, transmission of these MRSA strains through the food will contribute to the growing problem of antimicrobial resistance.

2.2.2.3 Campylobacter species

Campylobacter species are Gram-negative, non-sporeforming rods. Most species require a microaerobic atmosphere for optimal growth; however, some species grow aerobically or anaerobically. An atmosphere containing increased hydrogen appears to be a growth requirement for other species such as *C. sputorum, C. concisus, C. mucosalis, C. curvus, C. showae, C. rectus, C. gracilis*, and *C. hominis* (Nachamkin, 2003).

Campylobacters are a leading cause of bacterial enteritis worldwide and can be transmitted directly from animal to person, through ingestion of fecally contaminated water, food, or by direct contact with animal feces or contaminated environmental surfaces (Esteban *et al*., 2008). Species of the organism are primarily zoonotic, with a variety of animals implicated as reservoirs of infection, including a diverse range of domestic and wild animals and birds (Butzler, 1984; Altekruse *et al*., 1999). In addition, water and the environment play a significant but poorly understood role in the epidemiology of campylobacteriosis (Koenraad *et al*., 1997).

Several epidemiological studies in different countries have identified sources of *Campylobacter* enteritis in man to include animals, food, water, and milk products (Oporto *et al*., 2007; Esteban *et al*., 2008). Reports of *Campylobacter* enteritis in developing countries

(Padungton and Kaneene, 2005; Uaboi-Egbenni *et al*., 2008), underscores an urgent need to explore prevalence rates, antibiograms and haemolytic activities in animals because of the zoonotic nature of infections and for proper planning of effective prevention and control measures (Raji *et al*., 1997; Oporto *et al*., 2009).

A prevalence study in the Basque County (Northern Spain) identified 28.3% (34/120) of ovine and 18.0% (37/206) of bovine farms positive for *C. jejuni* (Oporto *et al*., 2007), and even higher rates (38.2%, 13/34) in free-range poultry farms (Esteban *et al*., 2008). In the United States, requirements for reporting incidence of culture-confirmed infections vary by state. The active foodborne disease surveillance program FoodNet (www.FoodNet.gov) provides uniform reporting from a panel of sentinel sites, giving an accurate incidence of diagnosed infections. The reported incidence of *Campylobacter* infections in the United States has been declining for several years, from 24.7 cases per 100,000 persons in 1997 to 12.7 cases per 100,000 in persons in 2005, a rate lower than reported for salmonellosis (Shepard *et al*., 2004; Fitzgerald *et al*., 2009).

Although the majority of Campylobacter infections are self-limiting, complicated cases may warrant antimicrobial therapy. Antimicrobial susceptibility data show an increase in the number of fluoroquinolone-resistant and, to a lesser extent, macrolide-resistant *Campylobacter* strains causing human infections (Gibreel and Taylor, 2006; Anderson *et al*., 2006). Antimicrobial treatment is indicated for systemic *Campylobacter* infections in immune-suppressed patients and for severe or long-lasting infections (Allos, 2001). Erythromycin is considered the drug of choice for treating *Campylobacter* gastroenteritis, and ciprofloxacin and tetracycline are used as alternative drugs (Nachamkin *et al*., 2000); however resistance of these species has been reported to these antibiotics (Pezzotti *et al*., 2003; Oporto *et al*., 2009) .

Isolates of *C. jejuni* and *C. coli* with resistance to various antimicrobial agents have been reported in both developed and developing countries (Hart and Kariuki, 1998; Van Looveren *et al*., 2001). A significant increase in the prevalence of resistance to macrolides among *Campylobacter* species has been reported since the 1990s, and this is recognized as an emerging public health problem (Altekruse *et al*., 1999). It has been suggested that resistance to macrolides is mainly found in isolates of animal origin; especially *C. coli* from pigs and also *C. jejuni* from chickens (Van Looveren *et al*., 2001).

2.2.2.4 Listeria species

Listeria species are Gram-positive, intracellular rods that are ubiquitously found in diverse environments such as soil, water, various food products, animals, and humans (Swaminathan and Smidt, 2007). *L. monocytogenes* is one of the deadly human foodborne pathogens responsible for listeriosis, a rare but fatal disease with a mortality rate of 20-30% in newborns, the elderly and immunocompromised individuals; however, some studies have also implicated *L. ivanovii*, albeit rarely (Guillet *et al*., 2010; Nyenje *et al*., 2012a). The organism is persistent in food industries, because it survives food-processing technologies that rely on acidic or salty conditions, and, unlike many pathogens, can continue to multiply slowly at low temperatures, allowing for growth even in properly refrigerated foods (Swaminathan and Smidt, 2007).

Apart from consumption of contaminated food, infection can also be transmitted, directly from infected animals to humans, as well as between humans and from mother to child in the uterus or during passage through the infected birth canal (Jacobson, 2008; Allerberger and Wagner, 2010). Different food sources particularly poultry, red meat and meat products have been associated with outbreaks as well as sporadic cases (Pichler *et al*., 2009; El-Malek *et al*., 2010). Other studies reported vegetable products especially cabbage as a source of

contamination and retrospective study, revealed that sheep manure from a flock diagnosed earlier having listeriosis was used to fertilize the crop (Schlech *et al*., 1982; Rocourt *et al*., 2001).

Listeriosis can be manifested in two forms, invasive or non-invasive (febrile gastroenteritis); in immunocompetent individuals, non-invasive listeriosis develops as a typical febrile gastroenteritis (watery diarrhoea, nausea and headache) while in immunocompromised adults, such as the elderly and patients receiving immunosuppressive agents, listeriosis can manifest as septicaemia or meningoencephalitis (Longhi *et al*., 2004). On the other hand perinatal listeriosis acquired by the foetus from its infected mother via the placenta can lead to abortion, birth of a stillborn foetus or a baby with generalized infection, and sepsis or meningitis in the neonate (Allerberger and Wagner, 2010).

Pathogenesis of *Listeria* starts with the entry of the bacterium through the intestines to the liver where it replicates until the infection is contained by the cell-mediated immune response. The mechanism by which *Listeria* causes diarrhoea is not entirely clear. However, it is believed that diarrhoea results from direct invasion of the organism to the intestinal mucosal epithelium (Schuppler and Loessner, 2010).

It is alleged that in normal individuals, the continual exposure to listerial antigens contributes to the maintenance of anti-*Listeria* memory T-cells (Werbrouck *et al*., 2006). However in deliberated and immunocompromised patients, there is unrestricted proliferation of the organism resulting in prolonged low-level bacteraemia; subsequently, the bacteria invade target organs (the brain and gravid-uterus). *L. monocytogenes* and *L. ivanovii* are facultative intracellular parasites hence they are able to survive in macrophages and invade a number of non phagocytic cells such as epithelial cells, hepatocytes and endothelial cells (Ramaswamy *et al*., 2007).

2.2.2.5 Vibrio parahaemolyticus

V. parahaemolyticus is the leading cause of acute gastroenteritis in humans after consumption of contaminated raw or undercooked seafood; it can also cause severe infections in immune-compromised (Hiyoshi *et al*., 2010). The organism was identified initially as a cause of foodborne illness in Osaka, Japan in 1951 where it caused 272 illnesses and 20 deaths; semidried juvenile sardines were the source of infection (Nair *et al*., 2007). Since then, the organism has been reported to account for half of all food poisoning cases in Japan and identified as a common cause of sea-foodborne illness in many Asian countries (Alam *et al*., 2002; Su and Liu, 2007). Similarly, high incidences of *V. parahaemolyticus* gastroenteritis have been noted in the United States in the summer season (Daniels *et al*., 2000; Potasman *et al*., 2002). In contrast to Asian countries, *V. parahaemolyticus* infection is less common in European countries (Nair *et al*., 2007). However, sporadic outbreaks have been reported in countries such as Spain, Italy and France (Di Pinto *et al*., 2008; Ottaviani *et al*., 2010). A small number of studies on the prevalence of *V. parahaemolyticus* in sea foods in particular shrimps have been reported on the African continent mostly from West Africa (Ndip *et al*., 2002; Eja *et al*., 2008; Adeleye *et al*., 2010).

Infection with the bacterium is manifested in three major syndromes: gastroenteritis, wound infections, and septicaemia with gastroenteritis being the commonest. Although the gastroenteritis is self-limited, the organism may cause septicaemia that is life threatening in individual having underlying medical conditions such as liver, heart and kidney diseases or immune disorders (Yeung and Boor, 2004). The exact virulence mechanisms of *V. parahaemolyticus* are not known; in particular, specific mechanisms that contribute to the ability of strains lacking recognized virulence factors (e.g. *tdh* and *trh*) to mount an infection are unknown (Han *et al*., 2007). Studies have linked the virulence of this organism to the presence of a TDH-related hemolysin (TRH), and thermostable direct hemolysin (TDH)

(Boyd *et al*., 2008). It is believed that TDH and TRH acts on cellular membranes as a pore forming toxin that alters ion balance in the intestinal cells thereby leading to secretory response and diarrhoea observed in gastroenteritis (Nair *et al*., 2007). TDH has also been associated with multiple biological activities including haemolysis, enterotoxicity, cytotoxicity and cardiotoxicity hence it has been considered a major virulence factor of this organism (Raimondi *et al*., 2000; Park *et al*., 2004).

2.2.2.6 Escherichia coli

E. coli is a Gram-negative rod grouped in the family Enterobacteriaceae, and is a successful gut coloniser in many host species (Tarr *et al*., 2005). Classification of *E. coli* strains is based on specific virulence factors and phenotypic traits; these include enteroheamorraghic *E. coli* (EHEC) or shiga toxin-producing *E. coli* (STEC) strains that produce verocytotoxin or shiga-like toxin, causative agent of haemorrhagic colitis (HC) and haemolytic-ureamic syndrome (HUS); enterotoxigenic *E. coli* (ETEC) strains which produces enterotoxin causing diarrhoea; enteroinvasive *E. coli* (EIEC) strains, the causative agent of dysentery-like illnesses; enteroaggregative *E. coli* (EAEC), strains do not secrete heat-labile enterotoxins but adhere to mucosal cells in an aggregative pattern, and diffusely adherent *E. coli* (DAEC) strains that adhere to the surface of epithelial cells (Tarr *et al*., 2005; Gyles, 2006; Wu *et al*., 2011). However, foodborne outbreaks have been particularly associated with EHEC and EAEC strains (Tarr *et al*., 2005; Wu *et al*., 2011).

Among the EHEC strains, *E. coli* O157:H7 has been widely recognized as the major cause of foodborne illness (Wu *et al*., 2011). The first devastating outbreak of EHEC (*E. coli* 0157:H7) occurred in Japan, 2764 confirmed cases were reported; the source was radish sprouts (Michino *et al*., 1999). Ever since, outbreaks and sporadic cases have been reported globally; in most of these outbreaks, contaminated meat, meat products, unpasteurized milk

and leafy green vegetables and fruits fertilized with contaminated animal manure was the source of contamination (Sartz *et al*., 2008; CDC, 2011).

In 2011, unusual outbreak of enterohaemorrhagic gastroenteritis and haemolytic uremic syndrome (HUS) related to infections with shiga toxin-producing *E. coli* O104:H4 (STEC O104:H4) occurred. First reported in Germany followed by France before spreading to other European countries and North America (Wu *et al*., 2011); raw tomatoes, cucumber and leaf salad was the source of contamination (Frank *et al*., 2011). This was the first and largest outbreak of infections due to serotype *E. coli* O104:H4 worldwide with 3167 EHEC and 908 HUS cases which claimed 50 lives (WHO, 2011).

Studies on the virulence of *E. coli* O104:H4 strain suggests that these strains have unique properties of both EHEC and EAEC genes, encoding the production of shiga-toxin (stx) and resistance to multi-antibiotics (Brzuszkiewicz *et al*., 2011; Ruggenenti and Remuzzi, 2011). The pathogenesis of EAEC strains includes: bacteria adherence to the intestinal mucosa using aggregative adherence fimbriae (AAF); the fimbriae allow bacteria to adhere to each other in a "stacked-brick" pattern and produce mucus, hence forming a biofilm on the surface of enterocytes; followed by the release of toxins and elicitation of inflammatory response, mucosal toxicity, and intestinal secretion (Brzuszkiewicz *et al*., 2011; Frank *et al*., 2011). Hence *E. coli* O104:H4 is a typical EAEC strain that forms AAF to enhance bacteria attachment to the intestinal wall and STEC/EHEC that produces stx.

Virulence factors of *E. coli* can be encoded by mobile genetic elements such as plasmids and bacteriophages which can be transferred horizontally. This is exemplified by the findings of Brzuszkiewicz *et al*. (2011), who reported that, the *E. coli* O104:H4 strain acquired the stx-producing gene from the stx-phage which is characteristic for EHEC strains, and speculated that the enhanced adherence factor may have facilitated the absorption of stx which resulted

in the higher percentage of HUS cases; the combination of the virulence factors (AAF, stx, extended-β-lactamases) and formation of the stacked-brick pattern may have led to a stronger gut colonisation and release of toxins (Ruggenenti and Remuzzi, 2011).

2.2.3 Parasites

Parasitic foodborne diseases are generally under-recognised; however they are becoming more common in humans worldwide, with infections in childhood, pregnancy and with those related to HIV/AIDS being of major importance (Khan *et al*., 2007; Dorny *et al*., 2009). Associated morbidity and mortality are high, with more than 58 million cases of childhood protozoal diarrhoea reported per year, at an estimated financial management cost of US$ 150 million (Savioli *et al*., 2006). Parasites of concern include *Giardia lamblia* (*G. lamblia*), *Entamoeba histolytica* (*E. histolytica*), *Cryptosporidium pavum* (*C. pavum*), *Toxoplasma gondii* (*T. gondii)* and *Trichinella spiralis* (*T. spiralis*). Infections are mostly asymptomatic, although some acute short lived and chronic conditions have been documented (Doyle, 2003).

2.2.3.1 Entamoeba histolytica

E. histolytica is an important cause of diarrhoea in people of the tropical and subtropical countries. Cases in the United States generally occur in immigrants, travellers returning from endemic areas, and in persons living in states along the border with Mexico (Doyle *et al*., 2003). According to the WHO, *E. histolytica* is the second leading parasitic cause of death (after malaria) and has been estimated to infect 50 million people worldwide of whom 40,000 - 100,000 die yearly (Babiker *et al*., 2009). Although most cases of *E. histolytica* remain asymptomatic, they excrete a large number of cysts in their feces hence serving as a source of infection (Doyle *et al*., 2003). In Mexico, it was observed that 340 asymptomatic carriers excreted an average of nearly 4,000 *Entamoeba* cysts/g feces (Garrido *et al*., 2002). Man is the major reservoir of *E. histolytica*, passing virulent cysts that are transmitted chiefly by

ingestion of contaminated food or water or through an infected food-handler; it also occurs when produce is freshened or crops are irrigated with contaminated water. Other vectors such as flies, cockroaches and other insects may also transfer cysts from feces to foods (Babiker *et al*., 2009).

Human infection usually begins with the ingestion of the cyst in food or water contaminated with human fecal material. Cysts survive the acidic pH of the stomach and pass into the intestine where the cysts undergo excystment and mature into trophozoites which are passed to the colon. In the intestine, many of the trophozoites encyst and both trophozoites and cysts are excreted along with the feces; cysts can survive for prolonged periods outside the host while the trophozoites survive only for a few hours (Ackers and Mirelman, 2006). Infections can range from non-invasive intestinal diseases which are often asymptomatic to invasive where the trophozoites penetrate the intestinal mucosa to other organs and produce an extraintestinal amoebiasis which are usually more serious and life-threatening (Ackers and Mirelman, 2006).

2.2.3.2 Cryptosporidium

Cryptosporidium are protozoan members of the Phylum Apicomplexa, affecting a wide variety of vertebrate hosts. In humans, *C. hominis* (anthroponotic origin) and *C. parvum* (zoonotic origin) are responsible for more than 90% of cryptosporidiosis which accounts for more than 3.1 million deaths each year among children less than 15 years of age (Fayer, 2004). *C. hominis* is more prevalent in North and South America, Australia, and Africa, whereas *C. parvum* causes more human infections in the United States and Europe, especially in the United Kingdom (Tumwine *et al*., 2005; Samie *et al*., 2006; Morse *et al*., 2007; Omoruyi *et al*., 2011).

In developing countries, cryptosporidiosis is most prevalent during early childhood, with as many as 45% of children experiencing the disease before the age of 2 years (Valenntiner-Branth *et al*., 2003); exemplified by most sub-Saharan countries, where cryptosporidiosis prevalence peaks among children aged 6 - 12 months and decreases thereafter. Experimental studies revealed that repeated exposure to *C. parvum* promotes an immunoglobin G (IgG) response that imparts partial protection against subsequent infection and illness (Chappell *et al*., 1999). Cryptosporidiosis is chronic and life-threatening among immunocompromised individuals but self-limiting in immunocompetent individuals (Paul and Gordon, 2002). It accounts for up to 6% and 24% of all diarrheal diseases in immunocompetent and in persons with AIDS respectively worldwide (Bialek *et al*., 2002).

The parasite is transmitted through the fecal-oral route or indirectly via contaminated water supply, food or environment (Miller *et al*., 2006). The ingested parasite in the form of oocytes, excyst in the gastrointestinal tract and release infective sporozoites, which attach to the apical membrane of the host epithelial cells where they mature into merozoites by asexual reproduction. The merozoites are released into the intestinal lumen where they can either infect other epithelial cells or mature into gametocytes those later releases the oocysts which are excreted in feces into the environment to start another life cycle (Morgan *et al*., 2002).

2.2.3.3 Toxoplasma gondii.

Toxoplasmosis is a widely prevalent disease caused by *T. gondii*, an obligate intracellular parasite that forms cysts in mammalian cells. *T. gondii* infects approximately one third of the global human population and a wide range of other mammalian and avian species (Marawan *et al*., 2008). The major sources of human infection are the ingestion of tissue cysts in raw or undercooked meat, food or water contaminated with sporulated oocysts or by transplacental transmission (Jiménez-Coello *et al*., 2012). In the United States, toxoplasmosis is the second leading cause of foodborne illness related deaths and fourth leading cause of foodborne

illness related hospitalizations (Scallan *et al*., 2011). It is also noted that South America, Asia and Africa have a higher prevalence of sero-positive individuals; high temperature and humid conditions in these countries may favour the persistence of viable sporulated oocysts in the environment (Ayi *et al*., 2009; Mercier *et al*., 2010; Jiménez-Coello *et al*., 2012).

The life cycle is complex involving two hosts; an intermediate host, usually warm-blooded animals and a definitive host (domestic cats). There are three infectious stages: tachyzoites, bradyzoites contained in tissue cysts, and sporozoites contained in sporulated oocysts (Alayande *et al*., 2012). The transmission cycle starts, with the shedding of oocysts by the definitive host in the feces. The oocysts sporulate and become infective within a few days in the environment. Intermediate hosts get infected after ingesting water or food contaminated with the cat feces. In the gut, oocysts transform into tachyzoites which later migrates to other parts of the body via the bloodstream and further develop into tissue cyst (bradyzoites) in skeletal, ocular muscle and neural tissue where they can persist for decades (Tenter *et al*., 2000). The mechanism of this persistence is unknown; however, some investigators believe that tissue cysts break down periodically, with bradyzoites transforming to tachyzoites that reinvade host cells and again transform to bradyzoites within new tissue cysts (Tenter *et al*., 2000).

2.2.3.4 Trichinella spiralis

Trichinella species are the causative agents of human trichinellosis, a zoonotic disease caused by the ingestion of raw or undercooked meat containing larvae of *Trichinella* nematodes. The most common sources of human infection are pig meat, wild game and horse meat. There are at least eight recognised species of *Trichinella*; the most commonly isolated specie is *T. spiralis* (Dorny *et al*., 2009). Trichinellosis affects as many as 11 million people worldwide (Hernandez-Bello *et al*., 2008). Numerous trichinellosis outbreaks have been noted in Asia,

particularly in China and Thailand. From 1964 to 2003, China experienced 247 deaths from trichinellosis and in Thailand 97 people died from 1962 to 2005 (Kaewpitoon *et al*., 2006). Between 2004 - 2005, 5,690 cases and 5 deaths were documented from 147 outbreaks, with wild game (bear, cougar or wild boar meat) and commercial pork products identified as the source of infection (Dupouy-Camet, 2009). In 2003, an outbreak in Poland involving 124 people was reported to have been caused by infected wild boar meat (Doyle, 2003).

The ingested *Trichinella* larvae encyst in muscle tissue and rapidly develop into adults in the intestine, where they mate and produce newborn larvae. The newborn larvae then migrate from the intestines through the lymphatic system to the blood stream, before invading striated skeletal muscle cells to complete the cycle (Movsesijan and Milosavijevic, 2010). The clinical signs of acute trichinellosis in humans are characterised by two phases. The first phase, whose symptoms include nausea, diarrhoea, vomiting, fatigue, fever and abdominal discomfort. Headaches, fevers, chills, cough, eye swelling, joints and muscle pains, itchy skin, diarrhoea, or constipation follow the first symptoms, and if the infection is heavy, patients may experience difficulty coordinating movements, and have heart and breathing problems (Dupouy-Camet, 2009; Bruschi, 2012).

2.2.3.5 Giardia intestinalis

G. intestinalis (also known as *G. lamblia* or *G. duodenalis*) are ubiquitous enteric protozoan pathogens that infect humans, domestic animals and wildlife worldwide. Giardiasis is linked to the socioeconomic level of a country, with prevalence ranging between 2 - 7% in most industrialized regions and reaching 40% in developing countries; most of these infection occur in children (Jůlio *et al*., 2012). A study in a refugee camp of Guma in Nigeria noted a prevalence of 40% in children (Nyamngee *et al*., 2009), while in Rwanda, a 60% prevalence was reported among children under the age of 5 years (Ignatius *et al*., 2012). In the United

States, *Giardia* is responsible for approximately 2.4 million infections annually, and they primarily occur in children day-care centres (Furness *et al*., 2000). The infection results from the ingestion of the cyst in fecally contaminated food or water or through person-to-person and to a lesser extent, animal-to-person transmission. The parasite has a two-stage life cycle: a reproductive trophozoite and an environmentally resistant cyst stage. An ingested cyst passes into the duodenum, where excystation occurs, releasing four trophozoites which multiply rapidly via asexual reproduction and colonise the small intestine. It is during the trophozoite stage that clinical symptoms occur, as a result of damage to the mucous membrane (Dawson, 2005).

Although often asymptomatic, *Giardia* infections may lead to acute or chronic diarrhoea with abdominal cramping, dehydration, nausea and/or vomiting, malabsorption, weight loss, and fatigue. The pathogenesis of *Giardia* is not completely understood due to the extensive variation seen in disease expression. Nonetheless, several pathogenic mechanisms have been implicated in giardial diarrhoea, including reduction in intestinal disaccharides and protease activities, disruption of microvillous brush border, villus shortening or atrophy, crypt hyperplasia, increased epithelial permeability, mucosal inflammation, bacterial overgrowth and intestinal hyper-motility (Adam, 2001; Mank, 2001; Koot *et al*., 2009).

2.2.4 Fungi

Filamentous fungi and moulds are able to produce an enormous number of secondary metabolites, including antibiotics and mycotoxins. The term mycotoxin refers to those secondary metabolites which, at a low concentration, are toxic to humans and animals (Sánchez-Hervás *et al*., 2008). Worthy of note is the fact that the existence of mycotoxin-producing fungi in plants is not always favourable to contamination with mycotoxins. In order for fungi to produce these secondary metabolites, they have to be stressed by some factor, such as nutritional imbalance, drought or water excess (Dutton, 2009). Mycotoxins have been implicated as causative agent of human foodborne intoxication, as well as human hepatic and extra-hepatic carcinogenesis (Wild and Gong, 2010); clinical symptoms include diarrhoea, liver and kidney damage, pulmonary oedema, vomiting, haemorrhaging and tumours (Bryden, 2012).

The most frequent toxigenic fungi are *Aspergillus*, *Penicillium* and *Fusarium* species (Sánchez-Hervás *et al*., 2008). The foodborne mycotoxins of greatest significance in Africa and other tropical developing countries are the fumonisins (FB), aflatoxins (AFs) and trichothecenes (Wagacha and Muthomi, 2008). These toxins contaminate various food stuffs, including maize, cereals, groundnuts and tree nuts feed, during production, harvest, storage or processing (Sánchez-Hervás *et al*., 2008); mycotoxins can also occur in milk, meat and their products as a result of animals consuming mycotoxin contaminated feeds (Wild and Gong, 2010). Since, the toxins frequently occur in maize, a staple food in most parts of Africa, Asia and Latin America; hence, the contamination translates to high-level chronic exposure in these countries (Wild and Gong, 2010).

2.3 Detection methods

The detection and enumeration of microorganisms in food is an essential part of any quality control or microbial safety in the food chain. Rapid and early detection of food contaminants is therefore imperative for the containment of foodborne pathogens (Andrea and Mariani, 2009). Microbial analysis is a valuable tool used to monitor the actual situation and trend in order to detect emerging risks, test compliance of the performance management based upon Hazard Analysis Critical Control points (HACCP); more so, accurate and ultimate microorganism identification and pathogen detection is essential for correct disease diagnosis, treatment and tracking of disease outbreaks (Jasson *et al*., 2010).

Although the implementation of preventive systems such as the HACCP has greatly improved food safety, the risk of foodborne diseases still remains, thus, underscoring the need for new detection methods that will significantly improve our food safety once incorporated in the HACCP (Bhunia, 2008). Several methods have been employed in the detection and characterisation of foodborne pathogens. They include: phenotypic characterisation (microscopy, traditional culture methods, biochemical and immunological tests), and genotypic characterization (nucleic acid detection methods *i.e*. PCR). Currently, the traditional microbiological culturing methods coupled to biochemical and serological identification is widely employed. The process requires dedicated laboratories, equipments and highly-trained personnel for detection and identification of each hazardous agent let alone the time it requires before any identification can be confirmed. Since more and more products nowadays contain multiple and processed ingredients, which are often shipped from different parts of the world, and share common production lines and storage spaces, food safety and environmental monitoring becomes a challenging task. However, recent advances in miniaturization of analytical systems and newly emerging technologies in particular, multiplex polymerase chain reaction (PCR), microarrays and biosensors techniques provide

control agencies and food industries with new possibilities for improved, more efficient monitoring of food and environmental contaminants.

2.3.1 Culture methods

In spite of numerous efforts by several researchers, only two foodborne viruses are cultivatable (HAV and rotavirus) in cell or tissue culture, therefore diagnosis of viruses has been made largely by electron microscopy (Koopman and Duizer, 2004). Traditional culture methods are mostly for bacteria identification. These methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in foods; they are sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of the microorganisms present in a food sample. Culture methods are often based on introduction of the homogenized food sample into an enrichment broth followed by plating on selective agar medium. The isolated colonies are confirmed using biochemical and serological tests. Hence, a complete series of tests is often required before any identification can be confirmed (Jasson *et al*., 2010).

However, culture medium preparation, inoculation of plates, colony counting and biochemical characterisation make these methods labour intensive, especially in the food industry where there is need for more rapid methods to provide adequate information on the possible presence of pathogens in raw materials and finished food products, for manufacturing process control and for the monitoring of cleaning and hygiene practices (Mandal *et al*., 2011). Furthermore, phenotypic properties which are crucial for the identification of microbes may not be constantly expressed; and if expressed, they may be difficult to read. The other drawback is cells which are viable but otherwise non-culturable which cannot be detected using this method. Therefore, extensive research has been carried out over the years to reduce assay time through the use of improved alternative methods for

detecting foodborne microorganisms and reduce the amount of manual labour by automated methods whenever possible (Jantzen *et al*., 2006; Jasson *et al*., 2010).

2.3.1.1 Quantitative culture methods

Quantitative methods involve the enumeration of the microorganisms present in a sample mostly performed by plate count method or the most probable number (MPN) method. In some cases, the number of microbes in a sample may be very high such that when inoculated into an agar medium, confluent growth is observed; hence it is necessary to dilute the sample so that discrete colonies can be isolated from the sample. On the other hand, the MPN method calculates the number of viable microorganisms by transferring subsamples of 3 serial dilutions to 9 or 15 tubes containing liquid culture medium. The tubes are incubated, and those that show growth (turbidity) are counted. The final result after multiplying by the dilution factor is compared to a standard MPN table to determine the MPN of bacteria in the product (Blodgett, 2010). Despite being more sensitive, MPN is less accurate, labour intensive and expensive; thus, it is mostly used to estimate bacteria in low levels (<10 per gram of food) (Betts and Blackburn, 2009).

2.3.1.2 Qualitative culture methods

Unlike quantitative methods, this procedure only detects the presence or absence of microorganisms in the sample. To increase the chances of isolation as most target cells typically occur in low numbers, and may be present in a mixed microbial population, the primary enrichment cultures are usually inoculated into secondary selective enrichment broths and incubated at elevated temperatures (37 °C or 42 °C for 18-24 h) before being struck onto selective or differential media plate. The presumptive colonies of the target microorganism based on colony pigmentation on a selective/differential media are confirmed biochemically and/or serologically (Betts and Blackburn, 2009).

2.3.1.3 Modified and automated culture methods

Several studies have documented the use of modified and automated methods. Some of these modified culture methods are based on the addition of substrates, chromogenic and fluorogenic in culture media that facilitates the rapid identification of presumptive colonies of the target microorganism. The fluorogenic enzyme substrates generally consist of a specific substrate for the specific enzyme such as sugar or amino acid and a fluorogen that converts UV light to visible light; for example Fluorocult *E. coli* O157:H7 agar for the isolation of *E. coli* O157:H7 (Manafi, 2000). While chromogenic enzyme substrates are compounds which act as the substrate for specific enzymes and change colour due to the action of the enzyme. For instance, *Yersinia enterocolitica* chromogenic medium (YeCM) (Weagant, 2008), modified mE medium (mEI) for the detection of *Enterococcus* (Rhodes and Kator, 1997), and *Salmonella* chromogenic medium for the isolation of *Salmonella* (Cassar and Cuschieri, 2003). These methods eliminate the use of subculture media and biochemical test (Manafi, 2000; Jasson *et al*., 2010). However, there is need to validate and evaluate them such that they may be recognized as reference methods if they are perceived as providing more accurate results than the traditional culture methods. Automation may be very useful in reducing the time required to prepare culture media, perform serial dilutions and count colonies (Jasson *et al*., 2010).

2.3.2 Microscopic evaluation

2.3.2.1 Direct microscopy

The method is rarely used to screen for parasites such as *Cryptosporidium* oocysts and *Giardia* cysts from food samples because of the problems encountered in extracting the cysts (which are mostly in low numbers) from food matrices. However, methods have been developed for certain 'high risk' foods, notably ready-to-eat salads and fresh produce. Some cysts such as *Giardia*, are very small, hence, their detection requires staining procedures

(Fayer, 2004). Diverse staining techniques have been developed; however procedures like the Ziehl-Neelsen, trichrome, auramine phenol and modified Kinyoun's stain are frequently used (Fayer, 2004). Currently immunological based techniques including direct fluorescent-antibody (DFA) testing which utilises fluorescent-labelled antibodies specific for cell wall antigens of *Giardia* cysts and *Cryptosporidium* oocysts are used to enhance visualization (Johnston *et al*., 2003).

2.3.2.2 Electron microscopy

Viruses and parasites are mostly diagnosed using the electron microscopy (EM) method (Tzipori and Ward, 2002). The EM method relies on the direct visualization of intact viral particles or parasites from food samples, sometimes incorporating immunostaining, which utilizes specific antibodies to bind target viruses before examination. However, the method is tedious, labour intensive and requires highly skilled personnel to differentiate between virus types.

2.3.3 Biochemical methods

The use of phenotypic and biochemical tests for the detection of bacterial pathogens of food origin have been the standard for many years. In the recent years, there have been new developments in biochemical test systems as many commercial multi-test systems are now available for use. Biochemical testing is based on the metabolic actions of the bacteria (Awong-Taylor *et al*., 2008). The tests can be grouped into three based on the activity: (1) carbohydrate utilization and oxidation fermentation tests (phenol red, triple sugar iron agar (TSI), Indole, methyl red, Voges-Proskauer, and citrate), (2) Enzyme tests (urease, gelatinase, and DNase), and (3) respiration tests (catalase, oxidase, and nitrate test) (Brown, 2007).

Biochemical test can be tedious and lengthy if individual test is done disjointedly. Interestingly, these test are now customized where almost all the significant biochemical test

could be found on cards, microtiter trays, or multichamber strips (e.g., Analytical Profile Index system, MicroScan Rapid GN Identification Systems, and Vitek GNI Card) (O'Hara *et al*., 1998; Mandal *et al*., 2011). While some studies have shown high accuracy rates associated with these systems (O'Hara *et al*., 2003; Croci *et al*., 2007), other studies have reported less satisfactory results (Israil *et al*., 2003; Martinez-Urtaza *et al*., 2004). Additionally, biochemically based systems may not be reliable for environmental isolates because of inadequate information on environmental bacteria in computerized databases (Croci *et al*., 2007).

2.3.4 Immunological assays.

The speedy detection of a pathogen is crucial, in particular where food samples with short shelf-lives are being analysed, or where the urgent administration of a suitable antimicrobial agent is required to treat a potential lethal infection. Furthermore some bacterial strains are viable but non-culturable; thus, development of accurate, rapid and sensitive immunoassay is essential for the detection of these non-culturable bacteria, viruses, parasites and toxins (Bryne *et al*., 2009). Therefore, immunology-based methods have been effectively engaged for the detection of bacterial cells, spores, viruses and toxins (Iqbal *et al*., 2000). The fundamental principle of antibody immunoassays is the binding of antibodies (produced by the body) to a target antigen (pathogen), followed by the detection of the antigen-antibody complex. The significant characteristic of an antibody is its ability to recognize only the target antigen in the existence of other antigens (organisms or other interfering food components). In addition, the successful use of antibodies to detect pathogens depends on the stable expression of target antigens in a pathogen, which are often influenced by temperature, preservatives, acids, salts, or other chemicals found in foods (Bryne *et al*., 2009). Despite the fact that these methods are less specific and sensitive than molecular techniques, they are

quicker, more robust and capable of detecting not only contaminating organisms but also their biotoxins that may not be expressed in the organism's genome (Iqbal *et al*., 2000).

Different antibody types are available for immunodetection; these embrace conventional and heavy chain antibodies as well as polyclonal, monoclonal or recombinant antibodies. However, most commercial immunoassays products available use recombinant and monoclonal antibodies because of their high sensitivity, specificity, reproducibility and reliability over a wide variety of microbes (Leonard *et al*., 2003). Examples of commercial immunological techniques commonly used in the detection of foodborne pathogens include the enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), enzyme-linked fluorescent assay (ELFA), bioluminescent enzyme immunoassay (BEIA), enzyme-linked immunomagnetic chemiluminescence (ELIMCL), immunochromatography (ICG) strip test, immuno-precipitation assay, agglutination test, radio-immunoassays (RIA), western blot assay and the recombinant immunoblot assay (RIBA) (Palumbo *et al*., 2003; Hunter, 2011).

Although most immunoassays are rapid, some such as ELISA are often lengthy; they encompass a number of steps; blocking, washing, incubation of primary and secondary antibodies and substrate development which can take several hours to complete. In addition others have demonstrated low affinity of the antibody to the pathogen and also potential interference from contaminants (Meng and Doyle, 2002). Barger *et al*. (2011) compared three immunoassays: radioimmunoprecipitation (RIP), electrochemiluminescence (ECL) and surface plasmon resonance (SPR) and found that samples that tested positive by the SPR immunoassay and negative by RIP and ECL were categorized to be of low antibody concentration. As such, some immunoassays are coupled with other methods for pathogen detection, *e.g*. immunomagnetic separation on magnetic beads is coupled with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) for detection

of staphylococcal enterotoxin B (Schlosser *et al*., 2007), combination of immunomagnetic separation with flow cytometry for detection of *L. monocytogenes* (Hibi *et al*., 2006).

However, in case of co-infections, more than one immunoassay may be used to detect the different microbes; this can be expensive hence rapid, sensitive and simultaneous detection kits which can detect three or more microbes including their toxins in a food sample have been developed (Lin *et al*., 2006; Wang *et al*., 2011). In their study, Wang *et al*. (2011) used separately coated streptavidin-conjugated with biotinylated anti-*Salmonella*, anti-*E. coli* and anti-*Listeria* antibodies mixed with a food sample to capture the three target bacteria. This multiplex immunoassay simultaneously detected *Salmonella* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* at levels as low as 20 to 50 CFU/mL in food samples in less than 2 hours without enrichment. Hence if multiplex immunoassays can be standardized and expanded to cover a wide range of foodborne pathogens in one test, they will be useful in mass screening and outbreak investigations.

2.3.5 Nucleic acid based assays

Many molecular methods have been developed and applied to the detection and characterization of foodborne pathogens in laboratories and food industries. These methods permit the detection of pathogen's nucleic acids (DNA and RNA) hence; the detection of pathogens is performed rapidly with high selectivity (Andrea and Mariani, 2009). Molecular methods mostly include: DNA banding pattern-based tests which utilises the polymerase chain reaction (PCR) technique, DNA sequence-based tests which are based on hybridization techniques and DNA microarray (Shi *et al*., 2010; Gehring and Tu, 2011).

2.3.5.1 PCR methods

Dating back to the 1990s, PCR has seen a remarkable increase in its applications; such that nowadays, the method is fundamental for the development of most molecular diagnostic methods for food safety and other fields (Andrea and Mariani, 2009). The technique relies on the amplification of primer sequence complementary to the sequence on the template DNA. Each amplification cycle consists of a heat denaturation phase when single strands are generated from a double-stranded DNA, an annealing phase when the primers bind to the single-stranded target sequences, and an extension phase when the DNA polymerase (Taq polymerase) makes a strand that is complementary to the template. This approach has been commonly used to detect a wide range of organisms that contaminate food, human and animal, and other environments (Batt, 2007; Shi *et al*., 2010).

Most comparative studies have reported PCR assays to be more sensitive over culture, microscopy and immunoassay methods in the detection of various food pathogens (Fratamico, 2003; Mateo *et al*., 2005; Zhang *et al*., 2008; Kawasaki *et al*., 2009). In their study, Fratamico (2003) found that the PCR-based assays were more sensitive than the culture method and the immunoassay in the detection of *Salmonella* from ground chicken, turkey and beef. Similarly, Zhang *et al*. (2008) reported that a modification of PCR using an immune-PCR assay increased the signal for the detection of shiga toxin 2 as it was able to detect as low 10 pg/mL of purified shiga toxin 2, compared to 1 ng/mL shiga toxin 2 detected by commercial EIA.

Despite its numerous advantages, the method is expensive to be implemented as a routine test and cannot distinguish between live and dead cells. In addition, the inhibition of PCR by food-derived inhibitors can lead to false negative results; thus, PCR application to foods will require effective methods for overcoming the effects of PCR inhibitors (Biswas *et al*., 2008).

There are two major types of PCR; standard (end-point) that requires the identification of the amplified fragment using gel electrophoresis and real-time, which quantify the amplification progress by monitoring changes in fluorescence within the PCR reaction vessel (Tichopad *et al*., 2003).

2.3.5.1.1 Standard PCR

Standard PCR methods are qualitative because the final yield of product is not primarily dependent upon the concentration of the target sequence in the sample. This technique is simple to implement. However, limited specificity has been observed in molecules of approximately the same molecular weight, as they cannot be distinguished (Tichopad *et al*., 2003). Some of the widely used standard PCR methods applied to detect food pathogens include, amplified fragment length polymorphism (PCR-AFLP), random amplified polymorphic DNA analysis (RAPD), pulsed-field gel electrophoresis (PFGE) (Markey *et al*., 2010), multiplex PCR in which more than one pair of a primer is used to amplify the different fragment of target gene simultaneously to save time and minimize the expense on detection of foodborne pathogens (Slavik *et al*., 2003; Kawasaki *et al*., 2009).

2.3.5.1.2 Real-time PCR

Real-time PCR combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel (Slavik *et al*., 2003). It allows the accumulation of amplified product to be detected and measured as the reaction progresses. The detection of PCR products is achieved by including SYBR-Green dye or other fluorescent labelled probes that emit lights during amplification. The increase in fluorescent signal is proportional to the amount of DNA. However, the specificity of the test largely relies on the use of a specific probe *e.g*. the Loop mediated isothermal amplification method has been used to detect foodborne pathogens (Tichopad *et al*., 2003).

2.3.5.2 Hybridization techniques

DNA hybridization (also known as gene probe assays) refers to the detection of DNA or RNA targets using complementary nucleic acid probes. This procedure usually requires the denaturation of double-stranded DNA (ds-DNA) probes which are subsequently labelled with a radioactive substance. The labelled gene probe is added and if the sample contains the same sequence as the gene probe, it will form a complex which is detected using Autoradiography. Gene Trak, which is suitable for detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* is one of the DNA hybridization tool-kit available on the market (Mandal *et al*., 2011).

2.3.5.3 DNA microarray

DNA microarray represents one of the latest advances in molecular technology, it has received considerable attention due to its ability to simultaneously analyse a very large number of nucleic acid sequence targets and detect multiple genetic targets or genomes from multiple pathogens on a single slide (Goji *et al*., 2012). The technique has great potential to be used for the discrimination of closely related strains by employing oligonucleotides specific for each target organism (Call, 2005). DNA microarray technology has been applied to foodborne pathogen detection (Kosti´c *et al*., 2010; Goji *et al*., 2012).

It is important to note that few pathogenic organisms are mostly present in a complex food matrix, underscoring the need for highly sensitive and specific detection methods. DNA microarray techniques are advantageous as they are capable of identifying multiple pathogens with very high specificity in a single experiment, while also demonstrating genetic differences and similarities among bacterial strains (Wang *et al*., 2007). For instance, Lin *et al*. (2011) set up a diagnostic test for the detection of a total of 23 bacteria and viruses and the method successfully discriminated real and artefacts. Similarly, Wang and colleagues presented an array-based assay for the identification of 23 foodborne pathogens (Wang *et al*.,

2007). The ability of microarrays, to miniaturize many different pathogen specific probes on a single support well accompanies their high sensitivity and specificity. Hence, it is easy to imagine the opportunities microarrays can offer, making them the technique of choice for pathogen detection and diagnostics in the upcoming future. Presently, the drawback of this technology is the need for specially trained operators and the expensive equipment required (Kostrzynska and Bachand, 2006).

2.3.6 Biosensors

Biosensors have recently been looked upon as attractive alternatives to the existing conventional methods. Biosensors in their simplest form are analytical devices that convert a biological response to a measurable electrical signal proportional to the concentration of the analytes. A biosensor consists of a bioreceptor or biorecognition element and a transducer. A bioreceptor can either be a tissue, microorganism, organelle, enzyme, antibody, *etc*, while the transducer may be optical, electrochemical, thermometric, *etc* (Su *et al*., 2010). These methods are very useful in foodborne pathogen detection because of their numerous advantages. For instance, they have sensitivity in the range as low as a ng/mL for microbial toxins; provide fast or real-time detection; besides, the miniaturization of biosensors allow for integration in food production equipment and machinery (Rasooly and Herold, 2006). The technique is also widely used to measure the cleanliness of surfaces that come into contact with food, including the presence of organic residues and microbial contaminants (Cunningham *et al*., 2011). The main disadvantage of biosensors is the instability of the biological sensing component, which tends to degrade and lose its effectiveness over short period of time (Hunter *et al*., 2011).

2.4 Treatment

Foodborne illnesses are mostly manifested as acute gastroenteritis and are usually self-limiting. However, in others, fluid replacement and supportive care may be essential. Oral rehydration is indicated for patients who are mildly to moderately dehydrate; whilst in more severe dehydration, intravenous therapy may be administered (Mølbak, 2005).

Worthy of note is the fact that many anti-diarrheal agents have potentially serious adverse effects in infants and young children; therefore their routine use in this age group is discouraged (CDC, 2004). For severe cases, extraintestinal disease, or for immunocompromised persons, antimicrobial therapy is prerequisite and should be based on: clinical signs and symptoms; organism detected in clinical specimens; antimicrobial susceptibility tests. Such information can also support public health surveillance of infectious disease and antimicrobial resistance trends in the community (CDC, 2004; Gilbert *et al*., 2004; Foley and Lynne, 2008).

A number of drugs have been recommended for severe extraintestinal foodborne disease; they include fluoroquinolones or third-generation cephalosporins, ampicillin, gentamicin, sulfamethoxazole/trimethoprim (Mølbak, 2005; Akoachere *et al*., 2009b). Since fluoroquinolones are reported to cause damage to the cartilage in children under the age of 16 years, third-generation cephalosporins including ceftriaxone are an alternative therapy for this age group (Gilbert *et al*., 2004).

Listeriosis is another deadly disease where intravenous antimicrobial therapy is paramount. Drugs like ampicillin, penicillin, erythromycin, sulfamethoxazole/trimethoprim are employed for invasive diseases whereas tetracycline, doxycycline and gentamicin are commonly used (Ruiz-Bolivar *et al*., 2011). On the other hand, viral infections are managed by supportive care whereas parasitic infection can be treated with metronidazole in cases of *E. histolytica*

and *G. lamblia*; spiramycin or primethamine plus sulfadiazinein in toxoplasmosis while cryptosporidiosis in adult is treated using paromomycin and in children, nitazoxanide is administered (CDC, 2004; Rossignol, 2010). We reported recently marked susceptibility of *L. ivanovii* and *E. cloacae* isolates from food against chloramphenicol, ciprofloxacin, streptomycin and sulfamethoxazole/trimethoprim hence these drugs could be considered in the treatment of infections caused by these organisms in the study area (Nyenje *et al*., 2012b).

The treatment of severe cases of food poisoning (SFP and botulism) involves supportive therapy, with mechanical ventilation where pulmonary involvement is expected. Administration of immune globulin to neutralize circulating botulism toxin can also assist (Arnon *et al*., 2006). Foodborne illnesses have fatal consequences in vulnerable groups, interestingly, some recommended prophylactic treatment have also shown positive effects against foodborne pathogens. For example, sulfamethoxazole/trimethoprim presently used in many transplant centres to prevent *Pneumocystis pneumonia*, is also effective against *L. monocytogenes* and *T. gondii.* Sulfamethoxazole/trimethoprim also appears to reduce the incidence of *Salmonella* infections after transplant, although resistance has occurred in some *Salmonella* species (Safdar and Armstrong, 2003; Conter *et al*., 2009; Morvan *et al*., 2010).

Antimicrobial resistance is a major challenge in the management of severe foodborne illness; even though much of the resistance experienced in human medicine is accredited to improper use of antibiotics in humans, antimicrobial use in animals selects for resistant foodborne pathogens that may be transmitted to humans as food contaminants (Mesa *et al*., 2006). Worthy of note is the fact that most of these foodborne pathogens are of food-animal origin (notably, *Campylobacter*, *Salmonella*, *Listeria*, *E. coli* O157 and *T. gondii*). In the United States, 14% of chicken samples contained ciprofloxacin-resistant *C. jejuni* two years after the introduction of fluoroquinolones use in poultry-farming which also saw an increase of

quinolone-resistant *C. jejuni* in human infections during the same period, suggesting that chickens were the possible reservoir (Collignon *et al*., 2009); therefore it is likely that the proper use of other antimicrobials in food animals may equally reduce the resistance to these drugs.

Outbreaks of a multidrug resistance *Salmonella* Typhimurium DT104 strain (chloramphenicol, ampicillin, streptomycin, sulfonamides, and tetracycline) have been documented (Davis *et al*., 2007; Sofos, 2008). *Salmonella* species resistant to nalidixic acid, gentamicin, kanamycin, sulfamethoxazole/trimethoprim and ciprofloxacin has also been reported (Savadkoohi and Kacho, 2007; De Paula *et al.*, 2010).

A few outbreaks involving community acquired MRSA in SFP have been reported (Jones *et al*., 2002). In France two MRSA strains were isolated from food incriminated in SFP (Kerouanton *et al*., 2007). MRSA colonize a number of animals including humans where they may serve as a reservoir for MRSA, leading to its persisting and spread in the community (Lee, 2006). It should also be noted that the resistance of MRSA infections are not only related to β-lactam antibiotics but also to other antibacterial drugs such as fluoroquinolones, gentamicin, clindamycin, erythromycin and sulfamethoxazole/trimethoprim (Huang *et al*., 2007; Kuint *et al*., 2007).

2.5 Prevention and control

Most of the foodborne pathogens are ubiquitous in nature; as such there is a possibility of cross-contamination between one or several products during processing. Preventive measures, such as the pasteurization of milk and dairy products, irradiation, healthier manufacturing processes, and attention to clean water have helped to minimize cases of tuberculosis, typhoid fever, and cholera that were once common causes of foodborne illnesses (Linscott, 2011).

Internationally, countries are now adopting the HACCP system; endorsed by the Codex Alimentarius Commission as a tool that can help prevent known hazards and reduce the risks that may occur at specific points in the food chain. Since the approach enforces procedural governance and rigorous documentation practices, HACCP serves not only as a model to assess risk, but also as an effective means to communicate risk control (WHO, 2007). In addition, quality control systems such as good agricultural practices (GAP) were recently recommended for farms, to provide basis for the development of best practices in the production of horticultural products (*e.g*. fruits, vegetables, potatoes, salads, *etc*.) (Codex Alimentarious Commission, 1997; Kokkinakis and Fragkiadakis, 2006).

Studies have demonstrated the effectiveness of HACCP and GAP implementation; For instance, in Greece, tomatoes produced with GAP exhibited low microbial levels when compared with those grown routinely. The study also found that salads from establishments applying HACCP methodology were safe. Hence it was concluded that the application of GAP and HACCP are critical for the quality of the products (Kokkinakis and Fragkiadakis, 2006). Similarly, in Jordan, low bacterial plate count was noted from home-made jam after applying HACCP system (Al-Saed *et al*., 2012).

GAP such as early harvesting; proper drying, sanitation, proper storage and insect management among others are some of the strategies employed to prevent fungal infections. Other possible interventions include biological control, chemical control and breeding for resistance strains (Wagacha and Muthomi, 2008). Dorner and Cole (2002) reported a reduction of 74 - 99.9% in aflatoxin contamination in peanuts in the United States upon introduction of atoxigenic strains of *A. flavus* and *A. parasiticus* into the soil used to cultivate crops. Other biological control measures address the potential use of microorganisms as

mycotoxin binders in the gastrointestinal tract of both humans and animals, thereby reducing the potential deleterious effects of exposure to these toxins (Kabak and Dobson, 2009).

At consumer's level, foodborne illnesses can be reduced by proper cooking of meat, poultry and eggs to temperatures that will kill bacteria; although, spores are heat resistant, steaming under pressure, grilling, roasting and frying of foods can destroy the vegetative cells and spores (Linscott, 2011). Additionally, refrigerating leftovers promptly and storing foods at recommended temperatures; avoiding cross-contamination of cooked and raw foods; washing of utensils and cleaning of surfaces before and after use with hot, soapy water; and frequently washing hands and/or using gloves when preparing food are all recommended (Gashaw *et al*., 2008).

Since most viral foodborne infections are transmitted feco-orally through infected persons who handle food that is not heated or ready-to-eat foods, emphasis should be on stringent personal hygiene during preparation. In addition to adequate heating, ultraviolet light or strong oxidizing agents can also inactivate viruses (Doyle, 2003). Water treatment for public consumption is a safe and highly effective preventative measure; additionally, the effective treatment of sewage minimizes the spread of enteric disease-causing organisms. For this reason, use of municipal water supplies is recommended for all food-handling facilities (Linscott, 2011).

2.6 Conclusion

Globally, foodborne illnesses are accountable for significant morbidity and mortality. It results from consumption of food contaminated with the pathogen, poisonous chemicals or toxins (Teplitski *et al*., 2009). These illnesses also play a key role in new and emerging infections as well as escalating antibiotic resistance trends. A range of new pathogens have emerged due to changing dynamics of the food industry. Therefore it is imperative to monitor

and investigate foodborne illnesses in order to control and prevent further outbreaks (Mans *et al*., 2010). Although there are recent advances in miniaturization of analytical systems and newly emerging technologies, foodborne diseases still remain a challenge, emphasizing the need for further development of new rapid methods that will advance in speed, sensitivity, limit of detection, simplicity, cost, quantitation, specificity, portability, and robustness.

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CHAPTER THREE

Foodborne Pathogens Recovered from Ready-to-eat Foods from Roadside Cafeterias and Retail Outlets in Alice, Eastern Cape Province, South Africa: Public Health Implication

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Foodborne pathogens recovered from ready-to-eat foods from roadside cafeterias and retail outlets in Alice, Eastern Cape Province, South Africa: public health implications.

Abstract

This study assessed the microbiological quality of various ready-to-eat foods sold in Alice, South Africa. Microbiological analysis was conducted on 252 samples which included vegetables, potatoes, rice, pies, beef and chicken stew. The isolates were identified using biochemical tests and the API 20E, API 20NE and API Listeria kits; results were analyzed using the one-way-ANOVA test. Bacterial growth was present in all the food types tested; high levels of total aerobic count were observed in vegetables, 6.8 ± 0.07 followed by rice, 6.7 ± 1.7 while pies had the lowest count (2.58 ± 0.24). Organisms isolated included: *Listeria* species (22%), *Enterobacter* species (18%), *Aeromonas hydrophila* (12%), *Klebsiella oxytoca* (8%), *Proteus mirabilis* (6.3%), *Staphylococcus aureus* (3.2%) and *Pseudomonas luteola* (2.4%). Interestingly, *Salmonella* species and *Escherichia coli* were not isolated in any of the samples. There was a statistically significant difference ($p < 0.05$) in the prevalence of foodborne pathogens from hygienic and unhygienic cafeterias. The results indicated that most of the ready-to-eat food samples examined in this study did not meet bacteriological quality standards, therefore posing potential risks to consumers. This should draw the attention of the relevant authorities to ensure that hygienic standards are improved to curtain foodborne infections.

3.1 Introduction

Foodborne diseases are an increasingly recognized problem involving a wide spectrum of illnesses caused by bacterial, viral, parasitic or chemical contamination of food. Although viruses account for half of all the foodborne illnesses, most hospitalizations and deaths related to foodborne infections are due to bacterial agents (Teplitski *et al*., 2009). Street sold foods are appreciated for their unique flavours and convenience. They also assure food security for low income urban population and livelihood for a significant proportion of the population in many developing countries (Ghosh *et al*., 2007). However, the unhygienic conditions in which these foods are prepared, stored and served raise a question regarding their microbiological quality. Researchers have investigated the microbiological quality of street vended foods in different countries; high bacterial counts and a high incidence of foodborne pathogens in such foods have been reported. In Ghana, bacterial counts of 5.13 - 6.36 $\log_{10}$ CFU g^{-1} were documented in the street foods of Kumasi (Feglo and Sakyi, 2012). Another study in Accra reported a total bacterial count range of 0.8 - 6.3 $\log_{10}$ CFU g^{-1}, Enterobacteriaceae count of 0.3 - 4.7 $\log_{10}$ CFU g^{-1} and 0.3 - 3.7 $\log_{10}$ CFU g^{-1} of *Staphylococcus aureus* (Mensah *et al*., 2002). In Egypt, Ismail (2006) studied the microbial quality of ready-to-eat meat sandwich and reported aerobic plate counts, Enterobacteriaceae, and *Enterococci* counts range of 2×10^3 - 4×10^6, 6×10^1 - 8×10^2 and 3×10^3 - 6×10^5 CFU g^{-1} respectively.

Contamination of food by enteric pathogens can occur from the farm if human sewage is used to fertilize the soils or if sewage water is used to irrigate the crops. Such risks are further increased if the food is mishandled during processing and preparations where pathogens could multiply exponentially under favourable conditions (Ghosh *et al*., 2007). However, no study has been conducted on the microbial quality of the food supplied by local food establishments in Alice. Therefore, the present study was carried out to assess the microbiological quality of various ready-to-eat foods supplied in Alice, in a bid to throw more light on the inherent risk associated with such foods.

3.2 Materials and methods

3.2.1 Study area

Alice is a rural settlement in the Nkonkobe municipality of the Eastern Cape Province of South Africa and situated in the geographical coordinates 32°50'36'' S, 26°55'00" E; with a population of about 9,788 and a student population of 8,548 (ASPIRE, 2011). The study was conducted between August and November, 2011. Two university restaurants and eight ready-to-eat food vending sites in Alice Town were sampled. These sites were chosen because they are very popular among students, workers, commuters, shoppers and passers-by. Members of these groups buy food from at least one of these outlets at one time or the other. They include two garages that were designated (G1 and G2), three supermarkets (S1, S2 and S3), four roadside cafeterias and two university restaurants (C1, C2, C3, C4, C5 and C6). The sites were classified as unhygienic cafeterias if they had no running water, poor hygienic conditions of the staff and surrounding *i.e.*, rubbish, sewage and other noxious substances present in the vicinity that can attract foodborne vectors such as flies, whereas hygienic cafeterias were termed those with running water, clean food preparation surfaces, toilets, clean environment and food-handlers who comply with food hygienic standards. CAC/GL

22R (1999), defined food hygiene as all conditions and measures necessary to ensure the safety and suitability of food at all stages.

3.2.2 Sample collection

A total of 252 cooked samples were purchased comprising of 42 batches of six food types which included beef and chicken stew, potatoes, rice, vegetables and pies all of which are popular foods from cafeterias in the study area. Samples were packed separately and transported to the Microbial Pathogenicity and Molecular Epidemiology Research Laboratory (MPMERG) of the University of Fort Hare for immediate processing.

3.2.3 Bacteriological analysis of the samples

3.2.3.1 Sample preparation, culture and bacterial count

Samples were prepared according to the method of Akoachere *et al*. (2009) with some modifications. Twenty five grams of each sample was weighed and homogenized by blending in 225 mL of sterile buffered peptone water. One millilitre of the homogenate was introduced into 9 mL of the buffered peptone water in a test tube, labelled 1:10 (10^{-1}) dilution and serially diluted to five other test tubes labelled 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}; the procedure was repeated for each sample and the blender was carefully cleaned and disinfected in between samples to prevent any cross contamination. One hundred microliters of each of the diluted sample was plated on Nutrient agar. The plates were incubated aerobically for 24 h at 37 °C. All discrete colonies were counted where possible and expressed as the $\log_{10}$ of colony forming units per gram (CFU g^{-1}). To improve recovery and detection, the tubes were incubated aerobically at 37 °C for 12 - 24 h after which a loopful of enrichment broth was cultured on Salmonella/Shigella, Mac Conkey, Eosin-Methylene Blue (EMB) and Colombia blood agar (Oxoid, Basingstoke, England) supplemented with 5% horse blood. The plates were incubated aerobically for 24 - 48 h at 37 °C.

3.2.3.2 Isolation and biochemical characterization of the isolates

Colonies were presumptively identified by colony pigmentation and Gram staining characteristics. Pure cultures were obtained by streaking a portion of an isolated colony on Nutrient agar and incubated aerobically at 37 °C for 24 h. The isolates were confirmed by oxidase, catalase, and coagulase activity. Isolates were further characterized biochemically using API 20E for enteric Gram-negative rods whilst API 20NE was used for the identification of non-fastidious and non-enteric Gram-negative rods. The confirmation of *Listeria* species was carried out using API Listeria kit (Biomerieux, Marcy-L'etoile, France). The tests were performed according to manufacturer's instruction for use. Briefly, a few single colonies from young cultures (18 - 24 h) were emulsified in 5 mL of sterile sodium chloride (0.85%) and the turbidity adjusted to the equivalent of the turbidity of 0.5 McFarland standards. The standardized bacterial suspension was distributed into the tubes of the test strip carefully to avoid the formation of bubbles. Anaerobiosis was created by overlaying with sterile mineral oil; the strips were then incubated in humid atmosphere for 18 - 24 h at 37 °C. Data interpretation was performed using the Analytical profile index (API) database (V4.1) with the apiweb^{TM} identification software.

3.3 Statistical analysis

Statistical analysis was performed using excel and SPSS version 19. The bacterial counts were expressed as mean ± Standard deviation using excel. One way ANOVA followed by Turkey's *post hoc* test was used to compare the bacterial counts in various food types and bacterial count from hygienic and unhygienic cafeterias. The mean difference was considered significant at $p < 0.05$.

3.4 Results and discussion

3.4.1 Bacterial count of isolates in the food samples

The mean bacterial count of the isolates in the food samples were expressed as $\log_{10}$ CFU g^{-1} for easy computation. Food were classified as acceptable if the bacterial count was less than or equal to 5 $\log_{10}$ CFU g^{-1} (NSWFA, 2009). The mean value of aerobic bacterial count on vegetables, rice, potatoes, beef, chicken stew and pies were 6.8 ± 0.07, 6.7 ± 1.7, 6.27 ± 0.18, 5.32 ± 3.14, 6.05 ± 0.12 and 2.58 ± 0.24 $\log_{10}$ CFU g^{-1} respectively (Table 3.1). The bacterial count of vegetables, rice, potatoes, beef and chicken stew was statistically significant when compared with pies ($p < 0.05$). A similar comparison was made for food from hygienic and unhygienic sources; the results revealed that there was statistically significant difference in the bacterial load of beef stew and rice from hygienic and unhygienic cafeterias ($p < 0.05$). However, no significance was observed for vegetables, chicken and potatoes samples ($p > 0.05$) (Table 3.2).

Table 3.1: Mean bacterial count of the food samples examined

Food types	Bacterial count range (log_{10} CFU g^{-1})	Mean bacterial count (log_{10} CFU g^{-1}) ± SD	*p*-value					
			V	R	C	B	PT	P
Vegetables (n=42)	6.3 - 6.8	6.8 ± 0.07	-	0.235	0.913	0.001	0.585	0.000
Rice (n=42)	4.3 - 6.7	6.7 ± 1.7	0.235	-	0.196	0.000	0.513	0.000
Chicken stew (n=42)	5.9 - 6.2	6.05 ± 0.12	0.913	0.196	-	0.001	0.513	0.000
Beef stew (n=42)	3.9 - 6.15	5.32 ± 3.14	0.001	0.000	0.001	-	0.000	0.000
Potatoes (n=42)	6.0 - 6.4	6.27 ± 0.18	0.585	0.513	0.513	0.000	-	0.000
Pies (n=42)	2.30 - 2.81	2.58± 0. 24	0.000	0.000	0.000	0.000	0.000	-

CFU g^{1}-, colony forming units per gram; SD- standard deviation; V, vegetables; R, rice; C, chicken stew; B, beef stew; PT, potatoes; P, pies; -, no comparison done. The mean difference is considered significant at $p < 0.05$.

Table 3.2: Mean bacterial counts of food obtained from hygienic and unhygienic cafeterias

Food types	Bacterial count (log_{10} cfu/g) ± Standard deviation		*p* -value
	Unhygienic	Hygienic	
Vegetables (n=42)	6.8 ± 0.07	6.4 ±0.7	0.128 (>0.05)
Rice (n=42)	6.7 ± 1.7	6.35 ± 0.07	0.000 (<0.05)
Chicken stew (n=42)	6.05 ± 0.12	5.95 ± 0.07	0.122 (>0.05)
Beef stew (n=42)	5.32 ± 3.14	4.32 ±3.01	0.002 (<0.05)
Potatoes (n=42)	6.27 ± 0.18	6.15 ± 0.21	0.171 (>0.05)
Pies (n=42)	ND	3.9 ± 0.7	ND

CFU g^{-1}, colony forming units per gram; ND, not determined; unhygienic cafeterias, vending sites without running water, toilets, fridges to store food and dirt environment; hygienic cafeterias, vending sites with running water, clean food preparation surfaces, toilets, clean environment and food handlers who comply with food hygienic standards. The mean difference is considered significant at $p < 0.05$.

Plate count of aerobic mesophilic microorganisms found in food is one of the microbiological indicators for food quality. The presence of aerobic organisms reflects existence of favourable conditions for the multiplication of microorganisms. In this study, all the sample types tested had mean contamination levels of ≥ 5.0 $\log_{10}$ CFU g^{-1} except the pies. The New South Wales Food Authority (NSWFA, 2009) recommends the standard limit for bacterial count of fully cooked ready-to-eat foods to be < 5.0 $\log_{10}$ CFU g^{-1}. Hence these foods could be of high risk in transmitting enteric pathogens. These findings corroborate previous works (Mensah *et al*., 2002; Christison *et al*., 2008; Bukar *et al*., 2010). In their study, Mensah *et al*. (2002) found a bacterial count of 6.3 ± 0.78 in salads sold on the streets of Accra. Likewise, Christison *et al*. (2008) also reported high bacterial prevalence in filled baguettes and salads.

Vegetables had the highest bacterial count of 6.3 - 6.8 $\log_{10}$ CFU g^{-1}. The findings are in agreement with other studies in Egypt, Turkey and Taiwan. Saddik *et al*. (1985) reported the upper limit of 6.69 $\log_{10}$ CFU g^{-1} aerobic counts of microorganisms on minimally processed vegetable samples in Egypt while Fang *et al*. (2003) reported an aerobic plate count on salad vegetable samples in Taiwan ranging from 3.30 - 8.64 $\log_{10}$ CFU g^{-1}. Furthermore, Vural and Erkan (2008) in Turkey had a range of aerobic plate counts from 6.43 to 7.63 $\log_{10}$ CFU g^{-1}.

Vegetables have been associated with foodborne outbreaks in many countries; they may be contaminated from the farm with human sewage, and from the irrigation water. Unsafe water used for rinsing the vegetables and sprinkling to keep them fresh are other possible sources of contamination (Bukar *et al*., 2010). As most of these produce are eaten raw or with minimal cooking, their microbial content may represent a risk factor for the consumer's health (Falomir *et al*., 2010). Some of these factors might be the possible source of contamination in the vegetables under study. A study in Morocco, reported the occurrence of pathogenic bacteria mostly of the Enterobacteriaceae family in vegetables irrigated by untreated wastewater. Although usually regarded as human pathogens, members of this family have

also been recognized as inhabitants of soil and plants. Thus, vegetables may serve as a reservoir from which these bacteria can colonize and infect a susceptible host (Karamoko *et al*., 2007).

Worthy of note is the fact that unhygienic cafeterias registered high bacterial counts; lack of sources of running water, refrigeration facilities, and post production operations and personal hygiene of the food handlers might be the possible contributing factors. It was also observed that in the unhygienic shops, vendors washed the utensils and dishes used for preparation and serving their food in buckets containing unclean water which were likely not replaced throughout the whole day. This might also be another reason for cross-contamination.

3.4.2 Prevalence of foodborne pathogens in the various food types.

Table 3.3 depicts the occurrence of possible pathogens in the 252 food samples tested. Bacterial growth was observed in all the food types; the most prevalent bacteria were *Listeria* species (22%), *Enterobacter* species (18%), *Aeromonas hydrophilla* (12%), *Klebsiella oxytoca* (8%) and *Proteus mirabilis* (6.3%). Vegetables and rice had the highest level of contamination with 108 (18%) isolates each. *Listeria ivanovii* was prevalent in pies (33%) followed by chicken (28%) while beef, rice and potatoes registered 14% each. *Enterobacter cloacae* was mainly isolated from beef stew (24%), pies and chicken (17%) each and rice (14%). *Aeromonas hydrophila* was detected in 10 (24%) of the vegetables, seven (17%) of rice and six (14%) of potatoes. *Klebsiella oxytoca* was present in seven (17%) of pies and potatoes, and in six (14%) of vegetables. It was also observed that the prevalence of the pathogens in the environment varied as *Proteus mirabilis* was isolated from seven (17%) of rice, four (10%) of chicken and three (7%) of beef obtained from one of the university cafeterias while none was isolated from the other shops. Interestingly, *E. coli* and *Salmonella* species which are common food pathogens were not isolated. The prevalence of foodborne pathogens from hygienic and unhygienic cafeterias was also compared. The unhygienic

cafeterias recorded the highest number of isolates 339 (54%) compared to 299 (47%) from hygienic cafeterias (Figure 3.1). However, *L. ivanovii* and *P. mirabilis* were the only isolates which were recovered more from unhygienic cafeterias than hygienic cafeteria.

Table 3.3: Bacteria distribution in the various examined food samples

Bacteria isolates	Chicken stew (n=42)	Beef stew (n=42)	Vegetables (n=42)	Rice (n=42)	Potatoes (n=42)	Pies (n=42)	Number(%) occurrence
Listeria ivanovii	11	6	8	6	6	14	51/252 (20%)
Listeria grayi	1	0	1	1	1	1	5/252 (2%)
Enterobacter cloacae	7	10	2	6	3	7	35/252 (14%)
Enterobacter sakazaki	1	0	4	0	1	0	6/252 (2.4)
Enterobacter gergoviae	0	0	0	0	0	2	2/252 (1%)
Enterobacter cancerogenus	0	0	1	0	1	1	3/252 (1.2%)
Proteus mirabilis	4	3	2	7	0	0	16/252 (6.3%)
Klebsiella oxytoca	2	2	6	1	7	3	21/252 (8.3%)
Aeromonas hydrophila	2	4	10	7	6	0	30/252 (12%)
Staphylococcus aureus	1	0	0	2	3	2	8/252 (3.2%)
Pantoea species	0	3	2	0	2	0	7/252 (2.8%)
Pseudomonas luteola	0	1	2	1	0	2	6/252 (2.4%)
Other Gram positive Bacilli	54	62	70	77	64	66	267/588 (45%)
Total isolates	**85**	**93**	**108**	**108**	**94**	**100**	**588**

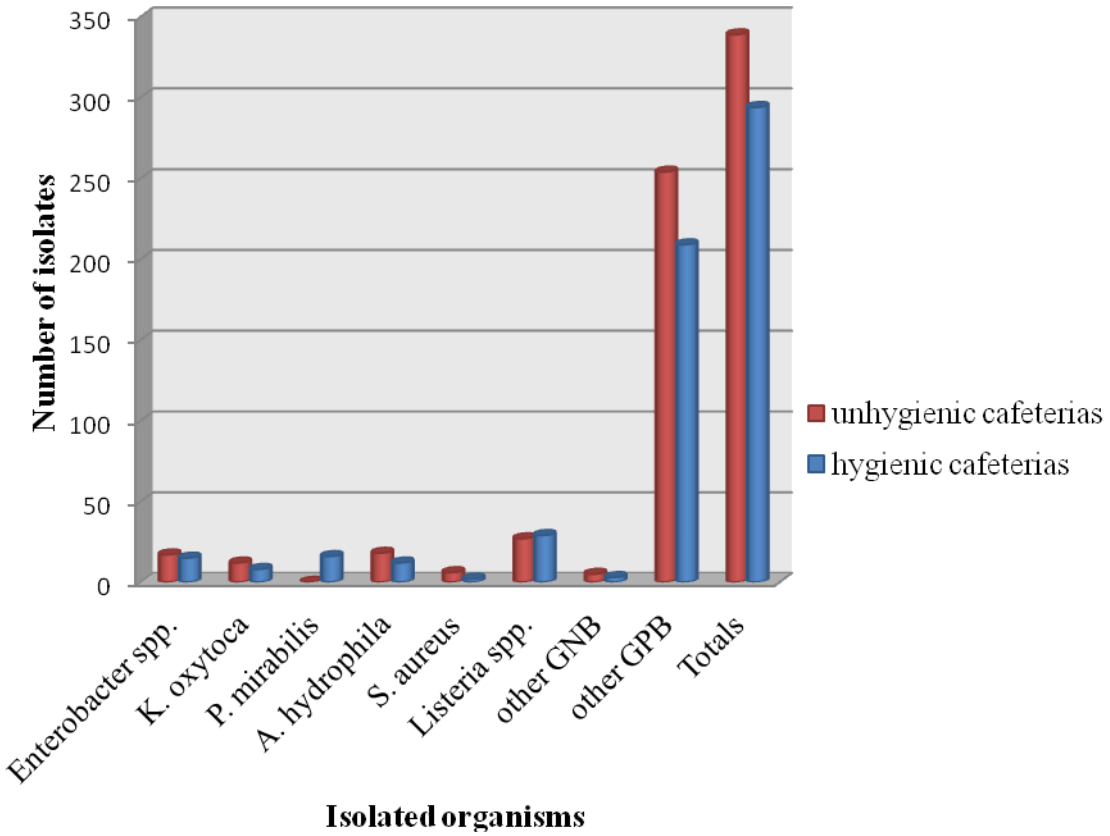

Figure 3.1: Bacterial contamination of food from hygienic and unhygienic cafeterias

The study found that ready-to-eat foods from roadside cafeterias contained more organisms if compared to that sold in standard fast foods centres and supermarkets which had running water and cement floors. Bukar *et al*. (2010) found that ready-to-eat rice sold on the streets of Kano contained more microorganisms if compared to that sold in standard fast food centres. The roadside cafeterias had no running water, hands and utensils washing was done in one bucket where the water was not regularly changed. This coupled with unhygienic surroundings like sewage, improper waste disposal system, might be the possible sources of food contamination in these sites.

Members of the family Enterobacteriaceae have been considered a potent cause of foodborne outbreaks (Centinkaya *et al*., 2008). In this study, Enterobacteriaceae represented 46% of the isolates. *E. cloacae* (14%) was the most prevalent among the isolated Enterobacteriaceae. The findings concurs the work of Falomir *et al*. (2010) who found *E. cloacae* and *K. oxytoca*, to be the most prevalent coliforms in ready-to-eat salads served in the dining halls of a pre-school and a primary school in Valencia city, Spain. The species of *Enterobacter* are the sixth most common cause of nosocomial infection in particular; *E. cloacae* have been implicated in a broad range of clinical syndromes. The literature is replete with descriptions of bacteraemia, meningitis, urinary tract infection, septicaemia, wound infection, central nervous system and gastrointestinal tract infections (Ghosh *et al*., 2007). Therefore the presence of these organisms in the foods under study might pose a health risk to children and individuals with underlying conditions.

Listeria species has been associated with a wide variety of food sources particularly poultry, red meat and meat products (El-Malek *et al*., 2010). The present study recorded a high occurrence of *Listeria* species in pies (33%) and chicken stew (28%). These findings are in line with those of other authors (Molla *et al*., 2004; Yucel *et al*., 2005; El-Malek *et al*., 2010). In Egypt, *Listeria* contamination rate in meat and chicken products was reported to be 41%

(El-Malek *et al*., 2010), lower than the 73.9% reported in Malaysia from imported frozen beef (Hassan *et al*., 2001) and 83.3% from raw minced meat in Turkey (Yucel *et al*., 2005). The bacteria can be endemic in food processing environments, because it survives food-processing technologies that rely on acidic or salty conditions and, unlike many pathogens, can continue to multiply slowly at low temperatures, allowing for growth even in properly refrigerated foods. Hence, their presence may be indicative of poor hygiene or cross contamination, which is considered to be a possible source of *Listeria* contamination in processed meat (Watson, 2009). In addition, minced/chopped meat products by their nature, undergo extensive processing and handling during their production thereby increasing the risk of contamination. This might explain the high prevalence of *L. ivanovii* in pies which were of either minced/chopped chicken or red meat.

Nevertheless, despite the high rates of contamination of certain foods with *Listeria* species, listeriosis is a relatively rare disease as compared with other common foodborne illnesses. However, because of its high case fatality rate of approximately 20-30%, listeriosis has been ranked second, after salmonellosis as the most frequent cause of foodborne infection-related deaths in Europe (Watson, 2009).

Clinical manifestations of listeriosis range from febrile gastroenteritis to more severe invasive forms, including sepsis, meningitis, rhombencephalitis and perinatal infections. Perinatal listeriosis can lead to abortion, birth of a stillborn fetus or a baby with generalized infection (granulomatosis infant-septica), and sepsis or meningitis in the neonate (Allerberger and Wagner, 2010).

L. monocytogenes is the most common causative agent of human listeriosis. However, *L. ivanovii*, an animal pathogen has been previously isolated, although rarely, from infected humans, indicating pathogenic potential for humans (Allerberger and Wagner, 2010; Guillet *et al*., 2010). Therefore the isolation of *L. ivanovii* in the present study might reflect a health risk to the consumers' particularly pregnant women.

The study registered a low *P. mirabilis* occurrence of 6.3%. It is noteworthy that all 16 isolates were obtained from one source (one of the university shops). *P. mirabilis* is a proteolytic bacterium implicated in food deterioration and spoilage. In addition to opportunistic infections, it can also cause food poisoning when consumed in contaminated food such as meat, vegetables, and seafood (Braide *et al*., 2011).

S. aureus was prevalent in 3.2% of the samples. The results were contrary to the findings of Ghosh *et al*. (2007) and Kumar *et al*. (2006) who reported high prevalence of *S. aureus* in street-vended foods. Differences in geographical location and personal hygiene of the food-handlers might help to explain this discrepancy. In their study, Ghosh *et al.* (2007) reported high prevalence of *S. aureus* from coriander sauce 91 (60%) and 129 (86%) from ready-to-eat salads in India. The authors attributed this high prevalence to poor hygienic conditions of the premises due to rubbish, sewage and other noxious substances present in the vicinity. Kumar *et al*. (2006) presented the high *Staphylococcal* count from fruit chaat (a drink made from a mixture of fruits and vegetables) samples obtained from mobile vendors without any covering compared to those samples from mobile vendors with coverings. The authors suggested that, covering to some extent acts as a shield for airborne bacterial pathogens.

Staphylococcus species are found on the skin and in the nose and throat of most healthy people; they are also widespread in untreated water, raw milk and sewage. When *S. aureus* is allowed to grow in foods, it can produce a toxin that causes illness. Although, cooking

destroys the bacteria, the toxin produced by *S. aureus* is heat stable and may not be destroyed even by heating, let alone by refrigeration. Foods that are handled frequently during preparation are prime targets for *Staphylococci* contamination (Ghosh *et al*., 2004).

The prevalence of *A. hydrophila* was at 12% with the organism isolated more from vegetables (10/42) than in the other food types. Other studies reported a prevalence of 4%, 26% and 41% Aeromonads in dairy products, vegetables and sea foods respectively (Neyts *et al.*, 2000; Mcmahon and Wilson, 2001). Although *A. hydrophila* is water based, waterborne outbreaks have not been reported, and waterborne transmission has not been well established as various studies have been unsuccessful in linking patient isolates of *A. hydrophila* with isolates recovered from the water supply (Borchardt *et al*., 2003). Nevertheless, this bacterium has gained public health recognition as an opportunistic pathogen. It has been implicated as a potential agent of gastroenteritis, septicaemia, cellulitis, colitis, and meningitis, and is frequently isolated from wound infections sustained in aquatic environments (Krovacek *et al*., 1992; Gavriel *et al*., 1998). Palumbo *et al*. (1985) found *Aeromonas* isolates universally present in all foods tested, including seafoods, raw milk, chicken, and meats such as lamb, veal, pork, and ground beef.

3.5 Conclusion

These findings demonstrate that ready-to-eat food sold in Alice Town constitutes a likely potential hazard to human health. The isolation of Enterobacteriaceae in ready-to-eat foods that are fully cooked is a good indicator of post-processing contamination or inadequate cooking. Therefore access to running water and health education to the vendors on personal hygiene, food safety and proper disposal of waste would improve food quality thereby reducing foodborne incidences.

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CHAPTER FOUR

Molecular characterisation of *Listeria ivanovii* and *Enterobacter cloacae* strains isolated from food samples in Alice, South Africa.

Molecular characterisation of *Listeria ivanovii* and *Enterobacter cloacae* strains isolated from food samples in Alice, South Africa.

Abstract

The present study was carried out to confirm *L. ivanovii* and *E. cloacae* strains using molecular techniques, and also to screen for the presence of virulence associated genes of these organisms, in an effort to determine their pathogenic potential as a guide of the risk they may pose to the community. DNA was extracted by use of the Quick-gDNA™ MiniPrep DNA extraction kit (Zymo Research Corporation, SA) according to the manufacturer's instructions for use. Standard PCR technique was utilized for the amplification of the different target genes; which included: virulence coding genes (*fyuA, ucaA, irp2 aer*, and *hlyA* for *E. cloacae*, and *iap, act, plcA* and *hlyA* for *L. ivanovii*); *hsp60* was used for the confirmation of *E. cloacae* while *iap60* was the target gene for *L. ivanovii*. A total of 97% (30/31) of the isolates were confirmed as *E. cloacae* complex while 45 (90%) of the samples tested positive for *Listeria* species by PCR. Two virulence genes (*ucaA* and *hlyA*) were detected in *E. cloacae* isolates and none from *L. ivanovii*. Sequence analysis of the *hsp60* (*E. cloacae*) PCR products recognized the presence of two sub-species; *E. cloacae* cluster III (75%) and *E. cloacae* cluster IV (25%), whilst analysis of the *iap60* gene sequence (*Listeria*) demonstrated that 56% (19/34) were *L. ivanovii*, 29.4% (10/34) *L. seeligeri* and14.7% (5/34) *L.welshemeri*. Both the *hsp60* and *iap60* sequences reported in this study were deposited in the GenBank database and accessible under the accession numbers KF768566 – KF768573 and KF800796 – KF800829 respectively. The presence of associated virulence genes in some of the strains of *E. cloacae* as well as different biovars of the organism is of public health concern as these pathogenic strains might cause foodborne illnesses in susceptible individuals who consume these foods.

4.1 Introduction

Foodborne diseases remain a major cause of morbidity and mortality in the general populace (Nyenje and Ndip, 2013). The illnesses result from the ingestion of food contaminated with pathogenic microbes or their toxins. Attributable to their pathogenicity, microbes are categorized into four; pathogens, opportunistic pathogens, commensal and beneficial microbes (Wang *et al*., 2009). A pathogen is a microorganism that is able to cause a disease in plants, animals, humans or insect; whereas opportunistic organisms can become pathogenic following a perturbation to their host (*e.g*., disease, wound, medication, immunodeficiency and ageing). These opportunists can emerge from among the ranks of normally commensal symbionts (Brown *et al*., 2012).

Microbes express their pathogenicity by means of their virulence (Todar, 2009). Virulence determinants are often acquired through horizontal transfer, which may explain why virulence traits are distributed sporadically among bacterial taxa or within some bacterial species. In many bacterial species, there is an association between species-specific genes and virulence genes. For instance, the *iap* gene is a virulence gene but some studies have used a fragment of the gene in detecting the *Listeria* genus (Bubert *et al*., 1992; Atil *et al*., 2011). Protein p60 encoded by the *iap* gene is essential for cell division and is regarded as an essential housekeeping protein for *Listeria* species; but also exhibit bacteriolytic activity (Wuenscher *et al*., 1993). However, studies have demonstrated that mutations in the *iap* gene leads to over expression of the p60 (Wuenscher *et al*., 1993; Pilgrim *et al*., 2003; Schimid *et al*., 2005).

The genus *Listeria* contains two pathogenic species; *L. monocytogenes* and *L. ivanovii*, the etiological agents of listeriosis. Listeriosis is a severe human foodborne infection characterized by gastroenteritis, meningitis, encephalitis, abortions, and perinatal infections

(Nyenje *et al*., 2012b). This Gram-positive bacterium is a facultative, intracellular pathogen that induces its own uptake into non-phagocytic cells and spreads from cell to cell using an actin-based motility process (Dussurget *et al*., 2004). These mechanisms correlate with the presence of virulence gene cluster, the *prfA* or the Listeria pathogenicity island (*LiPI*) which encodes a number of proteins that are necessary for intracellular survival and motility (Guillet *et al*., 2010). Other specific functions encoded in this cluster include haemolysin (*hly*), two phospholipases (*plcA* and *plcB*) and a metalloprotease (*mpl*), which all contribute to the escape of the bacteria from host cell vacuoles of infected cell to cytosol and subsequently spread from cell to cell; while genes such as those encoding for actin polymerizing protein (*actA*) and internalines (e.g., *inlA* and *inlB*) are essential for host cell invasion (Schmid *et al*., 2005). The presence of the pathogenicity island in the genomes of pathogenic bacterium suggests that, besides the host immunity, the serotype and the contamination level of the food product, other factors like the virulence potential of the organism plays a role in the ability to develop an infection (Schmidt and Hensel, 2004; Wilson, 2012). For instance, *Yersinia* and *Enterobacter* species, *E. coli*, and *K. pneumoniae* isolates containing the high pathogenicity island (*HPI*) have been reported to be more virulent than isolates lacking this island (Schubert *et al*., 2000; Paauw *et al*., 2009; Koczura *et al*., 2012).

Strains of *E. cloacae* are Gram-negative, facultative, anaerobic rod-shaped bacterium. Besides living ubiquitously in the terrestrial and aquatic environments, they also form part of the commensal gut flora and play an important role as pathogens of plants, insects and humans (Krzymin´ ska *et al*., 2009; Kremer and Hoffmann, 2012). Although recognized as the most common *Enterobacter* species causing nosocomial infections, little is known about the factors impacting their pathogenicity and virulence (Barnes *et al*., 1997). However, some studies have equally detected virulence gene associated with other Enterobacteriaceae and *Enterobacter* species from some strains of *E. cloacae*. Keller *et al*. (1998) demonstrated

broad distribution of serum resistance, aerobactin and haemagglutinin proteins, whereas Simi *et al*. (2003) found a low molecular enterotoxic haemolysin in seven out of 50 analyzed *E. cloacae* strains. Sevcik *et al*. (2003) described a paralytic toxin called saxitoxin in two species of the *E. cloacae* complex; the outer membrane protein (OmpX) has been directly linked to invasiveness of *E. cloacae* in rabbit ileal tissue (de Kort *et al*., 1994). In another approach, Paauw *et al*. (2009) executed a comparative genomic hybridization study to identify genes specific for an *E. hormaechei* outbreak strain; they detected the IncHI-2 plasmid pQC harbouring many resistance determinants and speculated that the presence of this plasmid may most likely lead to a greater likelihood of survival in the hospital environment.

Unfortunately, these studies mostly represented clinical isolates; hence there is dearth of information on environmental and foodborne isolates which are the possible sources of virulence and resistance genes to human through the food chain. Worthy of note is the fact that these studies demonstrated that some genotypes are significantly associated with certain infection sites (Kremer and Hoffmann, 2012); hence it can be equally hypothesized that different genotypes are associated with certain environments. Therefore this study was carried out to screen for the presence of virulence associated genes of *L. ivanovii* and *E. cloacae* from food in an effort to determine the likely pathogenic potential of these organisms and the potential risk they may pose to the community; but also further characterise these food isolates to ascertain the genotypes and biovars circulating in our environment as a possible tool for control measures.

4.2 Materials and methods

4.2.1 DNA extraction

DNA was extracted from 50 of the 51 *L. ivanovii* and 31 of the 34 *E. cloacae* strains (1 *L. ivanovii* and 3 *E. cloacae* strains could not be revived from the glycerol stocks) previously isolated from various ready-to-eat foods (Nyenje *et al*., 2012b). Isolates were centrifuged at 10,000 x g for 5 min, and DNA extracted from the pellets by use of the Quick-gDNA™ MiniPrep DNA extraction kit (Zymo Research Corporation, SA) according to the manufacturer's recommendations for use and stored at -20 °C until analysis. The DNA of reference strains *L. ivanovii* ATCC 19119 and *E. cloacae* ATCC 13047 was also extracted and used as positive controls.

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4.2.2 Molecular identification of E. cloacae strains by hsp60 analysis and their associated virulence genes.

Primers *hsp60*-F and *hsp60*-R were used to amplify a portion of the *hsp60* gene by PCR according to Mokracka *et al*. (2011). The PCR used master mix (Thermo Scientific), carried out in a total volume of 25 µL (12.5 µL master mix, 1.25 µL (0.5µM) each of foward and reverse primers, 2.5µL (0.1µg) DNA and 7.5 µL nuclease-free water). The primers used for the confirmation of *E. cloacae* and detection of the associated virulence genes are presented in Table 4. 1. The cycling conditions for *hsp60* included an initial denaturation of DNA at 95 °C for 3 min followed by 30 cycles each of 30 sec denaturation at 95 °C, 30 sec annealing at 67 °C and 1 min extension at 72 °C, followed by a final extension of 10 min at 72 °C. The associated virulence genes were amplified using the following conditions: initial denaturation at 94 °C for 3 min followed by 30 cycles each of denaturation at 95 °C for 30 sec, annealing at 56 °C for 1 min and extension at 72 °C for 1min, followed by a final extension of 10 min at 72 °C for *fyuA*, *aer* and *irp2* using primers in Table 4.1. The cycling conditions for *hlyA* were

as follows: 3 min of initial denaturation at 95 °C followed by 30 cycles each of 30 sec denaturation at 94 °C, 30 sec annealing at 60 °C and 1 min extension at 72 °C, whilst *ucaA* cycling conditions included: initial denaturation at 95 °C for 3 min, 30 cycles each of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1 min followed by final extension at 72 °C for 7 min. The resultant PCR products were separated by agarose gel electrophoresis (1.5% agarose stained with ethidium bromide), and visualized by ultra-violet trans-illuminator (UVP Gel Seq Software, England).

Table 4.1: Primer sequence for *E. cloacae* and their associated virulence genes

Target gene	Primer sequence (5' to 3')	Amplicon size (bp)	Reference
hsp 60	F - GGT AGA AGA AGG CGT GGT TGC R - ATG CAT TCG GTG GTG ATC ATC AG	341	Mokracka *et al.*, 2011
fyuA	F- GCGACGGGAAGCGATGATTTA R- TAA ATG CCA GGT CAG GTC ACT	547	Nosava and Titov, 2009
ucaA	F- GTA AAG TTG TTG CGC AAA C R- TTG AGC CAC TGT GGA TAC A	540	Sosa *et al.*, 2006
aer	F- CCT ATG GCC TGA GCG AGA AG R - CCA GTT CCA GTC CCA CCA CT	431	Nawaz *et al.*, 2010
hlyA	F- GGC CGG TGG CCC GAA GAT ACG GG R- GGC GGC GCC GGA CGA GAC GGG	597	Pablos *et al.*, 2009
irp2	F- AAG GAT TCG CTG TTA CCG GAC R- TCG TCG GGC AGC GTT TCT TCT	287	Nosava and Titov, 2009

4.2.3 Molecular detection of L. ivanovii and their associated virulence genes

The *iap* gene encodes the invasion associated protein p60, which is common to all *Listeria* species. The present study used *iap*-derived primers, Liv/Lis1B for the specific identification of *L. ivanovii* and Siwi2 for the identification of *Listeria* species closely related to *L. ivanovii* (*L. seeligeri* and *L. welshimeri*) (Table 4.2). PCR protocols were performed as described by Bubert *et al*. (1999) in a final volume of 25 µL (12.5 µL Master Mix (2x Thermo Scientific, SA), 1.25 µL (0.5µM) each of foward and reverse primers, 2.5µL (0.1µg) DNA and 7.5 µL nuclease-free water); amplification conditions for Siwi2 (*iap* derived primer) were as follows: 30 cycles, each at 94 °C for 45 sec, 50 °C for 1 min, and 72 °C for 3 min, whilst the cycling conditions for Liv/Lis1B included 35 cycles, each at 95 °C for 15 sec and 62 °C for 30 sec. On the other hand, the cycling conditions for the amplification of the putative virulence genes included an initial denaturation at 95 °C for 2 min followed by 35 cycles each of 15 sec denaturation at 95 °C, 30 sec annealing at 60 °C and 1 min 30 sec extension at 72 °C, followed by a final extension of 10 min at 72 °C. All the four set of primers for virulence-associated genes (Table 4.2) were amplified using similar PCR conditions. The electrophoresis of the PCR reaction was carried out using 1.5% (w/v) agarose gel stained with ethidium bromide, and was viewed under ultra-violet light.

Table 4.2: Primer sequence for *Listeria* species and their putative virulence genes

Primer	Primer sequence (5ʻ to 3ʾ)	Amplicon size	Reference
Siwi2	F- TAA CTG AGG TAG CGA GCG AA R- TTA TAC GCG ACC GAA GCC AAC	1.02 Kb	Bubert *et al*., 1999
Liv/Lis1B	F- CTA CTC AAG CGC AAG CGG CAC R - TTA TAC GCG ACC GAA GCC AAC	1 Kb	Bubert *et al*., 1999
plcA	F- CTG CTT GAG CGT TCA TGTCTC ATC CCC C R- ATG GGT TTC ACT CTCCTT CTA C	1.484 Kb	Notermans *et al.* 1991
iap	F- ACA AGC TGC ACC TGT TGC AG R- TGA CAG CGT GTG TAG TAG CA	131 bp	Furrer *et al.* 1991
hlyA	F- GCA GTT GCA AGC GCT TGG AGT GAA R- GCA ACG TAT CCT CCA GAG TGA TCG	456 bp	Paziak-Domanska *et al.* 1999
ActA	F- CGC CGC GGA AAT TAA AAA AAG A R- ACG AAG GAA CCG GGC TGC TAG	839 bp	Rawool *et al*., 2007

4.2.4 DNA sequencing

DNA sequencing was performed using a Big Dye Terminator DNA sequencing kit v3.1 (Applied Biosystems, UK). Direct sequencing was done with 2 μL of chromosomal DNA, 0.25 μL of primer (10 pmol per μL), 2 μL of Big Dye buffer and 2 μL of Big Dye. Cycle parameters included a denaturation at 96 °C for 10 sec, annealing at 50°C for 20 sec, and extension at 60°C for 4 min over 30 cycles, followed by Agencourt CleanSeq cleanup. Sequences were determined by electrophoresis with the ABI 3130xl DNA sequencer (Applied Biosystems, UK). Editing of the sequences was performed using Bioedit Alignment Editor, whilst phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software Version 5.2; sequences were aligned using ClustalW (Tamura *et al*., 2011).

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The significance of branching of the phylogenetic tree was evaluated by the bootstrap analysis of 500 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Felsenstein, 1985; Tamura *et al*., 2011).

Accession numbers

The sequences of this study have been deposited in the GenBank under these accession numbers:

Enterobacter cloacae hsp60 gene - KF 768566 - KF768573.

Enterobacter cloacae ucaA gene - KF 768595 - KF 768598.

Listeria ivanovii iap60 gene - KF 800796 - KF 800814

Listeria seeligeri iap60 gene - KF 800815 - KF 800824

Listeria welshimeri iap60 gene - KF 800825 - KF 800829

4.3 Results and discussion

4.3.1 Characterisation of E. cloacae strains by hsp60 and the presence of associated virulence genes.

A total of 97% (30/31) of the isolates were positive for the *hsp60* and was visualised as a band of 341 bp on agarose gel (Fig 4.1). Of the four putative genes targeted, *ucaA* and *hlyA* were the only genes detected in 40% (12/30) and 16.6% (5/30) of the strains respectively; they were observed as bands of 540 bp and 597 bp respectively (Fig 4.2 and Fig 4.3).

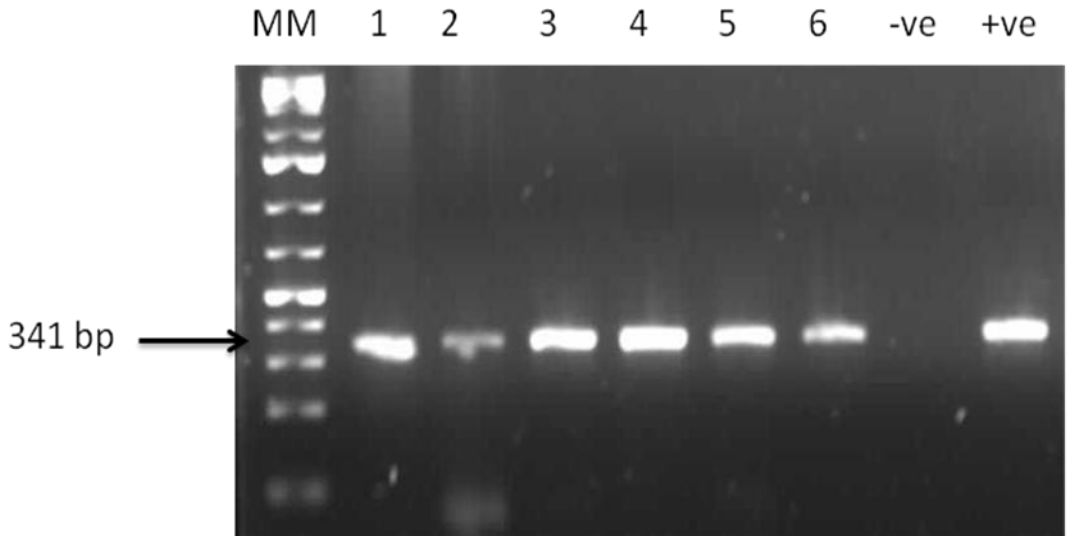

Figure 4.1: **Detection of amplified *hsp60* PCR product**. MM, 100 bp molecular marker; lanes 1- 6, positive samples; -ve, negative control; +ve, positive control (*E. cloacae* ATCC 13047).

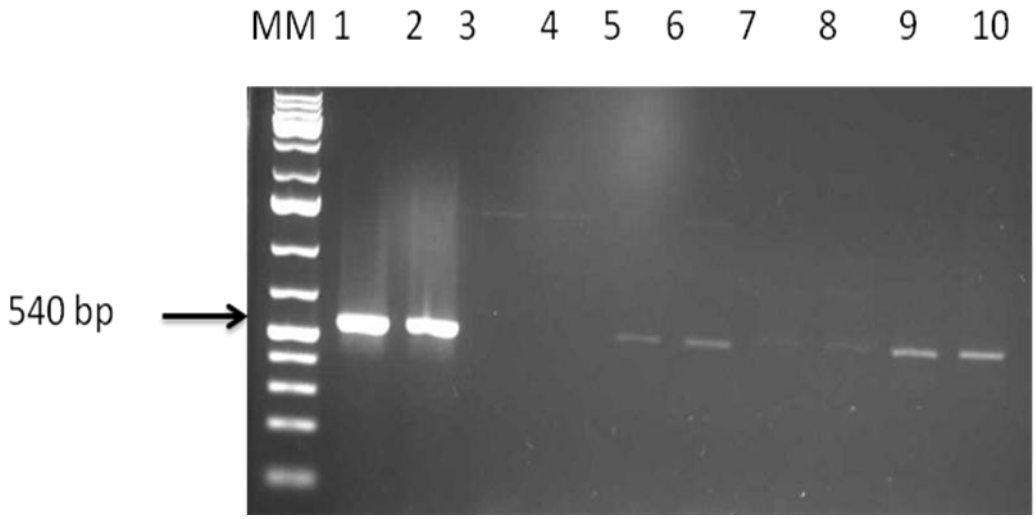

Figure 4.2: **Detection of amplified *uca A* PCR product**. MM, molecular marker 100bp; lanes 1, 2 and 5-10, positive samples; lane 3 and 4, negative samples.

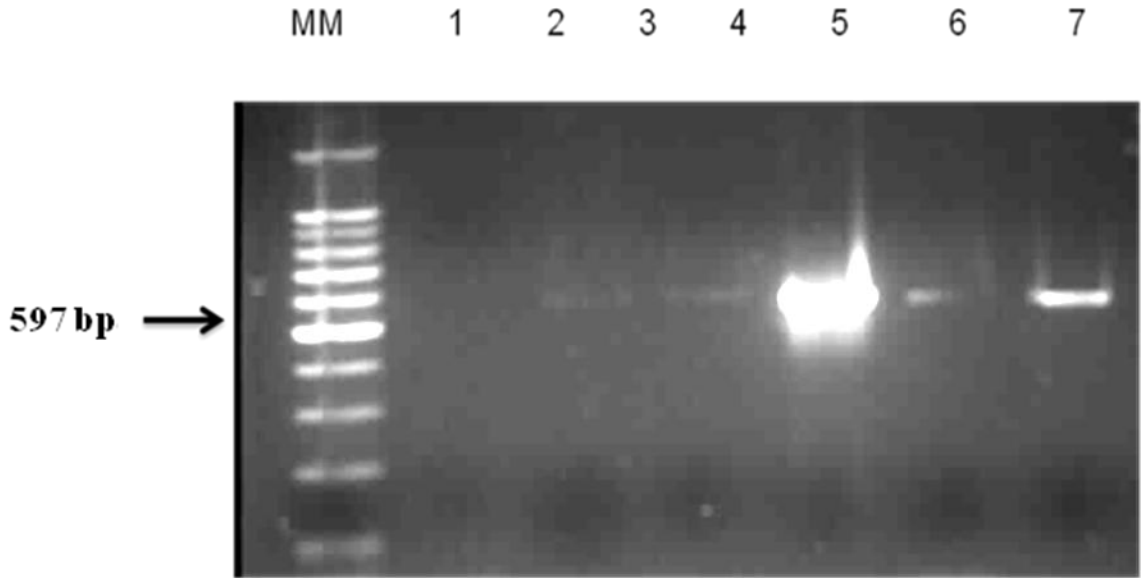

Figure 4.3: A 1.5% agarose gel electrophoresis separation of *hlyA* PCR products. MM, 100 bp molecular marker; lane 1, negative sample; lane 2- 7, positive samples.

4.3.2 Prevalence of species and genotypes

By analysing the *hsp60* sequence, each isolate was assigned to its respective species. A Neighbour joining tree was estimated including the 12 known genetic clusters of the *E. cloacae* complex (sequences retrieved from NCBI GenBank). Two of the twelve known genetic clusters of *Enterobacter cloacae* complex was reported in this study; six (75%) clustered around *E. cloacae* cluster III type strain rendering *E. cloacae* cluster III the most prevalent genotype of this study, while 2 (25%) clustered with *E. cloacae* IV strain (Fig 4.4). Most clusters had a good bootstrap support (>75 %) except cluster for EC12 which was weakly supported (54%) by bootstraps (Fig 4.4). Two strains, EC1 and EC12 demonstrated 98% similarity with *E. cloacae* cluster III during blast search; nonetheless, these strains belonged to the same clade with *E. cloacae* cluster III (Fig 4.4)

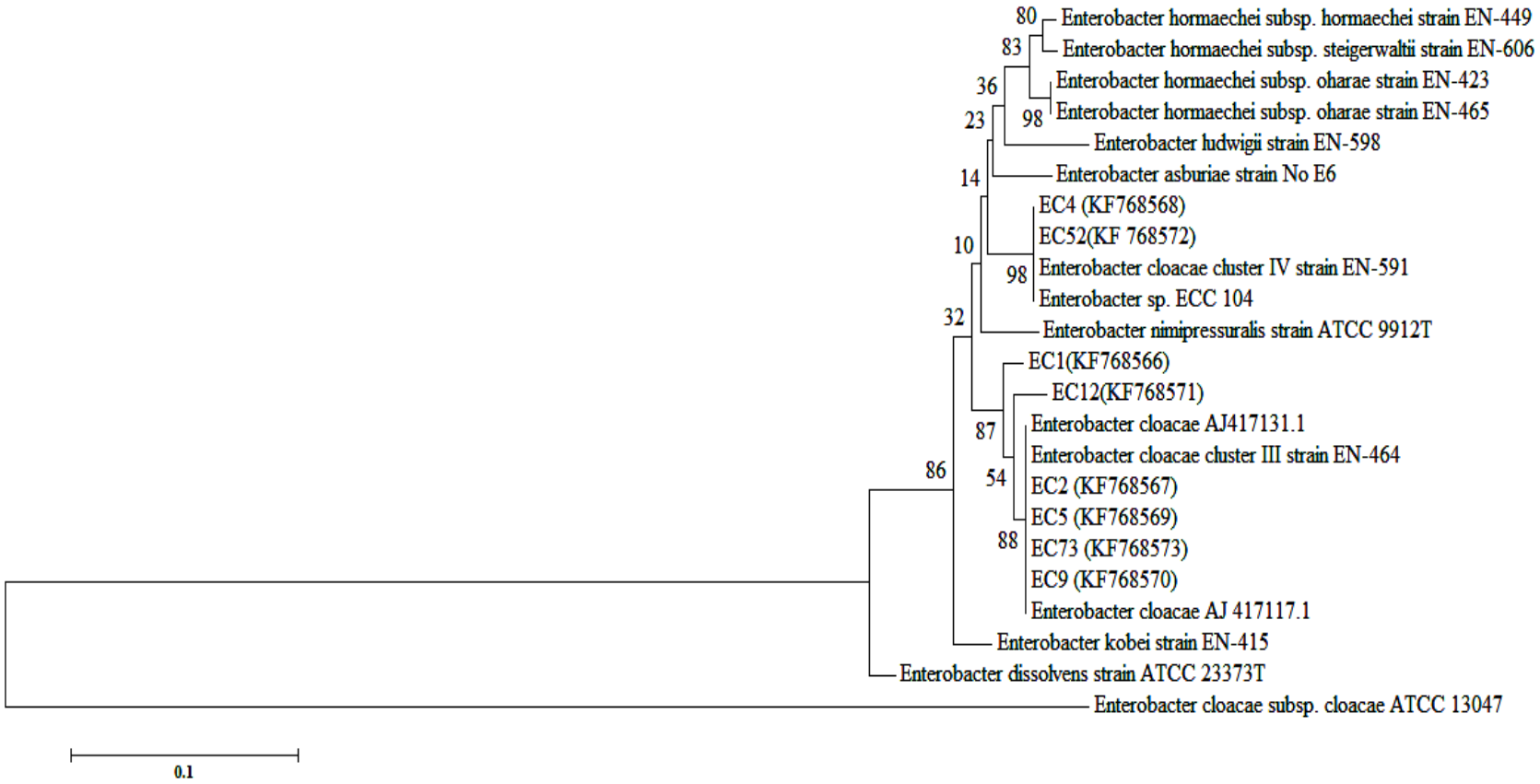

Figure 4.4: The Neighbour joining tree of the *hsp60* gene of *Enterobacter cloacae* complex: The strains from this study are designated with"EC (accession number)". The evolutionary history was inferred using the Neighbour joining method. The optimal tree with the sum of branch length = 0.28125826 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

By using commercial biochemical kits such as API 20E, *E. cloacae* complex strains are routinely identified as '*E. cloacae'*. However, according to the sequence of the *hsp60* gene, the complex is heterogeneous comprising of 12 closely related genetic clusters which differ in pathogenicity towards humans; they include: *Enterobacter asburiae*, *E. cloacae* subsp. *cloacae*, *E. cloacae* subsp. *dissolvens*, *E. cloacae* cluster III, *E. cloacae* cluster IV, *E. cloacae* cluster IX, *E. hormaechei* subsp. *hormaechei*, *E. hormaechei* subsp. *oharae*, *E. hormaechei* subsp. *steigerwaltii*, *E. kobei*, *E. ludwigii* and *E. nimipressuralis* (Hoffmann *et al*., 2005; Mokracka *et al*., 2011). Amongst these, *E. hormaechei* subsp. *oharae, E. hormaechei* subsp. *steigerwaltii, Enterobacter cloacae* subsp. *cloacae* and *E. cloacae* cluster III are the species most frequently recovered from clinical specimens and epidemic outbreaks (Hoffmann *et al*., 2005; Morand *et al*., 2009; Paauw *et al*., 2009). In this study, 75% (6/8) of the strains were *E. cloacae* cluster III. The study also noted that these clusters co-existed in the same environment, exemplified by the occurrence of *E. cloacae* cluster III and *E. cloacae* cluster IV in samples from supermarket 1 (Table 4.3).

Table 4.3: Distribution of the different *E. cloacae* variants and their sampling sites

Isolate	*E. cloacae* variant	Sampling site
EC2	*E. cloacae* cluster III	S1
EC4	*E. cloacae* cluster IV	S1
EC5	*E. cloacae* cluster III	S1
EC9	*E. cloacae* cluster III	C4
EC12	*E. cloacae* cluster III	C1
EC52	*E. cloacae* cluster IV	C5
EC73	*E. cloacae* cluster III	C1
EC1	*E. cloacae* cluster III	S1

S1, supermarket 1; C1, cafeteria 1; C4, cafeteria 4; C5, cafeteria 5.

While little is known about their virulence-associated properties, as Gram-negative pathogens, *E. cloacae* possess some virulence characteristics similar to other members of this family (Mezzatesta *et al*., 2012). Urinary tract infection is among the nosocomial infections caused by this organism; Wray *et al*. (1986) isolated and identified a protein, termed uroepithelial cell adhesin (*UCA*), from a uropathogenic isolate of *P. mirabilis* HU 1069 which was found to be responsible for the attachment of bacteria to uroepithelial cells. The present study identified 40% (12/30) of *E. cloacae* strains possessing the *ucaA* gene; implying that these foods contained virulent strains which may pose health risk to consumers especially the immunocopromised as these organisms are mostly opportunistic. In another study, Bijlsma *et al*. (1995) demonstrated that *UCA* synthesized by UTI-associated *P. mirabilis* strains isolated from dogs corresponds to thin fimbriae, the study further found that the greatest expression of this adhesion occurred during the maximal adherence capability of

bacteria to epithelial cells; suggest that the *UCA* protein acts as fimbriae, responsible for bacterial attachment to the uroepithelium. Several studies have reported on the role of fimbriae proteins (curli) in biofim formation (Olsén *et al*., 1993; Zogaj *et al*., 2003; Greetje *et al*., 2012). In our previous study, we demonstrated the ability of these isolates to form biofilm (Nyenje *et al*., 2013). In support of these findings was the study of Kim *et al.* (2012) which reported a good correlation between the phenotypic detection of curli fimbriae and the genotypic detection of the curli gene, suggesting the importance of these fimbriae in the formation and morphology of biofilms in *E. cloacae*.

Upon successful attachment to the epithelial cells, *Enterobacter* species produce many potential virulence factors, including enterotoxins, α-hemolysin (*hlyA*) and thiol-activated pore-forming cytotoxins similar to shiga-like toxin II (Kere´nyi *et al*., 2007). *HlyA* lyses cells by the creation of pores in the target cell membrane and affects erythrocytes, leukocytes and renal tubular cells (Kere´nyi *et al*., 2007). The virulence gene *hlyA* was detected in 16.6% of *E. cloacae* strains isolated from various foods in this study. The findings are in agreement with others (Kere´nyi *et al*., 2007; Rawool *et al*., 2007). Kere´nyi and colleagues (2007) reported *hlyA* prevalence of 83.9% in *Escherichia coli* strains that cause extraintestinal infections.

4.3.3 Listeria species and their associated virulence genes

Of the 50 *L. ivanovii* isolates detected by API Listeria, only 44% (22/50) of the isolates tested positive for *L. ivanovii* by PCR (using Liv/LisB primer) and was visualized as a band of 1Kb on agarose gel (Fig 4.5). Since *L. ivanovii* is closely related to *L. seeligeri* and *L. welshimeri* (Schimid *et al*., 2005), the study further used a primer (Swis2) which targets all the three species. It was found that 46% (23/50) of the strains that were previously confirmed to be *L. ivanovii* by API Listeria, but tested negative for *L. ivanovii* by PCR (using Liv/LisB) were

confirmed to be *Listeria* of these species (*L. seeligeri* and *L.welshimeri*) and was observed as a band of 1.02 Kb (Fig. 4.6), while 10% (5/50) tested negative for both Liv/LisB and Siwi2. Interestingly, none of the four associated virulence genes (*plcA*, *iap*, *hlyA* and *actA*) were detected from the isolates.

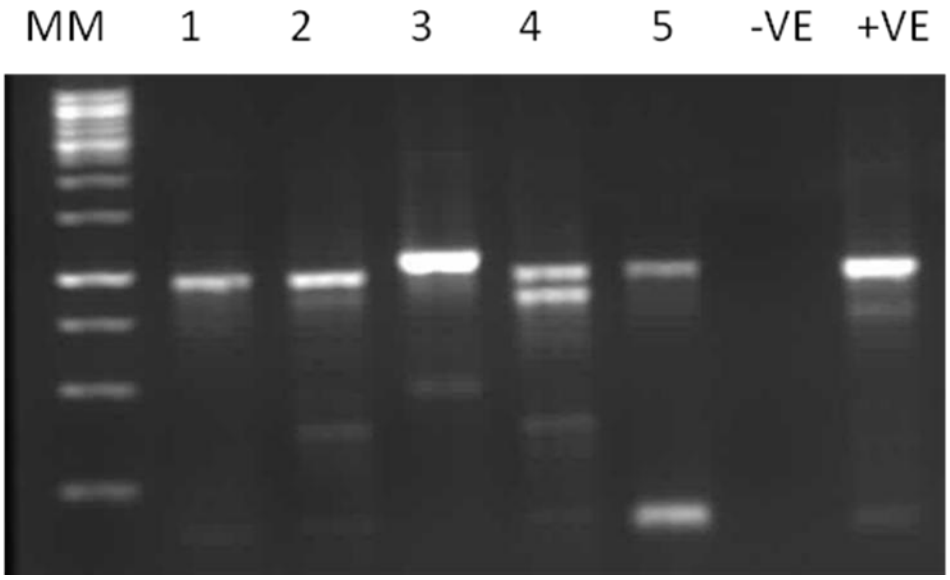

Figure 4.5: A 1.5% agarose gel electrophoresis separation of *L. ivanovii* PCR products observed as 1Kb band. MM, 1Kb molecular marker; lane 1-5, positive samples; -VE, negative control; +VE, positive control (*L. ivanovii* ATCC 19119)

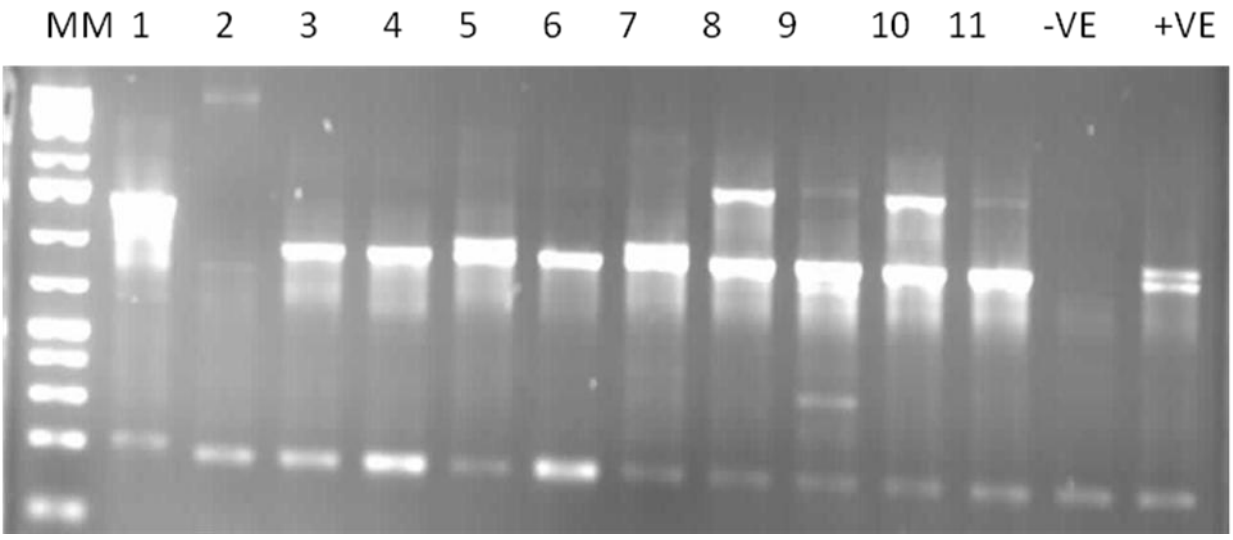

Figure 4.6: A 1.5% agarose gel electrophoresis separation of Siwi2 PCR products observed as 1.02 Kb. MM, 1Kb molecular marker; lane 1, 3-11, positive samples; lane 2, negative sample; -VE, negative control; +VE, positive control (*L. ivanovii* ATCC 19119)

Listeria species are closely related bacteria that share many biochemical characteristics. Biochemical tests measure the phenotypic characteristics of the bacteria; hence, their performance can be influenced by external factors that affect bacterial growth and metabolic mechanisms. For this reason, biochemical tests may present a challenge with regard to *Listeria* identification at species level. In this instance, molecular techniques that are intrinsically more specific and sensitive for the identification and differentiation of *Listeria* species are widely employed (Liu, 2006). Similarly in this study, some isolates that were previously confirmed to be *L. ivanovii* (99.9% identification) by API Listeria (biochemical test kit) were reported to be either of other closely related species (*L. seeligeri* and *L. welshimeri*) or non *Listeria* by PCR.

The present study further sequenced 34 of these PCR products; a Neighbour-Joining tree was estimated to determine the relatedness of the sequences of this study to those retrieved from the GenBank. Fifty-six percent (19/34) of the isolates clustered around *L. ivanovii* type

strains, 44% (15/34) clustered with *L. seeligeri* and 14.7% (5/34) grouped with *L. welshimeri* (Fig 4.5). The genus *Listeria* consists of closely related species; phyogenetic studies group them into two clades: *L. welshimeri*/*L. seeligeri*/*L. ivanovii*, while the other clade constitutes *L. innocua*/*L. marthii/L. monocytogenes* (den Bakker *et al*., 2010). However the placement of *L. welshimeri* has always been ambiguous. While some studies placed *L. welshimeri* basal in the *L. seeligeri*/*L.ivanovii* clade (Schmid *et al*., 2005), others place *L. welshimeri* in the latter clade (Volokhov *et al*., 2007; den Bakker *et al*., 2010). The current study accords the latter findings that place *L.welshimeri* in the *L. innocua*/ *L. marthii/L. monocytogenes* clade (Fig 4.7).

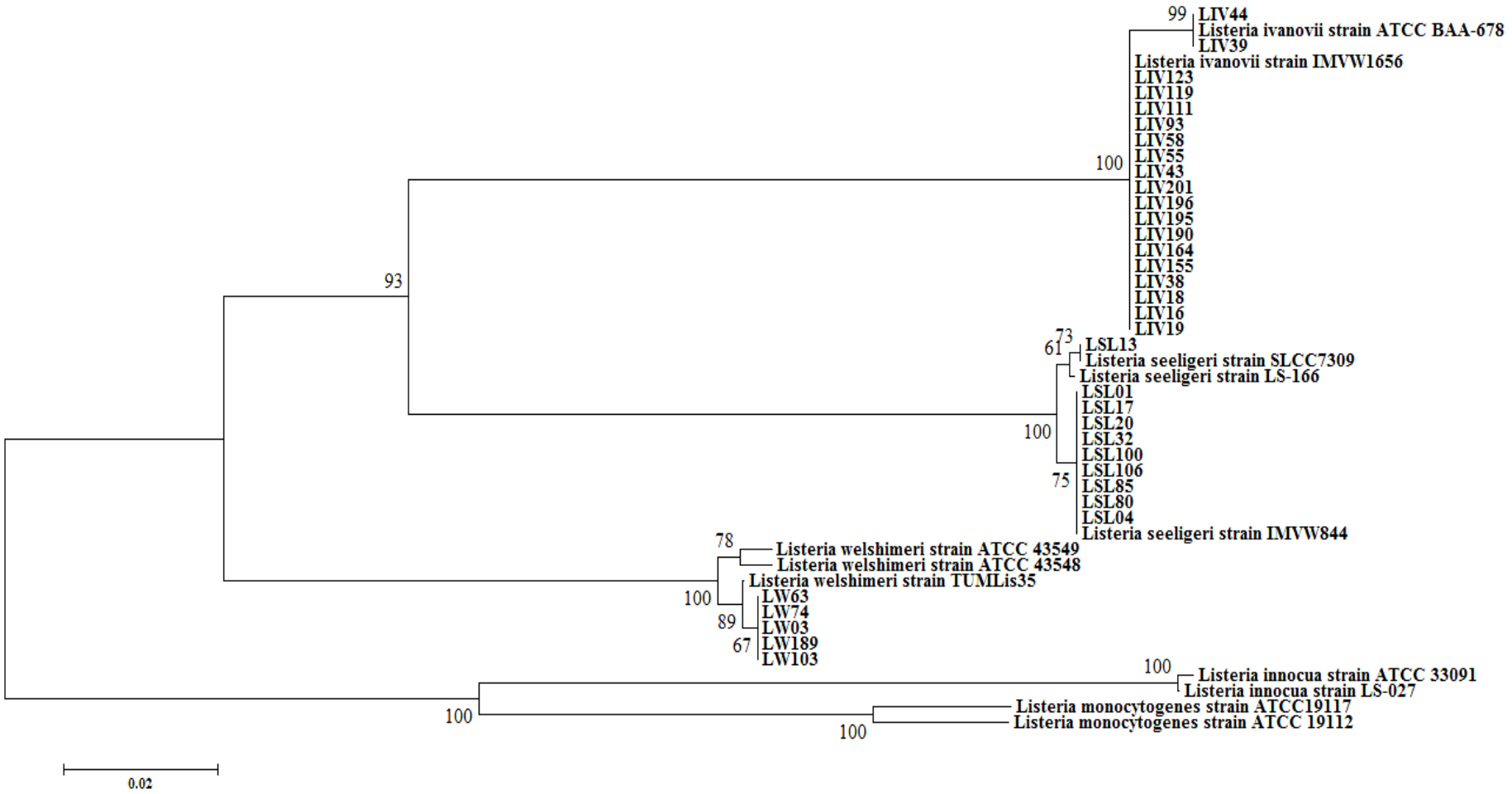

Figure 4.7: Evolutionary relationships of *Listeria* species taxa. The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 34 nucleotide sequences. Evolutionary analyses were conducted in MEGA5. The strains from this study are designated LIV, *Listeria ivanovii*; LSL, *Listeria seeligeri*; LW, *Listeria welshimeri.*

Even though some virulence properties like haemolytic activity on blood agar have been employed to differentiate the pathogenic and non-pathogenic *Listeria* species (Rocourt and Grimont, 1983; Johnson *et al*., 2004), isolates with atypical properties have been detected that require molecular identification (Allerberger, 2003). The findings of this study therefore signify the importance of molecular identification of *Listeria* species if proper diagnosis is to be made. This prompted us to sequence the Swis2 DNA products (34 products) in order to determine their specific species. The results of the sequence analysis demonstrated that 56% (19/34) were *L. ivanovii,* 29% (10/34) *L. seeligeri* and 14.7% (5/34) *L. welshemeri*. The findings are in agreement with another study in the same area which isolated these species from wastewater effluents (Odjadjare *et al*., 2010).

Virulence gene clusters such as the *prfA* cluster and clusters of internalin genes have been associated with the pathogenicity of the two pathogenic species of *Listeria*. The *prfA* virulence cluster encodes a number of proteins that are essential for intracellular survival and motility; they include *hlyA, plcA* and *ActA;* while the clusters of internalin genes; *inlA, inlB* and *inlC*, encode proteins required for invasion of different cell types, including human intestinal and epithelial cells (Johnson *et al*., 2004; Rajabian *et al*., 2009). The present study targeted genes encoded by the main *Listeria* virulence gene cluster, the *prfA* also known as the *LiPI*. Interestingly, all the strains tested lacked these virulence associated genes as they were not detected in any of the strains.

Although it is tempting to speculate that these strains are avirulent, a number of atypical *Listeria* strains and lineages have been reported; as well as several putative evolutionary intermediates, characterized by distinctive virulence gene presence or absence patterns (Johnson *et al*., 2004; Volokhov *et al*., 2007; den Bakker *et al*., 2010). For instance, while the non-pathogenic *L. innocua* is typically nonhemolytic and lacks the *prfA* cluster, a small

number of strains have been reported that contain the *prfA* cluster as well as *inlA*. Likewise, *L. seeligeri* strains that lack *prfA* virulence cluster have been reported (Volokhov *et al*., 2006). The full genome analyses study of den Bakker and colleagues (2010) which demonstrated that the evolution from a *Listeria* ancestor that contained all three virulence loci (*prfA, inlA* and *inlB*) yielded species and strains that have lost one or more of the key virulence genes lay credence to the findings of the current study. However, other studies have reported the presence of *inlC* as the only gene that differentiates pathogenic from non-pathogenic *Listeria* (Johnson *et al*., 2004; den Bakker *et al*., 2010). In their study, den Bakker *et al*. (2010) observed avirulence in mouse infection experiments from L. *monocytogenes* and haemolytic *L. innocua* strains that carried both the *prfA* cluster as well as *inlA* while lacking *inlC*. Nonetheless, the negative findings of the current study may also be attributed to the small sample size as only 50 strains were screened.

4.4 Conclusion

From the findings of this study, it can be inferred that the occurrence of these bacteria illustrate that ready-to-eat foods in these vending sites may act as a reservoir of pathogenic bacteria for human in the study area. Based on the observations outlined above, the need for a well-designed molecular approach to define pathogenic strains within the genus *Listeria* is warranted. However, some of these strains were reported to form biofilms *in-vitro* (Nyenje *et al*., 2012a); since biofilm formation is among the virulence factors of an organism, it can be equally speculated that these strains are pathogenic.

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CHAPTER FIVE

Current status on antibiogram of *Listeria ivanovii* and *Enterobacter cloacae* isolated from ready-to-eat foods in Alice South Africa

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Current status on antibiogram of *Listeria ivanovii* and *Enterobacter cloacae* isolated from ready-to-eat foods in Alice, South Africa.

Abstract

This study assessed the antimicrobial susceptibility of 51 *Listeria ivanovii* and 33 *Enterobacter cloacae* strains (based on API identification) isolated from various ready-to-eat foods sold in Alice, South Africa. The disc diffusion technique was used to screen for antimicrobial susceptibility against 15 antimicrobials; minimum inhibitory concentration (MIC) of five antibiotics was determined by the broth microdilution and M.I.C. Evaluator test strip methods. The efficiency of the two methods was also compared. All the strains of *E. cloacae* (100%) and 96% of *L. ivanovii* isolates were resistant to at least four or more of the antibiotics; nineteen antibiotypes were obtained based on the antibiotics used in the study. Antibiotype A5: AR PGR VAR ER APR was predominant in both *L. ivanovii* (23.5%) and *E. cloacae* (57.5%) isolates. Marked susceptibility of *L. ivanovii* was observed against chloramphenicol, ciprofloxacin, streptomycin and sulfamethoxazole/trimethoprim (100%) each while *E. cloacae* registered 100% susceptibility to ciprofloxacin only. Various percentages of susceptibility was reported to chloramphenicol and gentamicin (91%) each, nalidixic acid (97%) and streptomycin (94%). The MIC_{90} ranged from 0.004 - 7.5 µg/mL with *E. cloacae* being the most susceptible organism. Categorical agreement (interpreted as breakpoints of resistance and sensitive) between M.I.C. Evaluator strip and broth microdilution for amoxicillin, metronidazole and erythromycin was 100%; while categorical agreement for ciprofloxacin was 90%. The essential agreement (the doubling dilution difference) for erythromycin, ciprofloxacin and tetracycline were 90%, 70% and 15% respectively. The study demonstrated the presence of multi-resistant strains of bacteria in ready-to-eat-foods and speculates that these foods could serve as important vehicles transmitting multi-resistant bacteria to humans. Results also indicate a high efficiency of the

M.I.C. Evaluator strip method in determination of MIC as compared to broth microdilution method.

5.1 Introduction

Antimicrobial resistance is currently the greatest challenge worldwide. It decreases the effectiveness of drugs that decrease morbidity and mortality associated with serious and life-threatening infections and thus, compromising human health (Collignon *et al*., 2009). Food contamination with antimicrobial resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance. Since the last decade, the prevalence of antimicrobial resistance among foodborne pathogens has increased (Threlfall *et al*., 2000; Yucel *et al*., 2005), possibly as a result of selection pressure created by the use of antimicrobials in animal health (White *et al*., 2002).

Although foodborne diseases represent an important health problem, the international impact of foodborne illness is difficult to estimate as large numbers of illnesses remain underreported (WHO, 2002). On the other hand, these illnesses are sometimes indistinguishable from other diseases because they share common symptoms. Therefore, it is generally accepted in the scientific community, that the true incidence of foodborne diseases is unknown (Gashaw *et al*., 2008). However, in the United States, foodborne illnesses are estimated to be responsible for some 76 million cases and 3,000 deaths each year (Tauxe *et al*., 2010), whereas in the United Kingdom, foodborne diseases are estimated at 1.7 million per year (Adak *et al*., 2005). Several devastating outbreaks of foodborne diseases have been reported on the African continent, including acute aflatoxicosis in Kenya, which resulted in 317 cases and 125 deaths (Azziz-Baumgartner *et al*., 2005). Although cholera is preventable and treatable, Africa continues to be plagued with annual outbreaks. In 2005, a total of

131,943 cases and 2,272 deaths were reported to the World Health Organization with Africa accounting for 58% of the global incidence (WHO, 2006). In 2008, Zimbabwe, reported 98,424 cases and 4,276 deaths (WHO, 2009).

The most common clinical presentation of foodborne diseases takes the form of gastrointestinal symptoms which are generally mild and self-limiting; hence no antimicrobial therapy is required. However, therapy may be life-saving in patients with underlying illness (Mølbak, 2005). Antimicrobial resistance in some foodborne pathogens, for example *L. monocytogenes* has been documented, which has negatively affected the management of serious infections caused by these organisms (Angulo *et al*., 2004).

Listeria species are ubiquitous, Gram-positive bacteria that possess characteristics such as the ability to form biofilms, growth at low temperatures, and tolerance of osmotic stress. These factors inevitably contribute to its persistence in and adaptation to the environment. *L. monocytogenes* and *L. ivanovii* are reported to be the causative agents of listeriosis in both humans and animals, with the latter rarely occurring in humans (Yucel *et al*., 2005). However, Guillet *et al.* (2010) related the few cases of *L. ivanovii* infection to sporadic occurrence of the organism in nature, as the organism is isolated occasionally from animals or environmental sources including food. Furthermore, this organism has been increasingly isolated in food and different environments in some areas (Salihu *et al*., 2008; Odjadjare *et al*., 2010; Osaili *et al*., 2011; Nyenje *et al*., 2012). The bacterium was the most prevalent in wastewater and treated final effluents in the same study area of the present study (Odjadjare *et al*., 2010); similarly it was common in cooked food samples in the area (Nyenje *et al*., 2012) suggesting its prevalence in our environment. Listeriosis has been associated with mortality rates of up to 30% in infants and in patients with underlying diseases (Bertrand *et al*., 2005). The main route of transmission is through consumption of contaminated food. The

bacteria can also be acquired by the foetus from its infected mother via the placenta (Yucel *et al*., 2005). Listeriosis in immunocompetent adults develops as gastroenteritis which is normally self-limiting, however in immunocompromised individuals, the infection can manifest as septicaemia or meningoencephalitis whereas perinatal infections can lead to abortion, or sepsis and meningitis in the neonates (Allerberger and Wagner, 2010).

The first choice of treatment for listeriosis is a β-lactam antibiotic (*e.g*., penicillin or ampicillin), alone or in combination with an aminoglycoside (*e.g*., gentamicin). The association of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole, is considered to be a second choice therapy (Safdar and Armstrong, 2003; Bertrand *et al*., 2005; Conter *et al*., 2009). However, *Listeria* strains resistant to penicillin, ampicillin, erythromycin, streptomycin and tetracycline have been reported which dictates judicious use of these drugs (Charpentier *et al*., 1995; Bertrand *et al*., 2005; Srinivasan *et al*., 2005).

E. cloacae occur in water, sewage, soil, food, and as commensal microflora in the intestinal tracts of humans and animals. They are frequently isolated from clinical and food samples as opportunistic pathogens (Lund *et al*., 2000). These organisms are important nosocomial pathogens responsible for local and systemic infections in humans (Krzyminska *et al*., 2010). Outbreaks of *E. cloacae* have been reported in South Africa (Nierop *et al*., 1998; Brink *et al*., 2011). In 1998, an outbreak in the neonatal intensive care unit of a Gauteng hospital was reported and the investigation revealed that six of the isolates were from environmental sources, three from blood cultures of patients and one from the hands of a staff member (Nierop *et al*., 1998). Resistant strains among clinical isolates of these organisms have also been documented in hospitalized patients in Pretoria and Johannesburg (Brink *et al*., 2011). A wide variety of drugs are currently used in the treatment of infections caused by this organism. Such antibiotics include aminoglycosides, sulfamethoxazole/trimethoprim, fluoroquinolones and carbapenems. However, there are reports of an increasing prevalence of

antimicrobial-resistant Enterobacteriaceae, not only pathogenic but also commensal bacteria, suggestive that the community can act as a reservoir, and food can contribute to the spread of these resistant strains (Mesa *et al*., 2006). *E. cloacae* have been reported to be resistant to ampicillin, erythromycin, rifampicin and sulfamethoxazole (Haryani *et al*., 2008). Some strains have also been found to produce extended-spectrum β-lactamase (ESBL) and AmpC, conferring resistance to both third and fourth generation cephalosporins (Paterson, 2006).

Emergence of antimicrobial resistance bacteria has become a serious problem worldwide. While much of the resistance observed in human medicine is attributed to inappropriate use of antibiotics in humans, there is increasing evidence that antimicrobial use in animals selects for resistant foodborne pathogens that may be transmitted to humans as food contaminants (Li *et al*., 2007; Collignon *et al*., 2009). Therefore, the present study was carried out to determine the antimicrobial susceptibility profiles of *L. ivanovii* and *E. cloacae* isolated from ready-to-eat foods from Alice, South Africa, in an effort to assess the risks these foods may pose to the community.

5.2 Materials and methods

5.2.1 Antibiotic susceptibility test

5.2.1.1 Disc diffusion test

A total of 51 *L. ivanovii* and 33 *E. cloacae* strains (based on biochemical identification) previously isolated from various food samples (Nyenje *et al*., 2012) were screened for their antibiotic susceptibility using disc diffusion method as recommended by the Clinical and Laboratory Standards Institute CLSI (2006) on Mueller Hinton agar (Oxoid, Basingstoke,UK) with the following antibiotic discs: ampicillin (25 µg), erythromycin (15 µg), streptomycin (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), amoxicillin (20 µg), nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim (5 µg), vancomycin (10 µg), sulfamethoxazole/trimethoprim (1.25 µg), gentamicin (10 µg), neomycin (30 µg), penicillin G

(10 unit) and kanamycin (30 μg). All discs were obtained from Mast Diagnostics, Merseyside, United Kingdom.

Three to five well-isolated colonies from Nutrient agar (Oxoid, Basingstoke, UK) were transferred into 5 mL normal saline until its turbidity was equivalent to 0.5 McFarland standards. The suspension was streaked uniformly onto Mueller Hinton agar plate with a sterile cotton swab. Antibiotic discs were placed onto the surface of each plate (5 antibiotics/petri dish) using a sterile forceps. After incubation at 37 °C for 24 h, the diameter of growth inhibition zone surrounding each disc was measured and interpreted according to the CLSI (2007) recommendation. Currently, there are no interpretative criteria provided by CLSI for *Listeria* species. Therefore, the present study used the CLSI criteria for *Staphylococci* species (Li *et al*., 2007). *S. aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442 reference strains were used to test the efficacy of the discs.

5.2.1.2 Minimum inhibitory concentration (MIC_{90}) determination

Determination of MIC_{90} was done using the broth microdilution technique as previously described by Nyenje and Ndip (2011). Ciprofloxacin, tetracycline, chloramphenicol, kanamycin and nalidixic acid antibiotics which demonstrated marked susceptibility in the disc diffusion test and available in our laboratory at the time of the experiments were used. Tetracycline, nalidixic acid and chloramphenicol powders were dissolved in 0.2% Dimethyl sulfoxide (DMSO) whilst kanamycin and ciprofloxacin powders were dissolved in sterile distilled water as per manufacturer's instruction. Mueller Hinton broth (100 μL) (Oxoid, Basingstoke, UK) was dispensed into the wells except the first well of each row which contained 150 μL of Mueller Hinton broth and the last row of wells which contained distilled water. A 50 μL of the stock antibiotic was dispensed into the first well; a two-fold serial dilution was carried out up to well number 12 from which 100 μL was discarded. Twenty

microlitres of standardized bacterial suspension (0.5 McFarland standards) was added to each of the wells except the control wells (control wells contained broth only and distilled water only). An automatic ELISA microplate reader (SynergyMx, Biotek^{R} USA) adjusted to 570 nm was used to measure the absorbance of the plates before and after incubation at 37 °C for 24 h. The absorbencies were compared to detect an increase or decrease in bacterial growth and the values plotted against concentration. The lowest concentration of the antibiotic resulting in inhibition of 90% bacterial growth was recorded as the MIC_{90}. The following reference strains *S. aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442 were used as control organisms.

5.2.1.3 Minimum inhibitory concentration determination by the M.I.C. Evaluator.

Twenty *E. cloacae* strains were evaluated for their susceptibility to seven antimicrobials using the M.I.C. Evaluator method, following the manufacturer's guidelines (Oxoid, Basingstoke, England). Considering the role of commensal bacteria in the spread of antibiotic resistance; coupled with the fact that there were 20 M.I.C. Evaluator strips each of the five antibiotic powders present in our laboratory at the time of the experiments, *E. cloacae* was selected because it is one of the few commensal bacteria where screening of antibiotic resistance is recommended in health care facilities (Cornaglia *et al*., 2004). Briefly, a suspension equivalent to 0.5 McFarland standards turbidity was swabbed on Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates. M.I.C. Evaluator strips were placed aseptically and plates were incubated at 37° C for 18 to 20 h. The MIC was read where inhibition of growth intersected the strip. *S. aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442 reference strains were used to test the efficacy of the strips.

5.3 Analysis of the results

All MICs were interpreted using CLSI breakpoints (CLSI, 2010). The efficacy of the M.I.C. Evaluator and broth microdilution methods were compared using categorical and essential agreement, a method commonly used to compare different antimicrobial susceptibility testing methods. Categorical agreement is based on interpretive breakpoints of sensitive and resistant strains. The doubling dilution difference (essential agreement) in the MIC was calculated as: $\log_2$ (MIC by broth microdilution) – $\log_2$ (MIC by M.I.C. Evaluator method) whereby the agreement of the two methods within ±1 was the essential agreement (Rennie *et al*., 2012).

5.4 Results and discussion

5.4.1 Antimicrobial patterns by disc diffusion test

All *L. ivanovii* (51) isolates were resistant (total) to vancomycin, penicillin G and erythromycin, but were sensitive to chloramphenicol, ciprofloxacin, streptomycin and sulfamethoxazole/trimethoprim. Of the 51 isolates, 98% were susceptible to nalidixic acid and gentamicin; while susceptibilities of 82% and 55% were reported to trimethoprim and tetracycline respectively; the rest showed either intermediate or total resistance to these antibiotics. Ciprofloxacin was the most active drug. All *E. cloacae* strains (100%) were susceptible to ciprofloxacin while various degrees of susceptibility to nalidixic acid 32 (97%), streptomycin 31 (94%), gentamicin and chloramphenicol 30 (91%) each, were observed. However, the isolates showed marked resistance (100%) to amoxicillin, penicillin G, vancomycin and erythromycin, while 26 (79%) and 19 (58%) of the isolates were resistant to ampicillin and tetracycline respectively (Table 5.1). The results of this study corroborate other findings (Conter *et al*., 2009; Li *et al*., 2007; Chen *et al*., 2010). However these findings are also contrary to those of Odjadjare *et al*. (2010) who reported moderate sensitivity to ciprofloxacin (91%), chloramphenicol (87%) and streptomycin (65%) by strains recovered

from wastewater effluents in the same study area of the current study. This discrepancy may be due to the different sample sources of the isolates which may impact on their adaptation and response to different chemicals and antimicrobial agents. Previous studies on the antimicrobial susceptibility profiles of *Listeria* species have focused mainly on *L. monocytogenes* with a dearth of information in the literature on antibiotic susceptibility profiles for *L. ivanovii*. However, this animal pathogen is equally important to man as it has been isolated from infected humans, indicating pathogenic potential for humans (Guillet *et al*., 2010).

Table 5.1: Antimicrobial susceptibility profile of *L. ivanovii* and *E. cloacae* isolated from ready-to-eat foods.

Antibiotic	Number (%)					
	***L. ivanovii* (n=51)**			***E. cloacae* (n=33)**		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Chloramphenicol (30µg)	51 (100)	0 (0)	0 (0)	30 (91)	2 (6)	1 (3)
Kanamycin (30µg)	21 (39)	19 (37)	11 (22)	27 (82)	3 (9)	3 (9)
Amoxacillin (20µg)	3 (6)	0 (0)	48 (94)	0 (0)	0 (0)	33 (100)
Penicillin G (10 units)	0 (0)	0 (0)	51 (100)	0 (0)	0 (0)	33 (100)
Vancomycin (30µg)	0 (0)	0 (0)	51 (100)	0 (0)	0 (0)	33 (100)
Ciprofloxacin (5µg)	51 (100)	0 (0)	0 (0)	33 (100)	0 (0)	0 (0)
Streptomycin (10µg)	51 (100)	0 (0)	0 (0)	31 (94)	2 (6)	0 (0)
Sulfamethoxazole/trimethoprim (1.25µg)	51 (100)	0 (0)	0 (0)	26 (79)	0 (0)	7 (21)
Nalidixic acid (30µg)	50 (98)	1 (2)	0 (0)	32 (97)	1(3)	0 (0)
Trimethoprim (5µg)	42 (82)	4 (8)	5 (10)	25 (76)	0(0)	7 (21)
Erythromycin (15µg)	0 (0)	0 (0)	51 (100)	0 (0)	0(0)	33 (100)
Neomycin (30µg)	20 (39)	24 (47)	7 (14)	15 (45)	13 (39)	5 (15)
Tetracycline (30µg)	28 (55)	0 (0)	23 (45)	14 (42)	0 (0)	19 (58)
Ampicillin (10µg)	23 (45)	5 (10)	23 (45)	0 (0)	7 (21)	26 (79)
Gentamicin (10µg)	50 (98)	1(2)	0 (0)	30 (91)	1 (3)	2 (6)

Multidrug resistance was a common phenomenon observed in all the 51 (100%) *L. ivanovii* and 33 (100%) *E. cloacae* isolates. Nineteen antibiotic resistance patterns were obtained (Table 5.2). Antibiotype A5: AR PGR VAR ER APR (amoxicillin, penicillin G, vancomycin, erythromycin, ampicillin) was predominant in both *L. ivanovii* (23.5%) and *E. cloacae* (57.5%) isolates, followed by A3: AR PGR VAR ER (amoxicillin, penicillin G, vancomycin, erythromycin) which was obtained from seven (21.2%) of *E. cloacae* and 11 (22.5%) of *L. ivanovii*. The least resistant patterns for *E. cloacae* (3%) were demonstrated by A15 and A19 while for *L. ivanovii* (1.9%) A2, A4, A7, A11 and A12 respectively, demonstrated the same pattern.

The results indicated alarming multi-resistance frequencies to at least four or more of the test antibiotics. This is of major concern to the public as these foods may act as reservoir of resistant strains that can be transmitted to humans upon ingestion of the contaminated food. Similar patterns of resistance have been reported by other authors (Srinivasan *et al*., 2005; Haryani *et al*., 2008; Li *et al*., 2007). Srinivasan *et al*. (2005) found that all *L. monocytogenes* strains isolated from a dairy farm environment were resistant to ampicillin and rifampicin, and some to tetracycline, penicillin and chloramphenicol. Furthermore, Haryani *et al*. (2008) in their study observed that all *E. cloacae* strains studied were resistant to six or more antibiotics.

The results suggest that incidence of antibiotic resistance in *L. ivanovii* is relatively high. It is of great concern that the range of antibiotics to which resistance has been acquired is wide and the expanding range now includes a number of antibiotics used to treat listeriosis (penicillin, vancomycin, erythromycin and ampicillin). This may have future implications for the effective treatment of listeriosis if these resistant strains could be transferred to humans. Interestingly, the study reported high susceptibility of strains to gentamicin (98%) and sulfamethoxazole/trimethoprim (100%), drugs which are considered as second choice therapy

(Conter *et al*., 2009). These findings are in agreement with other reports (Srinivasan *et al*., 2005; Arslan and Ozdemir, 2008; Conter *et al*., 2009).

Table 5.2: **Antibiotypes of *E. cloacae* and *L. ivanovii***

No	Antibiotype	Number (%)	
		E. cloacae	***L. ivanovii***
A1	PG^R VA^R E^R	0 (0)	3 (5.9)
A2	PG^R VA^R E^R T^R	0 (0)	1 (1.9)
A3	A^R PG^R VA^R E^R	7 (21.2)	11 (22.5)
A4	K^R A^R PG^R VA^R E^R	0 (0)	1 (1.9)
A5	A^R PG^R VA^R E^R AP^R	19 (57.5)	12 (23.5)
A6	A^R PG^R VA^R E^R T^R	0 (0)	5 (9.8)
A7	K^R A^R PG^R VA^R E^R AP^R	0 (0)	1 (1.9)
A8	K^R A^R PG^R VA^R E^R T^R	0 (0)	3 (5.9)
A9	A^R PG^R VA^R E^R T^R AP^R	0 (0)	5 (9.8)
A10	A^R PG^R VA^R E^R NE^R T^R	0 (0)	2 (3.9)
A11	A^R PG^R VA^R W^R E^R T^R AP^R	0 (0)	1 (1.9)
A12	K^R A^R PG^R VA^R E^R NE^R AP^R	0 (0)	1 (1.9)
A13	K^R A^R PG^R VA^R E^R T^R AP^R	0 (0)	2 (3.9)
A14	K^R A^R PG^R VA^R E^R NE^R T^R	0 (0)	2 (3.9)
A15	A^R PG^R VA^R W^R E^R T^R AP^R	1 (3)	0 (0)
A16	A^R PG^R VA^R SXT^R W^R E^R T^R AP^R	3 (9)	0 (0)
A17	K^R A^R PG^R VA^R W^R E^R NE^R T^R AP^R	0 (0)	2 (3.9)
A18	K^R A^R PG^R VA^R SXT^R W^R E^R NE^R T^R AP^R GM^R	2 (6)	0 (0)
A19	C^R K^R A^R PG^R VA^R SXT^R W^R E^R NE^R T^R AP^R GM^R	1 (3)	0 (0)

A, amoxicillin; C, chloramphenicol; K, kanamycin; PG, penicillin G; VA, vancomycin; SXT, sulfamethoxazole/trimethoprim; W, trimethoprim; E, erythromycin; NE, neomycin; T, tetracycline; AP, ampicillin; GM, gentamicin.

The increases in antibiotic resistance among *Listeria* species are in line with the general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistances among many groups of bacteria. Multiple drug resistance in these organisms have been attributed to antimicrobial selective pressure and gene transfer mechanisms between and among *Listeria* species and close relatives of the bacteria such as *Enterococcus, Streptococcus* and *Staphylococcus* species (Safdar and Armstrong, 2003; Arslan and Ozdemir, 2008).

The present study observed 100% resistance of *E. cloacae* to amoxicillin, penicillin, ampicillin and erythromycin, while for neomycin and tetracycline it was 45% and 42% respectively. The findings are in agreement with those of Haryani *et al*. (2008), who observed 100% resistance to ampicillin and erythromycin; however, their study demonstrated various degrees of resistance to streptomycin (85.71%), ciprofloxacin and tetracycline (42.86%) and trimethoprim (28.57%) contrary to the findings of this study where ciprofloxacin recorded 100% sensitivity. Environmental factors as well as differing prescription practices may try to explain these discrepancies.

The emerging antibiotic resistance in the Enterobacteriaceae family is a significant problem that requires vigilance and intensified measures to control the further spread of resistance by these important Gram-negative pathogens. Worthy of note is the fact that most research has involved organisms that directly cause disease, focusing less on important contributions by commensal bacteria. Although these organisms generally do not cause disease in immunocompetent people, they can transfer resistance genes to other bacteria in the environment (Paterson, 2006). The European Community therefore recommended the monitoring of antimicrobial resistance in these organisms; *E. cloacae* is one of the few bacteria where monitoring in healthcare facilities is recommended (Cornaglia *et al*., 2004).

There are reports on similarities in virulence factors between human and food isolates of *Enterococcus* species, implying that, food commensals may be an overlooked aspect of antibiotic resistance (Chow *et al*., 1991). Several studies have tracked resistant zoonotic species using non-pathogenic *E. coli* as an indicator organism. De Francesco *et al*. (2004) demonstrated that commensal *E. coli* had higher rates of resistance than pathogenic multi-drug resistant *Salmonella* from the same herds, suggesting that commensal bacteria could be used to survey the prevalence of antibiotic resistant bacteria. On the other hand, food animal reservoirs are an important source of antimicrobial resistance, even though it might be difficult to quantify the exact burden, compared with human use.

5.4.2 Minimum inhibitory concentration (MIC_{90}) determination

Of the five antimicrobials tested, *E. cloacae* isolates were more susceptible to ciprofloxacin with MIC_{90} ranging as low as 0.004 - 3.75 μg/mL. The MIC_{90} of kanamycin, tetracycline, nalidixic acid and chloramphenicol ranged from 0.029 - 0.117 μg/mL, 0.029 - 3.75 μg/mL, 0.029 - 0.937 μg/mL and 0.058 - 0.937 μg/mL respectively (Table 5.3). On the other hand, the MIC_{90} for *L. ivanovii* isolates, ranged from 0.009 - 0.625 μg/mL for ciprofloxacin, 0.014 - 7.5 μg/mL for tetracycline, 0.029 - 3.75 μg/mL for chloramphenicol, 0.058 - 7.5 μg/mL for kanamycin and 0.058 - 7.5 μg/mL for nalidixic acid (Table 5.4).

In-vitro antibiotic susceptibility testing of isolates from patients remains an important guide to therapy for clinicians and a useful marker for epidemiological investigations. The adoption of quantitative determinations of antibiotic susceptibility (MIC determination), rather than qualitative methods (*i.e.*, disc diffusion test) may be of importance in identifying small, but potentially clinical significant changes, in antibiotic susceptibility (Giovanni *et al*., 2002). Interestingly, the study reported a MIC_{90} range as low as 0.004 - 7.5 μg/mL with *E. cloacae* being the most susceptible organism and ciprofloxacin the most active antibiotic. The

findings support the disc diffusion results in which all the isolates were markedly sensitive to this drug.

Table 5.3: **Minimum inhibitory concentration (MIC_{90}) of different antibiotics against *E. cloacae* isolates.**

Isolate no.	Ciprofloxacin	Tetracycline	Chloramphenicol	Kanamicin	Nalidixic acid
EC1-E	0.009	0.02	0.234	0.02	0.234
EC2-E	0.019	0.46	0.46	0.05	0.23
EC4-E	0.009	0.117	0.46	0.117	0.05
EC5-E	0.625	3.75	0.937	ND	0.23
EC7-E	0.004	0.117	0.23	0.117	0.117
EC09	0.004	0.117	0.46	0.05	0.029
EC12	0.156	0.46	0.46	0.029	0.23
EC52	0.009	0.23	0.937	0.117	0.23
EC53	0.625	ND	0.937	0.117	0.937
EC57	0.009	0.117	0.46	0.117	0.05
EC67	0.019	0.46	0.46	0.05	0.117
EC70	0.04	0.117	0.937	0.05	0.23
EC73	0.009	0.23	0.46	0.014	0.05
EC89	0.009	0.117	0.05	0.29	0.46
EC103	0.019	0.23	0.46	0.05	0.117
EC112	0.004	0.05	0.937	0.117	0.117
EC114	0.009	0.117	0.05	0.117	0.46
EC119	0.019	0.117	0.46	0.05	0.29
EC164	0.004	0.029	0.46	0.05	0.46
EC171	0.625	ND	0.46	ND	0.937
EC172	0.625	ND	0.937	ND	0.23
EC177	0.019	0.937	0.46	0.05	0.117
EC194	0.009	0.117	0.23	0.029	0.23
EC198	0.004	0.46	0.46	0.05	0.23
EC205	0.004	0.117	0.937	0.117	0.029
EC208	0.156	0.46	0.46	0.117	0.23
EC217	0.312	ND	0.23	0.029	0.46
EC220	0.009	0.46	0.23	0.029	0.23
EC221	0.004	0.23	0.46	0.117	0.29
EC225	0.009	0.234	0.937	0.117	0.117
EC235	0.312	ND	0.46	ND	0.937

ND, not determined.

Table 5.4. Minimum inhibitory concentration (MIC_{90}) of different antibiotics against *L. ivanovii* isolates.

Isolate	Ciprofloxacin	Tetracycline	Chloramphenicol	Kanamycin	Nalidixic acid
L1	0.312	0.937	0.468	0.468	1.875
L3	0.312	0.058	3.75	0.234	3.75
L4	0.078	0.117	3.75	0.234	0.468
L8	ND	0.117	0.468	0.468	3.75
L16	0.156	0.937	0.937	0.234	7.5
L17	0.156	0.058	0.468	0.234	0.468
L20	0.039	0.937	0.937	0.234	3.75
L27	0.039	0.117	0.937	0.937	0.468
L32	0.009	0.234	3.75	0.234	0.937
L37	0.625	0.117	0.468	0.468	0.937
L198	0.312	0.117	3.7	0.234	3.75
L38	0.156	0.234	0.468	0.117	1.875
L39	0.039	0.937	0.937	0.234	0.468
L41	0.019	0.058	0.937	0.937	1.875
L42	0.009	1.875	0.234	3.75	1.875
L43	0.625	7.5	0.468	7.5	0.058
L44	0.156	0.468	0.937	0.234	ND
L61	0.039	1.875	0.937	0.058	3.75
L74	0.312	0.937	0.468	0.117	7.5
L99	0.078	0.117	0.234	0.117	0.468
L109	0.009	0.058	0.468	3.75	0.117
L117	0.312	0.937	0.468	0.234	1.875
L127	0.019	0.117	1.875	0.117	0.468
L155	0.078	1.875	3.75	0.234	0.468
L160	0.625	0.234	0.468	0.117	1.875
L188	0.039	0.937	0.234	0.234	3.75
L190	0.039	0.937	0.937	0.117	0.937
L192	0.006	0.014	0.937	0.058	0.058
L63	0.312	0.937	0.234	0.468	1.875
L196	0.078	0.117	3.75	0.234	0.468
L55	0.009	1.875	0.468	3.75	1.875
L164	0.019	0.058	0.937	1.875	1.875
L123	0.156	0.058	0.468	0.234	0.468
L73	0.019	1.875	0.937	0.234	0.468

ND, not determined.

5.4.3 The comparison of M.I.C. Evaluator test strip and broth microdilution

Categorical agreement for M.I.C. Evaluator compared to broth microdilution for 20 strains of *E. cloacae* tested against five antibiotics are shown in Table 5.5. All the 20 (100%) strains demonstrated categorical agreement to amoxicillin, metronidazole and erythromycin. On the other hand, 18/20 (90%) and 12/20 (60%) strains were susceptible to ciprofloxacin and tetracycline using the broth microdilution method while with the M.I.C. Evaluator test, all strains (100%) and 11/20 (55%) were susceptible to ciprofloxacin and tetracycline respectively.

Table 5.5. **Antimicrobial susceptibility patterns of *E. cloacae* using broth microdilution and M.I.C.Evaluator test method.**

Drug	MICs (µg/mL)						
	Broth microdilution method			M.I.C.Evaluator test method			Break point S, R
	Range	S (%)	R (%)	Range	S (%)	R (%)	
T	0.014 - >30	12 (60)	8 (40)	2 - >265	11 (55)	9 (45)	< 2, > 2
CIP	0.001 - 2.5	18 (90)	2 (10)	< 0.002 - 0.06	20 (100)	0 (0)	≤ 1, ≥ 2
A	>256	0 (0)	20 (100)	16 - > 256	0 (0)	20 (100)	≤ 8, > 8
MET	> 256	0 (0)	20 (100)	> 256	0 (0)	20 (100)	≤ 32, > 32
E	32 - > 256	0 (0)	20 (100)	32 - >256	0 (0)	20 (100)	≤ 16, > 16

T, tetracycline; CIP, ciprofloxacin; A, amoxicillin; MET, metronidazole; E, erythromycin. All break points derived from CLSI standards.

The MIC results of the 2 methods (broth microdilution and M.I.C. Evaluator) were compared; agreement within ± 1 doubling dilution was 90% (18 strains), 70% (14 strains) and 15% (3 strains) for erythromycin, ciprofloxacin and tetracycline respectively (Table 5.6).

Table 5.6. **Distribution of differences in MICs antimicrobials by 20 strains of *E. cloacae* by the M.I.C.Evaluator test versus the broth microdilution method.**

Antimicrobial	**mean tubes difference** [a]	**No. of isolates with a MIC difference** [b] **of:**							**% agreement within ±1 log_2 dilution**
		<-2	-2	-1	0	+1	+2	>+2	
Tetracycline	2.314	0	0	0	1	2	10	7	15
Ciprofloxacin	-1.446	0	0	8	3	3	6	0	70
Amoxicillin	0.00	0	0	0	20	0	0	0	100
Metronidazole	0.00	0	0	0	20	0	0	0	100
Erythromycin	0.15	2	0	2	12	4	0	0	90

[a] mean difference in log_2 MICs (broth microdilution - M.I.C.Evaluator); [b] expressed in log_2 dilutions.

Agar dilution and broth microdilution are widely recommended quantitative antimicrobial susceptibility test methods, but they are tedious and time consuming to implement as routine test in many clinical laboratories; hence this study compared the broth microdilution method and the M.I.C. Evaluator method which has been validated for its high accuracy and easy performance for routine diagnostic use (Arroyo *et al*., 2005).

For comparison of the MICs, the doubling dilution difference in the MIC was calculated as: $\log_2$ (MIC by broth microdilution method) – $\log_2$ (MIC by M.I.C. Evaluator method). Thus, a MIC tube difference of one tube indicates that the broth microdilution MIC was 1 doubling dilution higher, that is, double the M.I.C. Evaluator MIC. The percentage of isolates with MICs measured by the two methods agreeing to within ±1 and ±2 doubling dilutions was calculated; essential agreement was reported as the percentage of isolates for which the M.I.C. Evaluator MIC was the same or one doubling dilution apart from the broth microdilution MIC (Reynolds *et al*., 2003).

According to Rennie *et al.* (2012), the United States Food and Drug Administration requires >90% essential and categorical agreement when two methods are compared. Interestingly, all the 20 strains demonstrated 100% categorical agreement to amoxicillin, metronidazole and erythromycin (Table 5.5), while erythromycin, ciprofloxacin and tetracycline registered 90%, 70% and 15% essential agreement respectively (Table 5.6). Although erythromycin registered 100% categorical agreement, when the doubling dilution difference of the two methods was compared, discrepancies were recorded surmounting to 10% hence the 90% essential agreement. Our findings are in agreement with those of Rennie *et al.* (2012) who reported essential agreements between 40 to 90% when broth microdilution was compared with M.I.C. Evaluator against clinical strains of anaerobes and other fastidious bacteria. Similarly, Bilal *et al*. (2007) demonstrated that all β-lactamase negative *H. influenzae* isolates which were resistant to

ampicillin registered high MICs on E-test than on broth microdilution method, with essential agreement varying from 42 – 68%. In another study, Arroyo *et al*. (2005) reported 16.5% (19/115 strains) essential agreement of *Acinetobacter baumannii* strains to colistin. Poor diffusion of polymyxins into the agar was associated with the discordance.

Seeking factors that impinge on optimum performance of the M.I.C. Evaluator/E-test against a range of organisms might be daunting; however, other studies reiterated the remarkable and convenient usage of the E-test and M.I.C. Evaluator especially in clinical management (Tristram, 2008; Rennie *et al*., 2012). Differences in antibiotics and microorganisms utilised may enhance some of the differences observed with the use of these methods. We speculate that similar factors could be the possible cause of poor concordance in the present study. In spite the fact that broth microdilution method is standardised and recommended method for use, it is prone to human errors through general manipulations of the assay compared to M.I.C. Evaluator which has clear breakpoints scale and easy to manipulate. Therefore, standardisation regarding the use of the M.I.C. Evaluator for testing *E. cloacae* is of importance.

5.5 Conclusion

The results demonstrate the presence of multi-resistant strains of bacteria in ready-to-eat foods. Their public health impact remains unknown, but point to the fact that these foods could be important vehicles transmitting multi-resistant bacteria to humans on a daily basis, implying that mitigation of drug resistance in foodborne bacteria is likely to be of benefit to human health. Since the recommended quantitative antimicrobial susceptibility tests are tedious and time consuming, M.I.C. Evaluator method could be a dependable, quick and definite alternative method for quantitative antimicrobial susceptibility testing. However, standardisation and further analysis regarding the suitability of the M.I.C. Evaluator is warranted.

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CHAPTER SIX

The distribution of β-lactamase genes among *Listeria ivanovii* and *Enterobacter cloacae* strains isolated in Alice, South Africa.

The distribution of β-lactamase resistance genes among *Listeria ivanovii* and *Enterobacter cloacae* strains isolated in Alice, South Africa.

Abstract

TEM-1 β-lactamase producing bacteria are widely spread; hence is now the most commonly encountered mechanism of resistance to the β-lactam antibiotics in Gram-negative bacilli. The prevalence and distribution of β-lactamase gene varies significantly with geographical regions. The current study determined the prevalence of β-lactamase and tetracycline resistant genes among the species of *Listeria* and *E. cloacae* strains. Standard PCR method was employed for the detection of β-lactamase genes (*TEM-1*, *SHV-1*; *CTX-M*) and tetracycline resistant gene (*TetO*) using specific primers for each gene. Fifty-six *bla*-$_{\text{TEM}}$ positive samples were sequenced and analysed. *Bla*-$_{\text{TEM}}$ was the only gene detected in 48 (96%) of *Listeria* species and 30 (100%) of *E. cloacae* strains. Analysis of the *bla*-$_{\text{TEM}}$ sequence demonstrated the occurrence of *bla*-$_{\text{TEM-1}}$ phenotype 2b. Seven percent (4/56) of the isolates had mutation of either substitution or insertion of a nucleotide. Two strains, EC53 and L32 had substitution type of mutation. *Bla*-$_{\text{TEM-1}}$ type was found to be prevalent in the study area. The detection of *bla*-$_{\text{TEM-1}}$ from *E. cloacae* and *Listeria* isolates implies that these strains have the ability to produce β-lactamase an enzyme that cleaves β-lactam antibiotics; this may create a dilemma in the management of infection caused by these organisms in our environment.

6.1 Introduction

Recognized as a universal problem of increasing magnitude, antibiotic resistance augment the morbidity and mortality caused by microbial infections, as well as the cost of treating these infectious diseases (Nyenje and Ndip, 2013). It is widely known that the "selective pressure" caused by overuse of antibiotics in human medicine and animal husbandry, is the main risk factor for the emergence of antibiotic resistance (Daikos *et al*., 2008; Colomer-Lluch *et al*., 2011). Additionally, in recent years there is a growing concern over the transmission of resistant bacteria via the food chain (Capita and Alonso-Calleja, 2013).

Antibiotics are widely used in food animals to treat, control, or prevent infectious diseases, and to improve the efficiency of feed utilization and weight gain, in an effort to maintain a consistent supply of healthy animals entering the food chain (Capita and Alonso-Calleja, 2013). For instance, it is estimated that half of all the antibiotics consumed in North America and Europe are used in food-producing animals; implying that antibiotic use in animals might have a major impact on the incidence of antibiotic resistance in humans (WHO, 2002). The primary concern has been the use of antimicrobials in food animals which select for resistance to antimicrobial agents in zoonotic intestinal bacteria, and subsequently these resistant genetic materials can be transmitted to humans via the food chain. It is also noted that commensal bacteria present in human or animal gut might transfer their resistance genes to existing susceptible flora, and thereby set the stage for a non-treatable infection at a later time in the individual's life (van den Bogaard and Stobberingh, 2000).

Microorganisms produce many antimicrobials in nature; however, these antibiotic-producing organisms have also become resistant to the antibiotics they produce, and the genes that confer such resistance can be transferred to other non-resistant bacteria. Therefore, the existence of antibiotics in the environment may provide long-term selective pressure for the emergence and transmission of these resistance-conferring genes in non-producing organisms (Canton, 2009).

The mobile genetic elements (MGEs) harbouring resistance determinants can readily be transferred horizontally between bacteria from animals, fish and humans; furthermore, such transfer can take place in natural environments such as the kitchen (Cabello *et al*., 2013). This mechanism has contributed greatly to the wide dissemination of antibiotic resistance genes in the environment. The co-existence of resistance genes with mobile elements such as plasmids, transposons, and integrons has been reported to facilitate the rapid spread of antibiotic resistance genes among bacteria (Dale and Park, 2004).

β-lactam antibiotics are considered to be of clinical efficacy and low toxicity; hence, they are broadly used as antimicrobials (Jain and Mondal, 2008). For instance, an aminoglycoside with penicillin or ampicillin (β-lactam antibiotic) is the drug of choice for listeriosis, while carbapenem is one of the drugs currently used in the management of infections caused by *Enterobacter* species (Conter, 2009; Nyenje *et al*., 2012). However, resistance to β -lactam antibiotics by both Gram-positive and Gram-negative bacteria has been widely reported (Srinivasan *et al*., 2005; Paterson, 2006; Jain and Mondal, 2008; Brink *et al*., 2011; Nyenje *et al*., 2012). The main mechanism of resistance of these organisms to β-lactam antibiotics is the ability to produce enzymes known as β- lactamase that destroy the β- lactam ring, which results in the loss by the antibiotic of its antimicrobial activity (Rubtsova *et al*., 2010).

β-lactamases represent a large group of genetically and functionally different enzymes of which extended spectrum β-lactamases (ESBLs) pose the greatest threat. ESBLs are often plasmid mediated and derived from mutations in the classic *TEM* (Temoneira) and *SHV* (Sulphydryl variable) genes by one or more amino acid substitution around the active site. ESBLs can also be mobilized from environmental bacteria (e.g. *CTX-M*) (Rodrı´guez-Ban˜o *et al*., 2008). Nonetheless, TEM-1 β-lactamase producing bacteria are widespread, and it is now the most commonly encountered mechanism of resistance to the β-lactam group of drugs in Gram-negative bacilli (Rupp and Fey, 2003; Pfaller and Segretii, 2006; Lal *et al*., 2007). DNA

sequencing of *TEM* gene has reported over 170 variations and point mutations, and is speculated that these mutations are the most responsible factor for resistance to β-lactams in these organisms (Livermore, 1995; Jain and Mondal, 2008).

ESBLs appear to be unique with geographical location. For example, *TEM*-10 has been responsible for several unrelated outbreaks in the United States and Europe, while *TEM*-3 is reported to be common in France (Nordmann, 1998; Barroso *et al*., 2000). The present study therefore was carried out to determine the prevalence of these resistant genes among the species of *Listeria* and *E. cloacae* strains and characterise their variants in an effort to establish baseline data on those common in the study area as a guide to empiric treatment.

6.2 Materials and methods

6.2.1DNA extraction and PCR conditions

The DNA samples previously extracted and stored at -20 °C were used. The amplification of the products used the methods as previously reported by Chen *et al*. (2010) and Cai *et al*. (2011). The 25µL final reaction mixture consisted of 12.5 µL, 2x Thermo Scientific PCR Master Mix (SA), 1.25µL (0.5µM) each of forward and reverse primers, 7.75µL nuclease-free water (Thermo Scientific, SA) and 2.5µL (0.1µg) of the DNA. The reaction tubes were placed in a Thermal cycler (Thermo Electron Corporation, MA distributed by the Scientific Group, SA). The cycling conditions for *CTX-M*, *SHV*, and *TEM* were as follows: initial denaturation at 95 ^{o}C for 5 min and 30 cycles each of denaturation at 95 ^{o}C for 30 sec, annealing at 56 ^{o}C for 40 sec and extension at 72 ^{o}C for 50 sec followed by a cycle of final extension at 72 ^{o}C for 10 min. While for *Tet O*, the PCR conditions were, initial denaturation at 95 ^{o}C for 5 min and 30 cycles each of denaturation at 94 ^{o}C for 1 min, annealing at 55 ^{o}C for 1min and extension at 72 ^{o}C for 2 min followed by 1 cycle of final extension at 72 ^{o}C for 10 min. Table 6.1 summarizes the primers used to amplify the targeted genes. Negative controls were added to each PCR run

including all reagents except template DNA which was substituted by nuclease water (Thermo Scientific, SA). Amplified DNA products (5µL) were electrophoresed on 1.5% agarose gel in 0.5x TAE buffer. The amplified bands were visualised under ultra-violet light.

Table 6.1: Primers used for the amplification of the target genes

Target gene	Primer sequence	Amplicon size (bp)	Reference
TEM	F- $^{5'}$GTC GCC GCA TAC ACT ATT CTC A$^{3'}$	258	Cai *et al*., 2011
	R- $^{5'}$CGC TCG TCG TTT GGT ATG G$^{3'}$		
SHV	F- $^{5'}$GCC TTG ACC GCT GGG AAA C$^{3'}$	319	Cai *et al*., 2011
	R- $^{5'}$GGC GTA TCC CGC AGA TAA AT$^{3'}$		
CTX-M	F- $^{5'}$CGG GAG GCA GAC TGG GTG T$^{3'}$	381	Cai *et al*., 2011
	R- $^{5'}$ TCG GCT CGG TAC GGT CGA$^{3'}$		
TetO	F- $^{5'}$AAT GAA GAT TCC GAC AAT TT $^{3'}$	781	Chen *et al*., 2010
	R- $^{5'}$CTC ATG CGT TGT AGT ATT CCA$^{3'}$		

6.2.2 DNA sequencing

DNA sequencing was performed as previously outlined in chapter four (page 130). The sequences of this study have been deposited in the GenBank under the accession numbers: *Enterobacter cloacae* TEM - KF 768574 - KF 768594; *Listeria* species TEM - KF 768599 - KF 768633.

6.3 Results and discussion

6.3.1 PCR amplification of bla-$_{TEM}$, bla-$_{SHV}$, bla-$_{CTX-M}$ and Tet O genes.

There was successful amplification of the expected 258 bp fragments (*bla*-$_{TEM}$) in 48 (96%) of *L. ivanovii* and 30 (100%) of *E. cloacae* strains which presented with penicillin resistance (Fig 6.1), while *bla*-$_{SHV}$, *bla*-$_{CTX-M}$ and *Tet O* genes were absent in all the test strains.

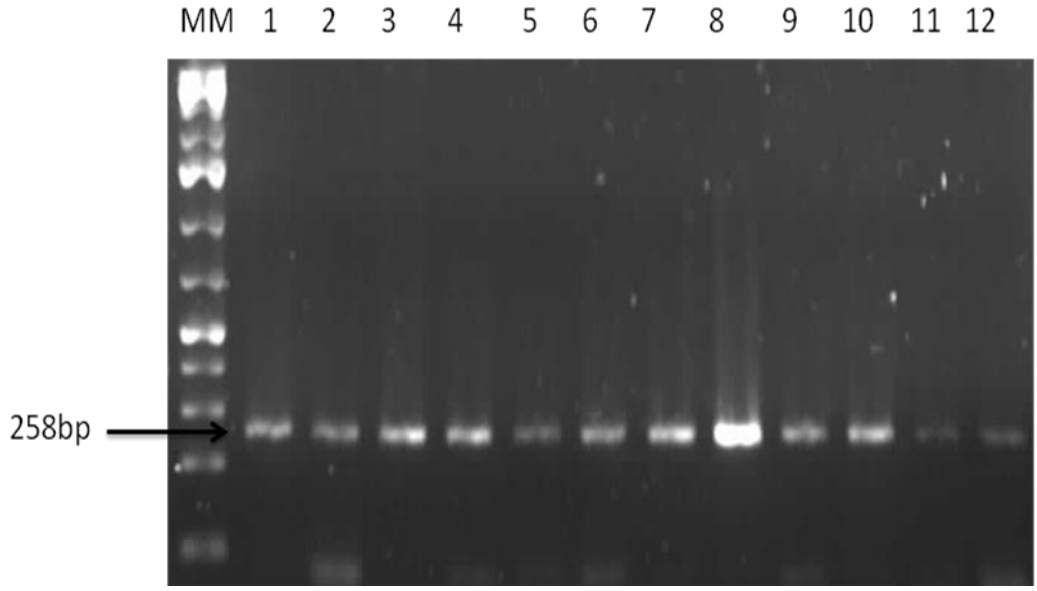

Figure 6.1: Detection of amplified *bla*$_{TEM}$ PCR product. MM, 100 bp molecular marker; lane 1-12, TEM positive samples.

Approximately two-thirds of antibiotics administered to humans are β-lactams; however, more and more frequent cases of clinical inefficiency of drug therapy by this group of antibiotics appear due to development of drug resistance among the microorganisms (Lachmayr *et al*., 2008). While *TEM*, *SHV* and *CTX-M* are the most occurring β-lactamase resistance genes; their prevalence and distribution varies significantly with geographical regions. In this study, *bla*-$_{TEM}$ was the only β-lactamase gene identified. Similarly, In Thailand, Nakayama *et al*. (2012) reported the *bla*-$_{TEM}$ gene in 90.6% (87/96) of penicillin resistant *Neisseria gonorrhoeae* isolates. Chaudhary and Payasi (2012) in India encountered a similar high prevalence range of *bla*-$_{TEM}$ (82-87%) in ESBL *Acinetobacter baumannii* clinical isolates from Middle East, African and Indian patients; whilst an environmental study from China observed predominance of the *bla*-$_{CTX-M}$ gene (80%) followed by the *bla*-$_{TEM}$ gene (39%) (Lu *et al*., 2010). Likewise, Europe has experienced a shift in the distribution of different ESBLs, with a dramatic increase of *CTX-M* enzymes over *TEM* and *SHV* variants. For example, in Sweden during the period from 2001 to 2006, 92% of consecutive non-duplicate ESBL-positive *E. coli* isolates expressed a *CTX-M*-type enzyme, with *CTX-M-1* being the predominant group (Fang *et al*., 2008). Similar results were found in multicenter studies performed between 2002 and 2004 in Finland (Nyberg *et al*., 2007).

While the production of β-lactamases is mostly common among Gram-negative bacteria, these enzymes have been identified in virtually all bacterial species, with notable exceptions being most *Enterococci* and Salmonellae (Hsieh, 2000; Parry, 2003; Lachmayr *et al*., 2008). This is in agreement to the findings of this study which detected *bla*-$_{TEM}$ in *L. ivanovii* strains (Gram-positive bacteria) (Fig 6.2). This could be in part that β-lactamase genes are typically plasmid-mediated, rather than chromosomal-mediated β-lactamases; this is of great concern given their easy spread via horizontal gene transfer (Rubtsov *et al*., 2010). The mobility of these genes is related to their association with transposons which have been implicated in the spread of *TEM* β-

lactamases to plasmids in *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Elwell *et al*., 1975; 1977).

Combination therapy of β-lactams (ampicillin, penicillin) with an aminoglycoside (erythromycin, gentamicin) or sulfonamide (sulfamethoxazole/trimethoprim) is a drug of choice for invasive listeriosis whereas a broad-spectrum β-lactam such as cefotaxime or ceftriaxone is commonly used to treat Gram-negative bacterial infections. The detection of *TEM* β-lactamase in *E. cloacae* and *Listeria* strains is therefore of human health concern if these organisms are transmitted via the food chain and causes infections; as this will mitigate the management of these severe infections.

Worthy of note is the fact that *E. cloacae* also forms part of the commensal gut flora in most animals including humans; therefore, the presence of *TEM* β-lactamase resistant genes in these commensals might create a pool of resistant genes which can be passed on to other strains (including pathogenic bacteria) in the gut via lateral gene transfer. β-lactamase resistant genes can also be found as components of multi-resistance transposons and on multiple plasmids within a given organism (Paterson *et al*., 2004); this explains why these strains were not only resistant to β-lactam antibiotics but also to other drugs like: vancomycin, erythromycin, tetracycline, neomycin, sulfamethoxazole/trimethoprim in a related study in our laboratory using these strains (Nyenje *et al*., 2012). Published studies attest to the fact that β-lactamase genes co-select with other resistances, especially to fluoroquinolones, aminoglycosides and sulfonamides and are co-transferred by conjugation. For instance, Zhang *et al*. (2008) reported the co-transfer of the *armA*, $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes in ESBL-producing *Klebsiella oxytoca*. Ma and colleagues (2009) complemented to these findings when they isolated ESBL *Klebsiella pneumoniae* bearing *CTX-M*, *SHV*, *armA* and *rmtB* genes.

6.3.2 Sequence analysis of the bla$_{TEM}$ gene

NCBI nucleotide BLAST search using the partial *bla*$_{\text{TEM}}$ gene sequences demonstrated the occurrence of *bla*-$_{\text{TEM-1}}$ phenotype 2b in all the strains sequenced. To determine their phylogenetic affiliation with those in the GenBank, a Neighbour-Joining tree was estimated and it was noted that the strains of this study clustered with *bla*-$_{TEM}$ of other members of the bacteria family (*e.g*. *E.coli* and *K. Pneumonia*) in a single cluster. Long lines were observed with mutant strains depicting an evolutionary distance (Fig. 6.2). Although β-lactamase resistance is mostly associated with Gram-negative bacteria, the current study noted the presence of *bla*-$_{TEM}$ in *Listeria* species that were related to *bla*-$_{TEM}$ of members of *E. coli*, *Klebsiella* spp., and *E. cloacae* (Fig. 6.2). The findings of this study attest to the fact that the co-existence of antimicrobial resistance genes with mobile elements such as plasmids facilitates the swift spread of antibiotic resistance genes among bacteria since β-lactamase resistance genes are often plasmid mediated (Dale and Park, 2004).

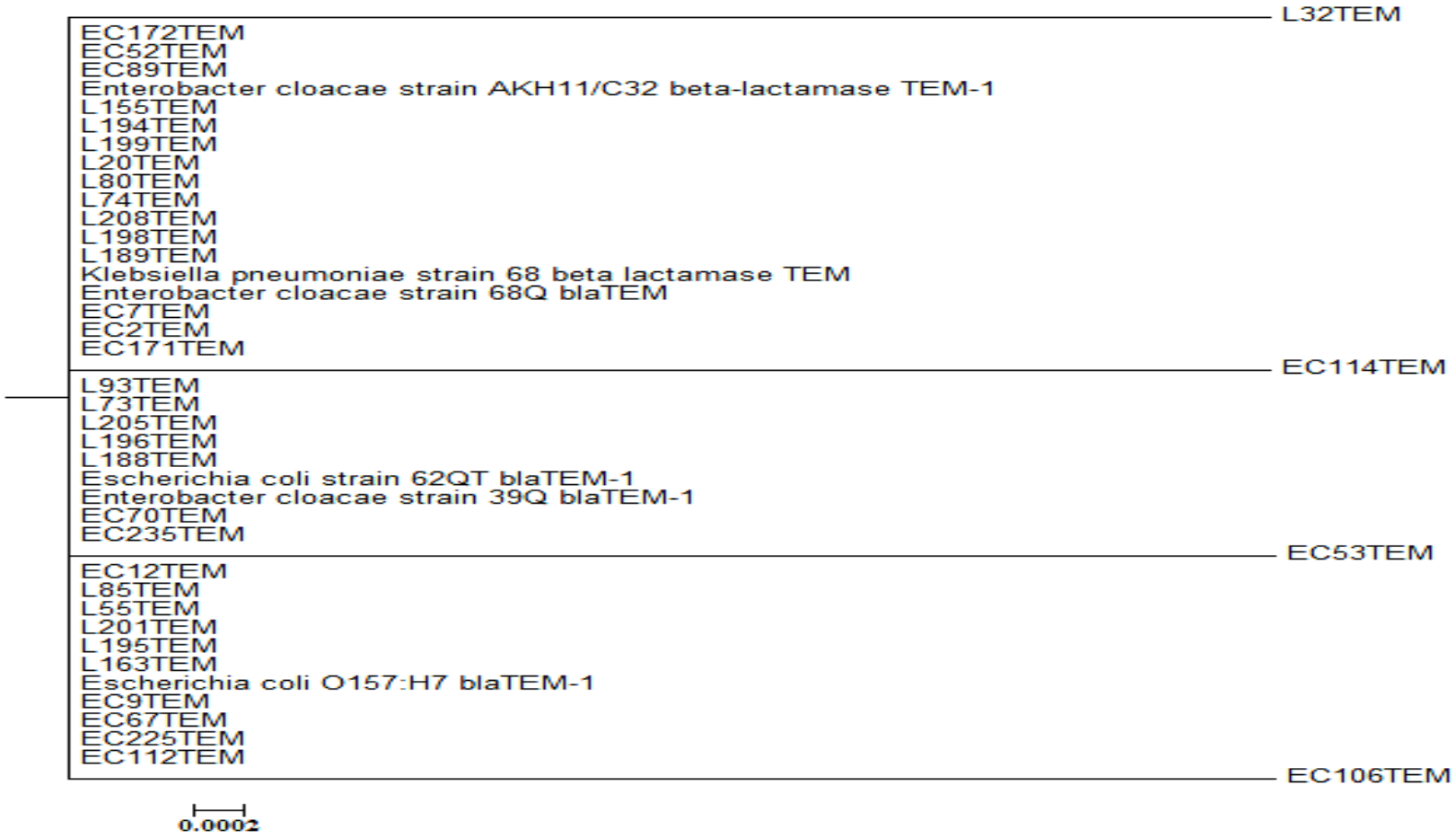

Figure 6.2: TEM Neighbour-joining distance tree constructed in MEGA5 using the ClustalW aligned, partial *bla-$_{TEM}$* gene sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method.

6.3.3 Characterization of bla_{TEM} genes for possible mutations.

Four isolates (EC53, EC106, EC114 and L32) had mutation of either substitution or insertion of a nucleotide. Of particular interest were strains EC53 and EC106 which had an amino acid substitution, Ile_{173}→Leu and Thr_{105}→Tyr respectively, whilst EC 114 and L32 strains had silent type of mutations where the nucleotide substitution/or insertion did not affect the amino acid translation code (Table 6.2). The numbering of the mutation positions is according to the Amber scheme of numbering (Amber *et al*., 1991).

Table 6.2: Detection of mutations in the *bla*-TEM gene in *E. cloacae and L. ivanovii* strains.

Strain number	Change in nucleotide sequence	Change in amino acid sequence
EC53	Substitution of "C" (CTA) for "A" (ATA) at position 102	Ile_{173}→Leu
EC106	Insertion of "G" (GTC) at Position 4	Thr_{105}→Tyr
EC114	Substitution of "G" (CCG) for "A" (CCA) at position 6	Silent mutation (Pro_{107}→Pro)
L32	Substitution of "A" (ACT) for "G" (GCT) at position 183	Silent mutation (Leu_{169}→Leu)

Studies have demonstrated that replacement/deletion of just a few amino acid in a protein sequence alter the enzyme structure resulting in significant broadening of the antibiotic spectrum subjected to hydrolysis (Woodford and Ellington, 2007). Similarly in this study, mutation in the amino acid sequence was observed in two strains (EC53 and EC106); suggestive of a possible change in total charge of the protein that may lead to alteration of the β-lactamase enzyme structure and function.

Information available in the beta-lactamase database shows some variations in the amino acid substitution at a single position; for example, isoleucine residue at position 173 has been reported to be substituted for valine (Zarnayová *et al*., 2005), while other studies have documented lysine or threonine substitution at the same position (http://www.lahey.org/studies). However, the present study reported leucine substitution (Fig 6.3) which has not been described in the β-lactamase database that was last updated on the 5th of November, 2013 (http://www.lahey.org/studies).

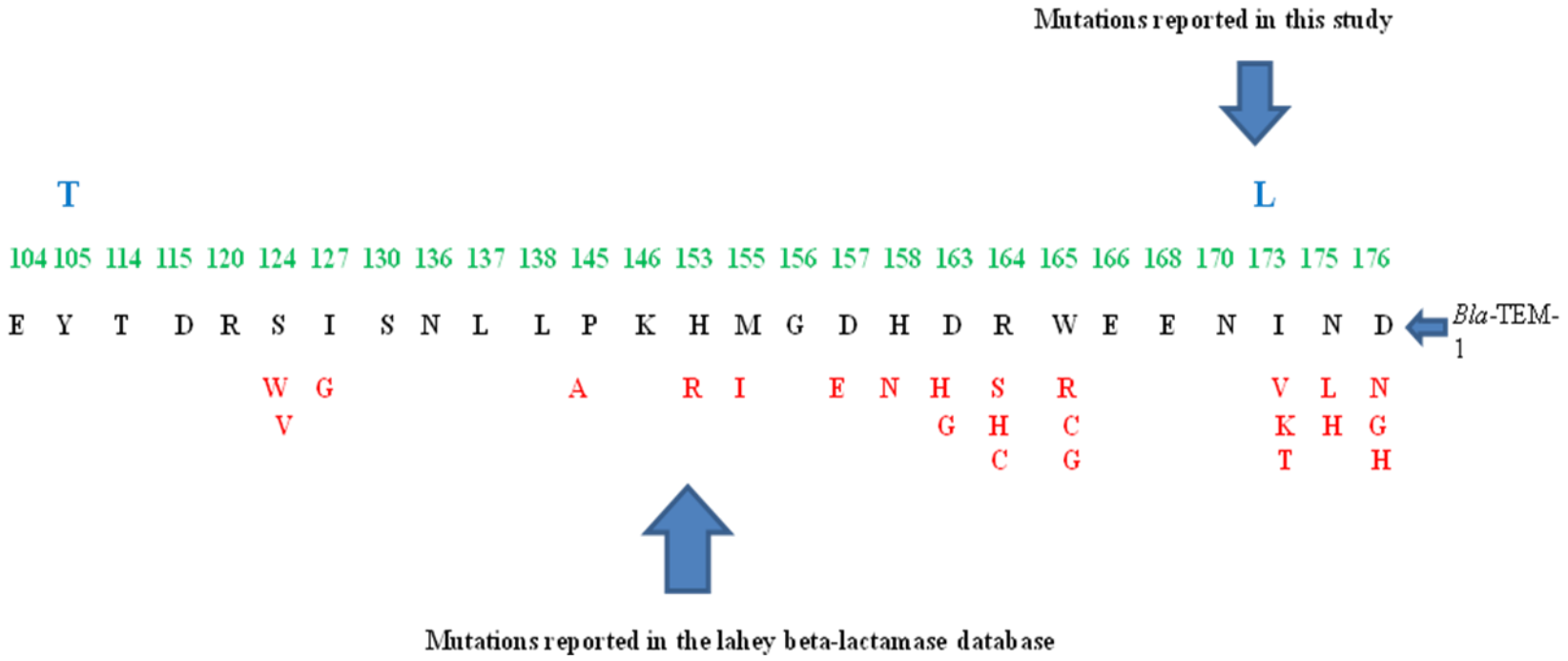

Figure 6.3: Positions and types of amino acid substitutions in TEM type beta-lactamases

Upper row in blue, mutation positions reported in this study corresponding to the *bla*-$_{TEM}$ gene; second row figures in green, amino acid numbering positions according to Amber scheme of numbering; third row in black, position of amino acid in TEM type β-lactamases; the last rows in red, positions of amino acid mutations described in the database.

The evolution and spread of β-lactamase mutation differ geographically; keeping pace with the introduction of novel antibacterials and prescription practices, where mutations might occur during the course of treatment (Jones *et al*., 2009). In addition, some microorganisms are able to produce chromosome-encoded β-lactamases, for example, *Klebsiella pneumoniae* produces β-lactamase *SHV-1* and *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Serrata* spp., and *Pseudomonas aeruginosa* produce C class β-lactamases. All these factors contribute to differences observed in the β-lactamase mutation (Jones *et al*., 2009).

6.3 Conclusion

The prevalence and distribution of β-lactamase gene varies significantly with geographical regions. *Bla*-$_{\text{TEM-1}}$ 2b type was found to be prevalent in the current study area. In spite of β-lactamase gene mostly associated with Gram-negative bacilli, their plasmid-mediated nature facilitates their spread via horizontal gene tranfer, exemplifed by the detection of *bla*-$_{\text{TEM-1}}$ from L. *ivanovii* a Gram-positive bacteria. The detection of *bla*-$_{\text{TEM-1}}$ from *E. cloacae* and *L. ivanovii* isolates implies that these strains have the ability to produce β-lactamase an enzyme that cleaves β-lactam antibiotics. The attendant implications and consequences could bear on human health if these organisms are transmitted via the food chain; causing infections which could be difficult to treat using this group of drugs.

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CHAPTER SEVEN

Biofilm formation and adherence characteristics of *Listeria ivanovii* strains isolated from ready-to-eat foods in Alice, South Africa.

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Biofilm formation and adherence characteristics of *Listeria ivanovii* strains isolated from ready-to-eat foods in Alice, South Africa.

Abstract

The present study was carried out to investigate the potential of *L. ivanovii* isolates to exist as biofilm structures. The ability of *L. ivanovii* isolates to adhere to a surface was determined using a microtiter plate adherence assay whereas the role of cell surface properties in biofilm formation was assessed using the coaggregation and autoaggregation assays. Seven reference bacterial strains were used for the coaggregation assay. The degree of coaggregation and autoaggregation was determined. The architecture of the biofilms was examined under scanning electron microscope. A total of 44 (88%) strains adhered to the wells of the microtiter plate while 6 (12%) did not adhere. The coaggregation index ranged from 12 to 77% while the autoaggregation index varied from 11% to 55%. The partner strains of *Staphylococcus aureus, Streptococcus pyogenes*, *Plesiomonas shigelloides,* and *Shigella sonnei* displayed coaggregation indices of 75% each, while *Samonella* Typhimurium, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa* registered coaggregation indices of 67%, 58%, and 50%, respectively. The ability of *L. ivanovii* isolates to form single and multispecies biofilms at 25 °C is of great concern to the food industry where these organisms may adhere to kitchen utensils and other environments leading to cross-contamination of food processed in these areas.

7.1 Introduction

In nature, bacterial cells are most frequently found in close association with surfaces and interfaces, in the form of multi-cellular aggregates embedded in an extracellular matrix generally referred to as biofilms (Donlan, 2009). Biofilms are usually heterogeneous, they contain more than one type of bacterial species, but they can be homogeneous in cases such as infections and medical implants (O'Toole *et al*., 2000). Microbial biofilms pose a challenge in clinical and industrial setting especially in food processing environments where they act as a potential source of microbial contamination of foods that may lead to spoilage and transmission of foodborne pathogens (Milanov *et al*., 2009; van Houdt and Michiels, 2010; Adetunji and Isola, 2011).

Environmental conditions in food production areas including the presence of moisture, nutrients and inocula of microorganisms from the raw materials might favour the formation of biofilm. Furthermore, when food processing equipment are not easily cleaned due to its design and food particles not completely removed, the particles aid in the formation of biofilms by providing a coat that not only provides the biofilm with nutrients but also a surface to which it can easily stick on (Kamila and Katarzyna, 2011). Once biofilms have formed on food processing surfaces, they are hard to eliminate often resulting in persistence and endemic population (Vestby *et al*., 2009).

Biofilms offer their member cells several benefits, including channelling nutrients to the cells and protecting them against harsh environments. In particular, it has been noted that cells within biofilms are more resistance to antibiotics, disinfectants, as well as to host immune system clearance than their planktonic counterparts (Morakawa, 2006; van Houdt and Michiels, 2010). Several mechanisms account for this increased antibiotic resistance, including the physical barrier formed by exopolymeric substances, a proportion of dormant

bacteria that are inert toward antibiotics, and resistance genes that are uniquely expressed in biofilms (Kavanaugh and Ribbeck, 2012). Outbreaks of pathogens associated with biofilms have been related to the presence of *Yersinia enterocolitica*, *C. jejuni*, *E. coli* O157:H7, and the species of *Listeria, Salmonella* and *Staphylococcus*. These bacteria are of special significance in ready-to-eat and minimally processed food products, where microbiological control is not conducted in the terminal processing step (Kamila and Katarzyna, 2011).

L. monocytogenes and *L. ivanovii* are potential pathogens of listeriosis, a rare but serious disease with a high mortality rate in susceptible individuals (Leclercq *et al*., 2010; Graves *et al*., 2010; Guillet *et al*., 2010). *Listeria* strains have been reported to survive for months to years in food-processing environments and thus, colonize various food products leading to food contamination (De Nes *et al*., 2010). *L. monocytogenes* biofilms in food processing plants have been widely studied (Schuppler and Loessner, 2010). However, there is a dearth of information on the ability of *L. ivanovii* to form biofilm; this might be due to the fact that it rarely causes human illnesses due to its low prevalence in the environment. Nonetheless, recent studies in the study area have reported high prevalence of the organism in wastewater effluents and various ready-to-eat foods (Odjadjare *et al*., 2010; Nyenje *et al*., 2012), suggestive that the organism might be endemic in the area. Therefore the present study was carried out to investigate the ability of *L. ivanovii* isolates to exist as biofilm structures, in an effort to establish the factors for this endemicity.

7.2 Materials and methods

7.2.1 Biofilm formation and quantification.

The biofilm forming ability test was done in accordance with the method of Stepanovi´c *et al*. (2000). *L. ivanovii* strains previously isolated from ready-to-eat foods were cultured on Nutrient agar (Oxoid, Basingstoke, England) and plates were incubated at 37 °C for 24 h. Few single colonies were suspended in sterile saline to a turbidity standard comparable to a 0.5 McFarland. The suspension was vortexed for 1 min from which 20 µL was pipetted into a 96-well U-bottomed microtiter plate (Greiner Bio-one GmbH, Frickenhausen, Germany) containing 180 µL of brain heart infusion (BHI) broth (Oxoid, Basingstoke, England). The plates were incubated aerobically for 24 h at room temperature (25°C ± 2°C).

After incubation, the contents of the wells were decanted into a waste container and each well was washed three times with 200 µL of sterile normal saline. Following every washing step, the wells were emptied by carefully aspirating the content into a waste container and the plates were left to dry overnight in an inverted position before they were fixed with hot air at 65°C for 1 h. Plates were stained with 150 µL of 1% crystal violet for 30 min; the excess stain was aspirated and plates rinsed off by placing them under running tap water until the washings were free of the stains. The plates were left to dry at room temperature in an inverted position overnight before resolubilizing the dye bound to adherent cells with 150 µL of 33% (v/v) glacial acetic acid; the optical density (OD) of each well was measured at 595 nm using a microtiter plate reader (SynergyMx, Biotek^{TM} Winooski, VT, USA). Reference strains of *P. aeruginosa* ATCC 15442 and *S. aureus* NCTC 6571 were used as positive controls while negative control well contained broth only. Tests were performed in triplicates on three occasions, the results averaged and biofilms quantified as non-adherent, weakly adherent, moderately adherent or strongly adherent.

7.2.2 Autoaggregation and coaggregation assays

Twelve (three each non adherent, weakly adherent, moderately adherent and strongly adherent) *L. ivanovii* isolates and seven reference strains (*S. aureus* NCTC 6571, *S. pyogenes* A ATCC 49399, *S.* Typhimurium ATCC 13311, *P. aeruginosa* ATCC 15442, *P. shigelliodes* ATCC 51903, *A. hydrophila* ATCC 35654, *S. sonnei* ATCC 29930) were used for these assays. The bacteria strains were grown separately in 20 mL of BHI broth at 37 °C for 48 h. Cells were harvested by high-speed centrifugation (11,000 x g for 10 min), washed twice in 3mM NaCl containing 0.5 mM $CaCl_2$. Subsequently, the cells were re-suspended in the same solution (3mM NaCl containing 0.5 mM $CaCl_2$), centrifuged at 650 x g for 2 min, the supernatant was carefully aspirated and discarded into a waste container. The OD of the cell suspension was measured and adjusted to 0.3 using an automated spectrophotometer (Optima Scientific V-1200) at a wavelength of 660nm; the cell suspension was used for coaggregation assay. Equal volumes (1mL each) of the coaggregating partners were mixed and the OD (OD_{Tot}) of the mixture was immediately read at 660 nm before incubation at room temperature for 1 h. Subsequently, the tubes were centrifuged at 2,000 rpm for 2 min and the OD of the supernatant (OD_s) measured at the same wavelength (660nm) (Basson *et al*., 2007).

The degree of coaggregation of the paired isolates was determined using the equation

$$\% \text{ coaggregation} = (OD_{Tot} - OD_s) \times 100 / OD_{Tot}$$

For autoaggregation assay, the individual bacterial suspension adjusted to an OD of 0.3 was incubated at room temperature for 1 h and the cell suspension centrifuged at 2000 rpm for 2 min. The supernatant (2 mL) was transferred into a cuvette and the OD measured at 660nm.

The degree of autoaggregation was calculated as follows:

$$\% \text{ autoaggregation} = (OD_0 - OD_{60}) \times 100 / OD_0$$

OD_0 refers to the initial OD of the organism, and OD_{60} is the OD of the supernatant after 60 min of incubation.

7.2.3 Characterization of biofilm formation using scanning electron microscope.

The biofilms were further examined using scanning electron microscope (SEM) according to the method previously described by Greetje *et al*. (2012) with some modifications. A representative of the biofilm forming strain-population was studied. Briefly, a microscope cover slip (22 x 22 mm) on a glass slide was placed in a petri-dish half-filled with BHI broth. Subsequently, a few colonies of *L. ivanovii* were transferred into the BHI broth and incubated at 25°C for 72 h. For the coaggregation assay, the partner isolate was added after 1 h of incubation. The cover slips were washed three times with normal saline before fixing with 2.5% (w/v) glutaraldehyde solution for 1 h. Subsequently, the samples were dehydrated in a series of 20, 40, 60, 80 and 99.5% ethanol solution for 30 min in each concentration. Finally the sample were post fixed in 1% Osmium tetroxide (OsO4), critical point-dried using CO_2 and sputter-coated with Gold palladium using Elko 1B.3 ion coater before viewing with the SEM (Japan Electron Optical Laboratories JSM-6390LV, Tokyo, Japan).

7.3 Data analysis

Tests were done in triplicate on three separate occasions and the results averaged. The cutoff OD (ODc) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control. Isolates were classified as follows: $OD \leq OD_C$ = non-adherent, $OD_C < OD \leq (2 \times OD_C)$ = weakly adherent; $(2 \times OD_C) < OD \leq (4 \times OD_C)$ = moderately adherent and $(4 \times OD_C) < OD$ = strongly adherent (Basson *et al*., 2007).

7.4 Results and discussion.

7.4.1 Microtiter adherence assay

The biofilm formation ability of 50 *L. ivanovii* strains is summarized in Table 7.1; variations in biofilm formation were observed. A total of 44 (88%) strains adhered to the wells of a microtiter plate while 6 (12%) did not adhere. The majority of the isolates demonstrated weak (44%) and moderate (34%) adherence while only 5 (10%) strains strongly adhered to the wells. The optical density range of non adherent was 0.332 - 0.503 and strong adherent 2.32 - 3.846.

Table 7.1: Biofilm formation by *L. ivanovii* isolates (n=50) following incubation at 25 °C.

Biofilm formation	Number (%)	OD Range	Mean OD ± SD
Non adherent	6 (12)	0.332 - 0.503	0.431 ± 0.055
Weak adherent	22 (44)	0.545 – 1.083	0.785 ± 0.175
Moderate adherent	17 (34)	1.105 – 2.084	1.432 ± 0.354
Strong adherent	5 (10)	2.32 – 3.846	3.045 ± 0.887
Total biofilm	44 (88)	0.545 – 3.846	1.754 ± 0.763

OD, optical density; SD, standard deviation. The results are the mean of three independent experiments carried out in triplicates.

The potential of bacteria to form biofilms is affected by a number of factors including strain characteristics, physical and chemical properties of the solid phase, temperature, composition of growth medium, and the presence of other microorganisms (Stepanovi´c *et al*., 2007). Previous works have observed low biofilm quantities with tryptic soy broth (Rickard *et al*., 2000; Kennedy and O'Gara, 2004); therefore this study used BHI broth which has been shown to strongly influence biofilm development in many organisms such as *Staphylococcus* and *Listeria* species (Kennedy and O'Gara, 2004; van der Veen and Abee, 2011). The present study observed that 88% of the strains were able to form biofilm at 25 °C and four biofilm phenotypes were demonstrated. This is of great concern to the food industry especially in the tropics whose room temperature usually falls between 22 °C and 28 °C; implying that with favourable conditions, these organisms at room temperature may grow and adhere to kitchen utensils or the environment if not properly cleaned, hence creating a source for cross-contamination. The attached cells in part also form a substrate for other microorganisms less prone to biofilm formation; this will lead to an increased survival rate of pathogen and further spreading during food processing. The findings are in agreement with those of Di Bonaventura *et al*. (2008) who reported biofilm formation of *L. monocytogenes* at low temperatures (4 °C, 12 °C, and 22 °C) on a glass. However, hydrophobicity was found to be higher at 37 °C than at 4 °C, 12 °C, and 22 °C.

7.4.2 Coaggregation and autoaggregation

Coaggregation occurred to varying degrees between all the seven partner strains and *L. ivanovii* isolates. The coaggregation index ranged from 12% - 77% while autoaggregation ranged from 11% - 55%. Some strains which strongly adhere to the wells were equally able to stick to each other (autoaggregation of 32% - 55%) and followed by moderate and weak adherent 35% - 41% and 30% - 46% respectively, while non adherent cells registered the least autoaggregation 11% - 20%. On the other hand, moderate adherent strains had slightly

high coaggregation index range of 44% - 77% followed by strong adherent 41% - 77% and the least was non adherent with 12% - 40% (Table 7.2). It was also observed that 95% of the moderately adherent strains had coaggregation index of > 50% while weak and strong adherent had 90% each (Table 7.2). The partner strains *S. aureus*, *S. pyogenes*, *P. shigelloides*, and *S. sonnei* displayed coagreggation indices of 75% while *S.* Typhimurium, *A. hydrophila* and *P. aeruginosa* registered coaggregation indices of 67%, 58% and 50% respectively. Isolate Liv 38 had the highest coaggregation range of 65% - 77% and the least was Liv 194, 12% - 28 % (Table 7.3).

Table 7.2: *L. ivanovii* isolates with coaggregation indices >50% among the four biofilm phenotype

Biofilm phenotype	%Autoaggregation range	%Coaggregation range	Coaggregation indices >50%
Non adherent	11 - 20	12 - 40	0
Weak adherent	30 - 46	37 – 75	90
Moderate adherent	35- 41	44 -77	95
Strong adherent	32 - 55	41 – 77	90

Table 7.3: Coaggregation indices of selected biofilm forming *L. ivanovii* isolates with seven different reference strains partners

Isolate (biofilm phenotype)	%Autoagg.	Coaggregation indices (%)							
		Partner strains							
		Range %	*S. aureus* NCTC 6571	*S. pyogenes* A ATCC 49399	*S. Typhimurium* ATCC 13311	*P. aeruginosa* ATCC 15442	*P. shigelloides* ATCC 51903	*A. hydrophila* ATCC 35654	*S. sonnei* ATCC 29930
Liv 188 (NA)	19	18 - 37	31	28	18	37	24	27	19
Liv 119 (NA)	20	13 - 40	25	22	13	40	16	25	28
Liv 194 (NA)	11	12 - 28	19	12	17	28	13	20	16
Liv 37 (WA)	42	53 - 74	67	68	74	67	53	67	70
Liv 18 (WA)	30	37 - 70	54	51	45	37	70	40	66
Liv 01 (WA)	46	67 - 75	68	70	72	71	72	67	75
Liv 03 (MA)	41	61 - 71	74	67	71	61	64	68	71
Liv 155 (MA)	35	57 - 70	57	62	60	58	58	66	70
Liv 99 (MA)	36	44 - 77	51	55	52	53	77	44	60
Liv 16 (SA)	47	41 - 67	67	66	61	41	53	62	56
Liv 38 (SA)	32	65 - 77	72	73	77	69	71	65	72
Liv 41 (SA)	55	49 - 69	61	59	67	49	66	57	69
C. indices >50%		50 - 75	75	75	67	50	75	58	75

NA, non adherent; WA, weak adherent; MA, moderate adherent; SA, strong adherent; Autoagg, autoaggregation; C. indices, coaggregation indices >50%.

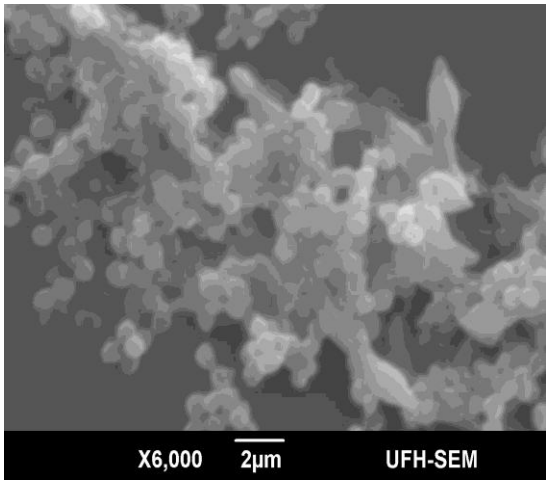
Autoaggregation and coaggregation are of great importance in biofilm formation; they integrate biological structures, by mediating the juxtapositioning of species next to favourable partner species within taxonomically diverse biofilms. Autoaggregation is a process whereby a strain within the biofilm will utter polymers to boost the integration of genetically identical strains; these interactions are enhanced by increased hydrophobicity (Basson *et al*., 2007). In the present study, isolates displayed variations in their autoaggregating abilities suggesting differences in strains and serotypes.

Rickard *et al*. (2003) defined coaggregation as a process by which genetically different bacteria become attached to one another via specific molecules. It was observed that *S. aureus, S. pyogenes* A, *P. shigelloides,* and *S. sonnei* were the strong partners while *P. aeruginosa*, a strong biofilm producer, recorded the least potential to coaggregate. The findings are in agreement with those of Jacobs and Chenia (2011).

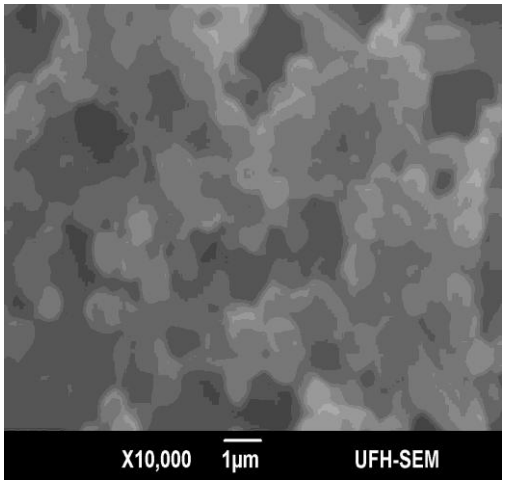
However, coaggregation results were contrary to SEM images where strong biofilms were observed in single species than in multispecies biofilms. Worthy of note is the fact that in autoaggregation assays, the individual isolates were grown separately, mixed, and incubated for only 60 min before the OD was read; while with the SEM the partner isolate was added after 2 h of initial growth and the mixture was incubated for 72 h. Previous studies have demonstrated the ability of *Listeria* species to grow on surfaces with other microorganisms, both Gram-positive and Gram-negative species, in a mixed species biofilm in food processing environments (Habimana *et al*., 2010; van der Veen and Abee, 2011). However, van der Veen and Abee (2011) using plate counts and fluorescence microscopy showed that the cell count of *L. monocytogenes* was more than the partner strain, *Lactobacillus plantarum* cells. These findings are in agreement with our findings where few cells of the partner organism were apparent in a strong biofilm structures under SEM.

Studies on *Flavobacterium* species observed that isolates which were unable to autoaggregate or showed low aggregation indices displayed varying levels of coaggregation with diverse aquatic bacteria (Basson *et al*., 2007). In this study, the non adherent strain (Liv188) displayed both autoaggregation and coaggregation characteristics entailing that some of the strains though cannot attach to a solid surface as primary colonizers may interact with already formed organisms later as biofilm partners. Microorganisms can adhere to a surface where it acts as primary colonizers or as later biofilm partners by establishing interactions with other microorganisms (Kolenbrander, 2000). Cell surface components (flagella, pili, adhesin proteins, capsules, and surface charge) are the major contributors to attachment and coaggregation in biofilms (Jacobs and Chenia, 2011).

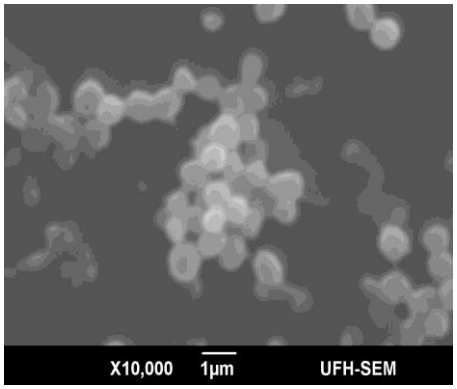
In order to evaluate the architecture of the biofilms, SEM was used. Figure 7.1 shows the scanning electron micrographs of autoaggregates and coaggregates biofilms of *L. ivanovii* and their coaggregates partner *S. aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442. The different biofilm phenotypes were clearly distinguishable; the strong and moderate adherent strains were seen as densely packed colonies while the weak adherent strains, few cells were stuck together and the cell morphology was clear (short thick rods). However, contrary to the microtiter results, it was observed that *L. ivanovii* isolates preferred to grow in single species than multi-specie biofilm.



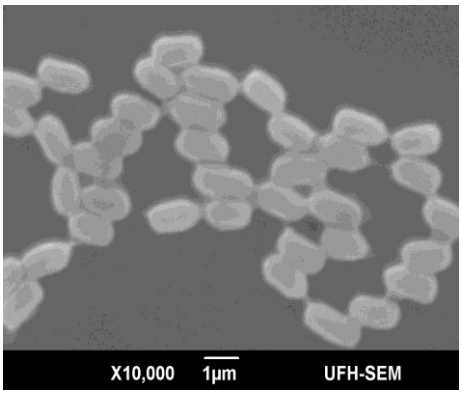
(a)



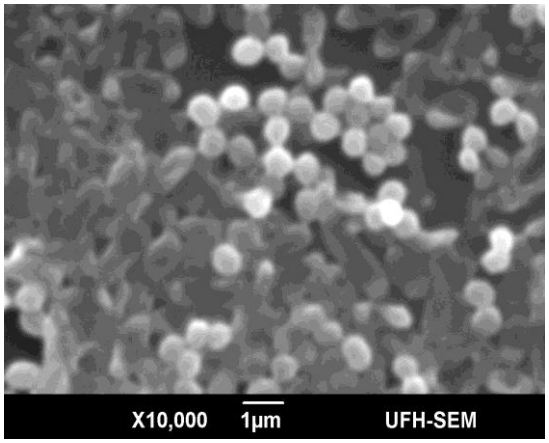
(b)



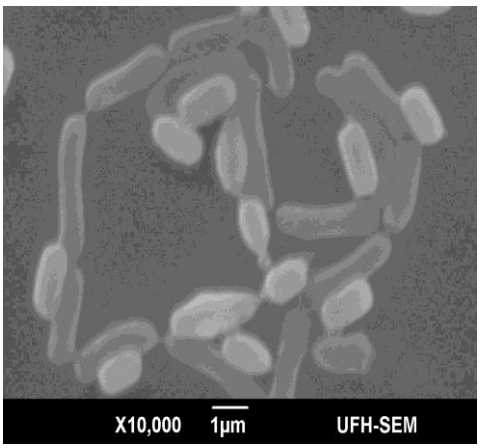
(c)



(d)



(e)



(f)

Figure 7.1: Scanning electron micrographs of autoaggregates and coaggregates biofilms of *L. ivanovii* and their coaggregates partner *S. aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442. (a) and (b) strong adherent autoaggregate; (c) moderate adherent autoaggreagate; (d) weak adherent autoaggreagate; (e) coaggregates of *L. ivanovii* and *S. aureus*; (f) coaggregates of weak adherent *L. ivanovii* and *P. aeruginosa*.

As crystal violet basically stains the number of cells that have attached to the wells of the microtiter, SEM analysis was employed to evaluate the architecture of the biofilms. Unlike weak biofilms where the morphology of single colonies were distinguishable, moderate and strong biofilms showed the presence of densely packed colonies of pleomorphic organisms (very short rods and coccobacilli); this could be in part that the cells were smaller due to competition hence they had to adjust for survival. This could explain the high level of resistance observed in biofilms as nutrient and oxygen depletion within the biofilm cause some bacteria to enter a stationary state, in which they are less susceptible to growth dependent antimicrobial killing. Also some bacteria might differentiate into a phenotypically resistant state and express biofilm-specific antimicrobial resistance genes that are not required for biofilm formation but contributes to the survival of organisms in the biofilm.

7.5 Conclusion

The study demonstrated the ability of *L. ivanovii* isolates to form single and multispecies biofilms at 25°C with strong biofilms from single species. This is of great concern to the food industry where these organisms may adhere to kitchen utensils and the environment leading to cross-contamination. Some strains could not adhere to a surface but could autoaggregate and coaggregate implying that preventing primary adhesion would prevent biofilm formation in these strains. Future studies are required to determine the antimicrobial susceptibility of the strains that form biofilms as well as determine the virulence genes expression of adherence traits in these biofilms, to throw more light on their pathogenic potential in our environment.

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CHAPTER EIGHT

Evaluation of different growth media and temperature on the suitability of biofilm formation by *Enterobacter cloacae* strains isolated from food samples in South Africa.

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Evaluation of different growth media and temperature on the suitability of biofilm formation by E*nterobacter cloacae* strains isolated from food samples in South Africa.

Abstract

The study evaluated the effects of growth medium, temperature, and incubation time on biofilm formation by *E. cloacae* strains. The ability to adhere to a surface was demonstrated using a microtiter plate adherence assay; the coaggregation and autoaggregation assays were also evaluated. The architecture of the biofilms was examined under SEM. All the strains adhered to the wells of the microtiter plate when incubated for 48 h irrespective of the growth medium and incubation temperature. It was also noted that 90% and 73% of strains prepared from Nutrient broth and cultured in Brain Heart Infusion broth (BHI) and Tryptic Soy broth (TSB) respectively were able to form biofilm, in contrast to 73% and 60% strains from Nutrient agar and cultured in BHI and TSB respectively grown under similar conditions. However, no statistically significant difference was observed when the two methods were compared. The coaggregation index ranged from 12% - 74% with best coaggregate activity observed when partnered with *S. pyogenes* (54% - 74%). The study signifies the suitability of BHI and TSB medium for the cultivation of *E. cloacae* biofilm; however, temperature and incubation time significantly affect biofilm formation by these bacteria.

8.1 Introduction

Enterobacter cloacae a Gram-negative bacteria, belongs to the family Enterobacteriaceae. The bacterium comprises part of the normal flora of the gastrointestinal tract of 40% - 80% of the population and is widely distributed in the environment (Peterson *et al*., 2005). The organism has also been isolated from food processing plants, fresh vegetables, rice, meat and meat products (Shaker *et al*., 2007; Haryani *et al*., 2008; Falomir *et al*., 2010; Nyenje *et al*., 2012a). *E. cloacae* are capable of causing opportunistic infections including pneumonia, wound, skin and soft tissue, ophthalmic, bloodstream and urinary tract infections particularly catheter-related where it has been reported to form biofilm in dwelling catheters of hospital patients (Storti *et al*., 2005; Mokracka *et al*., 2011).

Biofilms are defined as matrix-enclosed bacterial populations, which adhere to each other and to surfaces; observations have conclusively shown that biofilm bacteria predominate numerically and metabolically, in virtually all ecosystems and are difficult to eliminate (Donlan, 2002). Bacterial biofilms are known to be linked to medical conditions such as cystic fibrosis, periodontitis, and nosocomial infections from the use of catheters and prosthetic heart valves (Delle-Bovi *et al*., 2011). However, biofilm formation in food processing plants has also been documented; both pathogenic and food spoilage microorganisms have been isolated from such bacterial communities (Schlegelová *et al*., 2010).

It is well known that bacteria, including foodborne pathogens grow predominantly as biofilms in most of their natural habitats, but also in food processing, catering and the domestic environment (Kusumaningrum *et al*., 2003). Biofilms may generate a persistent source of contamination, leading to serious hygienic problems and also economic losses due to food spoilage (Brooks and Flint, 2008). Poor sanitation of food-contact surfaces is believed

to be an essential contributing factor of foodborne disease and development of bacterial biofilms. The formed biofilms are a source of cross contamination as cells will continuously detach and contaminate food once it passes over contaminated surfaces or through aerosols originating from contaminated equipment. This may seriously affect the quality and safety of the processed food and pose a potential risk to the consumer (Chmielewski and Frank, 2003).

E. cloacae and other species of *Enterobacter* have been reported to cause foodborne illnesses through consumption of a variety of foods, including infant foods (Shaker *et al*., 2007). In our recent study, *E. cloacae* was reported to be the second most prevalent organism from ready-to-eat foods (Nyenje *et al*., 2012a); implying that the organism might be one of the emerging foodborne pathogens that can be endemic in food processing environments. Studies on the ability of *E. cloacae* to form biofilms have focused mostly on clinical isolates (Donlan, 2001; Revdiwala *et al*., 2012) where authors have hypothesised that their ability to persist in these environments as well as their virulence, is a result of their capacity to colonize medical devices (Revdiwala *et al*., 2012). However, in the food industry, studies have demonstrated the ability of *Cronobacter sakazakii*, formerly known as *Enterobacter sakazakii* to adhere to feeding bottles, feeding tubes and other food processing surfaces with sparse information on *E. cloacae.* Modern food processing supports and selects for biofilm forming bacteria on food-contact surfaces due to mass production, lengthy production cycles and vast surface areas for biofilm development (Lindsay and von Holy, 2006).

Biofilm formation depends on an interaction between three main components: the bacterial cells, the attachment surface and the surrounding medium (van Houdt and Michiels, 2010). However, environmental factors such as pH, temperature, osmolarity, O_2 levels, nutrient composition and the presence of other bacteria also play important roles (Stepanovic *et al*., 2003; Di Bonaventura *et al*., 2007). The integration of these influences ultimately determines the pattern and behaviour of a given bacterium with respect to biofilm development (Goller

and Romeo, 2008). Hence, biofilms are very diverse and unique, not just to the microorganism, but to the particular environment in which they are being formed. This makes *in-vitro* characterization of biofilms difficult and requires the establishment of laboratory conditions that mimic the natural setting being studied.

The ability of these organisms to form biofilms at ambient temperature (25 ^{o}C), which is mostly room temperature in tropical countries implies that, under favourable conditions, they may be able to stick to kitchen utensils and other environments creating a reservoir for cross-contamination and enhance antimicrobial resistance of organisms to disinfectants and other antimicrobial agents. Therefore the present study aimed to determine the effects of temperature, nutrient availability and incubation time on attachment and biofilm formation by *E. cloacae* strains at ambient (25 ^{o}C) and body (37 ^{o}C) temperature in BHI and TSB media; in an effort to ascertain the suitable biofilm growth conditions for this bacterium as a guide to its containment in the food industry. BHI was chosen as one of the nutrient-rich laboratory medium, and TSB a less-rich (compared to BHI) culture medium which are frequently used in biofilm investigation.

8.2 Materials and methods

8.2.1 Bacterial strains

Bacterial strains used in the study consists of *E. cloacae* isolates previously isolated from various food sources (Nyenje *et al*., 2012a) and reference strains*: S. aureus* NCTC 6571, *S. pyogenes* A ATCC 49399, *P.shigelloides* ATCC 51903, *P. aeruginosa* ATCC 15442, *S.* Typhimurium ATCC 13311, *A. hydrophila* ATCC 35654 and *S. sonnei* ATCC 29930. These strains have been reported to form biofilms (Qadri *et al*., 1998; Hammer *et al*., 2005; Bridier *et al.*, 2011; Diez-Garcia *et al*., 2012); and were also available in our laboratory at the time of the experiments.

8.2.2 Microtiter plate assay

E. cloacae isolates were screened for their adherence to polystyrene microtiter plate wells following 24 h and 48 h incubation at 25 °C or 37 °C, in both BHI and TSB. The method previously described by Nyenje *et al*. (2012b) was employed which made use of the agar and broth techniques; for the agar method, *E. cloacae* isolates were cultured on Nutrient agar (Oxoid, Basingstoke, England) and plates incubated at 37 °C for 24 h. Few single colonies were suspended in sterile saline to a turbidity standard comparable to a 0.5 McFarland. The suspension was vortexed for 1 min from which 20 µL was pipetted into a 96-well U-bottomed microtiter plate (Greiner Bio-one GmbH, Frickenhausen, Germany) containing 180 µL of BHI or TSB (Oxoid, Basingstoke, England). While the inoculum for broth technique, isolates were grown in Nutrient broth (Oxoid, Basingstoke, England), incubated for 24 h at 37 °C. Subsequently, the bacterial suspension was diluted 1:100 in BHI or TSB; an aliquot of 200 µL of diluted bacterial suspension was added to each well. The negative control wells contained 200 mL of broth only per well. The plates were incubated aerobically at different temperatures (25 °C and 37 °C) for 24 h and 48 h. At the end of incubation period, the wells were carefully washed, stained with 1% crystal violet and read using a micrototer plate reader as previously stated in the previous chapter (page 209). Reference strains of *P. aeruginosa* ATCC 15442 and *S. aureus* NCTC 6571 were used as positive controls, while negative control well contained broth only.

8.2.3 Autoaggregation and coaggregation assays

A representative of the different biofilm phenotypes were examined for their ability to coaggregate with the following reference strains: *S. aureus* NCTC 6571, *S. pyogenes* A ATCC 49399, *S.* Typhimurium ATTC 13311, *P. aeruginosa* ATCC 15442, *P. shigelloides* ATCC 51903, *A. hydrophila* ATCC 35654, *S. sonnei* ATCC 29930. SEM was used to visualize the architecture of the formed biofilms. The method previously describe by Nyenje *et al*. (2012b) was employed.

8.3 Statistical analysis

Statistical analysis was performed using SPSS version 19. One way ANOVA followed by Turkey's *post hoc* test was used to compare the agar and broth methods, BHI and TSB liquid media. The incubation temperatures and periods were also compared; the mean difference was considered significant at $p < 0.05$.

8.4 Results and discussion

8.4.1 Microtiter adherence ability

Tests were performed in triplicates on three occasions, the results averaged and standard deviations were calculated. The cut-off was defined as three standard deviations above the mean OD of the negative control (ODc). Isolates were classified as follows: $OD \leq OD_C$ = non-adherent, $OD_C < OD \leq (2 \times OD_C)$ = weakly adherent; $(2 \times OD_C) < OD \leq (4 \times OD_C)$ = moderately adherent and $(4 \times OD_C) < OD$ = strongly adherent (Basson *et al*., 2007).

E. cloacae isolates displayed four different biofilm phenotypes (non, weak, moderate and strong adherent) after incubation for 24 h in both BHI and TSB broth (Table 8.1). The OD of non adherent (NA), weak adherent (WA), moderate adherent (MA) and strong adherent (SA) obtained when the bacteria were grown in BHI ranged from 0.359 - 0.486, 0.552 - 1.159, 1.092 - 2.14 and 2.192 - 4.22 respectively. On the other hand, the OD obtained when the

bacteria were grown in TSB ranged from 0.352 - 0.618, 0.62 - 1.583, 1.25 - 2.414 and 2.542 - 3.938 for NA, WA, MA and SA respectively (Table 8.2). It was observed that all the strains (100%) adhered to the wells of the microtiter plate when incubated for 48 h irrespective of the growth medium (BHI or TSB) and incubation temperature (Table 8.1). On the other hand adherence ability was demonstrated by 73% (22) and 60% (18) of the strains obtained from inoculums prepared in Nutrient agar and grown in BHI and TSB respectively, compared to 90% (27) and 73% (22) prepared in Nutrient broth grown in BHI and TSB respectively, when the plates were incubated at 25 °C for 24 h (Table 8.1). All the strains (100%) from Nutrient agar cultures grown in BHI were able to form biofilm when incubated at 37 °C for 24 h compared to 28 (93%) from Nutrient broth grown in BHI; no difference was noted from TSB cultures, as 93% of the strains from both Nutrient agar and broth formed biofilm (Table 8.1). However, no statistical significance (*p*-value >0.05) was observed when the two methods (agar and broth) were compared; thus it can be suggested that *E. cloacae* strains for biofilm formation assays may be cultivated either in broth or on solid medium.

Worthy to note is the fact that infecting bacteria are often surface-associated, and their cell surface can therefore be expected to be more similar to that of bacteria grown on solid medium than to that found grown in liquid media (Donlan, 2001). Bacterial cells grown on solid medium differ in expression of cell- associated molecules compared to those grown in liquid medium (Kiers *et al*., 2001; Basson *et al*., 2007). Probably the presence of slime layers that are regarded as soft polyelectrolyte layers surrounding the bacteria decreases the energy barrier due to electrostatic repulsion in the interaction of the organisms with negatively charged substrata and, thus, plays an important role in their adhesion (Kiers *et al*., 2001). In their study, Kiers *et al*. (2001) found that cell surface softness of *Staphylococcus epidermidis* first grown on agar medium increased by a factor of two, compared to the cell surface softness for the strains grown in liquid medium. Therefore, preparation of the inoculum from

broth or agar may directly influence the biofilm production, since adhesion is the first step in biofilm formation.

It was also noted that a good number of strains grown in Nutrient broth, 90% and 73% cultured in BHI and TSB respectively were able to form biofilm, in contrast to 73% and 60% strains prepared from agar grown under similar conditions. However, no statistical significance was observed (p >0.005) when the two methods were compared.

Composition of the medium has been documented to influence the ability of bacteria to produce biofilm under *in-vitro* conditions; in particular the presence of glucose in the growth medium (i.e. standard TSB medium commonly contains 0.25% glucose) has been reported to enhance biofilm formation (Stepanovic *et al*., 2007). Some studies have shown preference for BHI to TSB, although some strains of *Staphylococcus*, *Vibrio* and *Pseudomonas* species have been reported to produce greater biofilm quantities in TSB while others do so in BHI (Stepanovic *et al*., 2007; Hoštacká *et al*., 2010). The present study compared the two growth media and observed no statistical significance (p >0.005). However, a slightly higher number of *E. cloacae* strains grown on BHI demonstrated the ability to adhere to the wells of the microtitre plate, implying that although both BHI and TSB media could support the development of *E. cloacae* biofilms *in-vitro*, some strains will produce strong biofilms on BHI than TSB. These findings are in agreement with other studies (Mathur *et al*., 2006), which found that supplementation (enriching) of TSB medium with glucose increased the ability of *Staphylococci* to form biofilm; while Knobloch *et al*. (2002) recommended the use of the two media to confirm a biofilm positive isolate.

All the *E. cloacae* strains (100%) demonstrated adherence characteristics at low temperatures of 25 °C when incubated for 48 h in comparison to the range of 60% - 90% of the strains that had the same characteristics when incubated for 24 h only; the range increased with high temperatures (37 °C) where 93% - 100% of the strains demonstrated the adherence ability when incubated for 24 h (Table 8.1). Statistical significant adherence was observed (p <0.005) when the two incubation periods were compared, which may imply that long incubation times and high temperatures influence biofilm formation.

Previous works have indicated that temperature, nutrients and other components in media affect attachment of microorganisms to surfaces of various materials (Hood and Zottola, 1997; Iversen *et al*., 2004). The present study observed strong biofilms from plates incubated at 37 °C (Table 8.1). This could be attributed to the fast growth rate of the bacteria at higher temperatures (Hoštacká *et al*., 2010). Similarly, Iversen *et al*. (2004) reported strong adherence of their test bacteria at high temperatures (>40 °C). Contrary to these findings were the observations by Di Bonaventura *et al*. (2007) who reported a higher biofilm production at a lower temperature (32 °C) than the higher temperatures of 37 °C; which may further suggest that biofilm formation varies with temperature and organism. Hence from the findings of the current study it can be deduced that *E. cloacae* strains will produce strong biofilms at higher temperatures.

Table 8.1: The effects of temperature, incubation time and growth medium on the biofilm formation of *E. cloacae* isolates

B. phenotype	Parameters number (%)															
	BHI [a]				TSB [a]				BHI [b]				TSB [b]			
	24 h		48 h		24 h		48 h		24 h		48 h		24 h		48 h	
	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
NA	8(27)	0	0	0	12(40)	2(7)	0	0	3(10)	2(7)	0	0	8(27)	2(7)	0	0
WA	18(60)	10(33)	10(33)	5(17)	14 (47)	17(57)	18(60)	4(13)	20(67)	11(37)	12(40)	4(13)	19(63)	14(47)	6(20)	2(7)
MA	4(13)	18(60)	16(53)	0	4 (13)	10(33)	11(37)	10(33)	7(23)	14(47)	15(50)	9 (30)	3 (10)	11(37)	16(53)	11(37)
SA	0	2(7)	4(13)	25(83)	0	1 (3)	1 (3)	16(53)	0	3(10)	3(10)	17(57)	0	3(10)	8(27)	17(57)
T. adh. (%)	22 (73)	30 (100)	30 (100)	30 (100)	18 (60)	28 (93)	30 (100)	30 (100)	27 (90)	28 (93)	30 (100)	30 (100)	22 (73)	28 (93)	30 (100)	30 (100)

BHI, brain heart infusion broth; TSB, tryptic soya broth; NA, non adherent; WA, weak adherent; MA, moderate adherent; SA, strong adherent; T. dh., total adherent; B. phenotype, biofilm phenotype; BHI [a], colonies from Nutrient agar and grown in BHI broth; TSB [a], colonies from Nutrient agar and grown in TS broth; BHI [b], inoculums from Nutrient broth and grown in BHI; TSB [b], inoculums from Nutrient broth and grown in TSB.

Table 8.2: The mean optical densities and standard deviations of the different biofilm phenotypes.

Parameters	Biofilm formation							
	Non-adherent		Weakly adherent		Moderately adherent		Strongly adherent	
	OD range	Mean OD ± SD	OD range	Mean OD ± SD	OD range	Mean OD ± SD	OD range	Mean OD ± SD
25 ^{0}C BHI (24 h)[a]	0.448 - 0.486	0.470 ± 0.02	0.572 - 0.990	0.733 ± 0.13	1.30- 2.14	1.406 ± 0.15	ND	ND
25 ^{0}C BHI (48 h)[a]	ND	ND	0.611- 1.06	0.875 ± 0.13	1.12- 2.01	1.344 ± 0.25	2.192-2.769	2.543 ± 0.39
25 ^{0}C TSB (24 h)[a]	0.352- 0.618	0.512 ± 0.08	0.626 - 1.131	0.785 ± 0.15	1.365-1.458	1.405 ± 0.07	ND	ND
25 ^{0}C TSB (48 h)[a]	ND	ND	0.625 - 1.237	0.984 ± 0.18	1.625-1.789	1.507 ± 0.231	ND	ND
37 ^{0}C BHI (24 h)[a]	ND	ND	0.590 - 1.074	0.885 ± 0.12	1.164- 2.022	1.504 ± 0.34	2.224-3.327	2.775 ± 0.77
37 ^{0}C BHI (48 h)[a]	ND	ND	0.580 - 0.784	1.872 ± 0.32	1.331- 2.13	1.872± 0.32	2.35- 4.22	3.034 ± 0.62
37 ^{0}C TSB (24 h)[a]	0.421- 0.464	0.442 ± 0.03	0.662 - 1.081	0.861 ± 0.169	1.297- 1.997	1.591 ± 0.199	ND	ND
37 ^{0}C TSB (48 h)[a]	ND	ND	0.988 - 1.583	1.483 ± 0.493	1.93 - 2.485	2.149 ± 0.190	2.556-3.635	2.955± 0.503
25 ^{0}C BHI (24 h)[b]	0.359- 0.462	0.420 ± 0.05	0.565 - 1.083	0.832 ± 0.14	1.098 -1.988	1.509 ± 0.340	ND	ND
25 ^{0}C BHI (48 h)[b]	ND	ND	0.729 - 1.048	0.855 ± 0.13	1.436 - 2.144	1.728 ± 0.584	2.253 - 3.24	2.660 ± 1.13
37 ^{0}C BHI (24 h)[b]	0.44- 0.481	0.460 ± 0.02	0.552 - 1.111	0.803 ± 0.17	1.123 - 2.039	1.577 ± 0.36	2.405- 3.27	2.837 ± 0.61
37 ^{0}C BHI (48 h)[b]	ND	ND	1.01 - 1.159	1.074 ± 0.06	1.392 - 2.129	1.697 ± 0.24	2.247-3.805	2.93 ± 0.546
25 ^{0}C TSB (24 h)[b]	0.421- 0.559	0.508 ± 0.04	0.62 - 1.117	0.834 ± 0.16	1.319 -1.774	1.548 ± 0.22	ND	ND
25 ^{0}C TSB (48 h)[b]	ND	ND	0.711-1.066	1.034 ± 0.18	1.364 - 2.414	1.706 ± 0.36	2.568-3.382	2.929 ± 0.36
37 ^{0}C TSB (24 h)[b]	0.503- 0.535	0.519 ± 0.2	0.623 - 1.153	0.877 ± 0.2	1.561 - 2.413	1.802 ± 0.2	2.542-2.66	2.601 ± 0.08
37 ^{0}C TSB (48 h)[b]	ND	ND	0.841- 1.076	0.958 ± 1.66	1.25 - 2.19	1.831 ± 0.54	2.612-3.938	3.261 ± 0.54

OD, optical density; SD, standard deviation; ND, not determined; [a], colonies from Nutrient agar; [b], inoculums from Nutrient broth; BHI, brain heart infusion broth; TSB, tryptic soy broth.

8.4.2 The capability of E. cloacae strains to autoaggregate and coaggregate with partner organisms

All *E. cloacae* strains were able to coaggregate with the seven partner strains; the coaggregation index ranged from 12% - 74% with best coaggregate activity observed when partnered with *S. pyogenes* (54% - 74%) (Table 8.3). It was also noted that most of the strains which weakly adhered to the wells had a slightly high coaggregation index range of 56% - 74% followed by moderate adherent 12% - 70% and the least was non adherent with 54% - 66% (Table 8.3). Of particular interest was a weakly adherent isolate EC 235 which demonstrated the strongest coaggregation with all the partners. On the other hand, all the strains were able to adhere to each other, with autoaggregation range of 27% - 89% (Table 8.3).

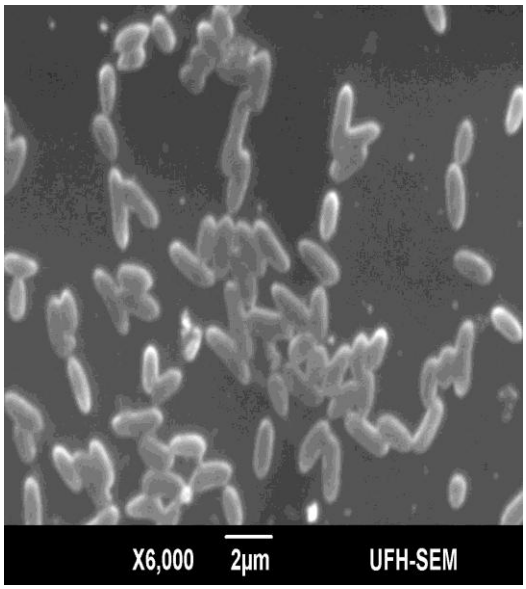
Table 8.3: Coaggregation indices of selected biofilm forming *E. cloacae* strains with seven different reference strains partners

Isolate (biofilm phenotype)	%Autoagg.	Coaggregation indices (%)							
		Partner strains							
		Range %	*S. aureus* NCTC 6571	*S. pyogenes* A ATCC 49399	*S.* Typhimurium ATCC 13311	*P. aeruginosa* ATCC 15442	*P. shigelloides* ATCC 51903	*A. hydrophila* ATCC 35654	*S. sonnei* ATCC 29930
EC 52-2 (NA)	27	55 - 66	55	66	63	55	64	61	58
EC 205 (NA)	38	54 - 66	54	63	66	62	62	64	61
EC 12-2 (WA)	45	56 - 69	69	69	59	56	68	69	ND
EC 70 -2 (MA)	89	12 - 70	57	61	29	70	12	64	56
EC 89 -2 (MA)	30	31 - 62	43	54	62	40	53	31	23
EC 235 (WA)	34	65 - 74	68	74	74	72	72	65	69

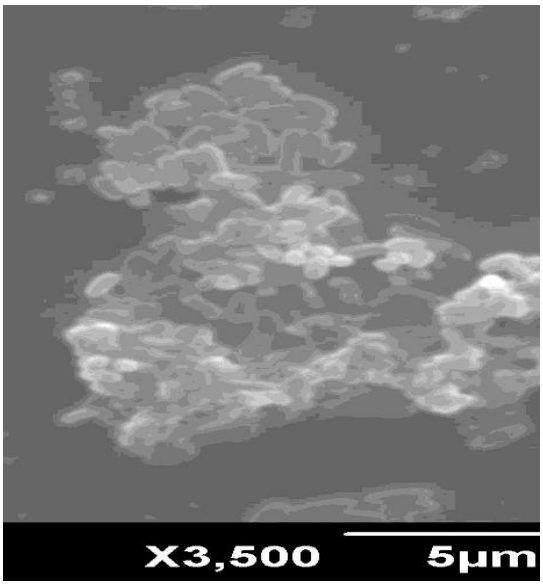
NA, non adherent; WA, weak adherent; MA, moderate adherent; Autoagg, autoaggregation.

First recognized between bacterial isolates from human dental plague, coaggregation is the highly specific recognition and adhesion of different bacterial species to one another by specific molecules. Coaggregation in biofilms is important in development of mix-culture biofilms; it enables cells to withstand the highly changing environment, which on the other hand can adversely affect mono-dispersed cells. The results of this study revealed that *E. cloacae* isolates which weakly adhered to the microtiter plate demonstrated the strongest coaggregation with all the seven partner strain; these findings suggest the possibility of these strains to act as bridging organisms in multi-generic biofilms due to their ability to coaggregate with diverse coaggregating partners. Such bridging organisms are believed to carry complementary receptors recognized by functionally similar adhesins on cells from distinct genera (Malik *et al*., 2003).

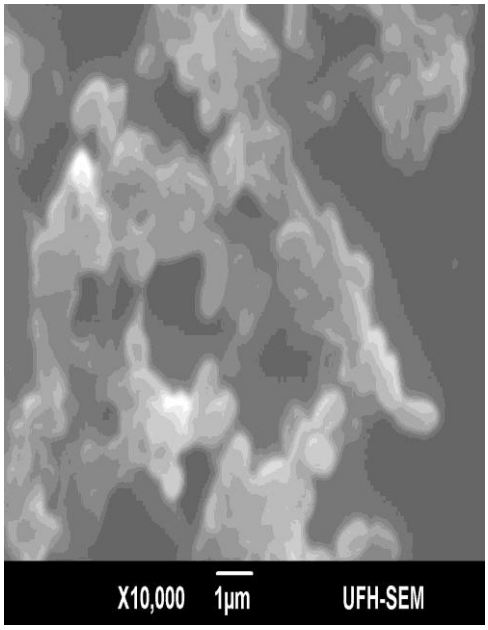
Figure 8.1 shows the scanning electron micrographs of *E. cloacae* autoaggregates and its coaggregate partners *S. aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442. The different phenotypes were clearly distinguishable; cells from the strong adherent strains were seen wedged together while for the moderate and weak adherent strains, few cells were stuck together. The SEM graphics of coaggregate assay demonstrated weak coaggregation with *S. aureus* and strong coaggregation with *P. aeruginosa.* It was also observed that *E. cloacae* strains coaggregated with organisms important in food spoilage and/or intoxications, i.e., *S. aureus*, *S.* Typhimurium, *S. sonnei* and *P. aeruginosa* implying that under favourable conditions, *E. cloacae* in food processing plants can enhance the bridging of organisms in multi-generic biofilms.


X6,000 2µm UFH-SEM

(a)


X3,500 5µm

(b)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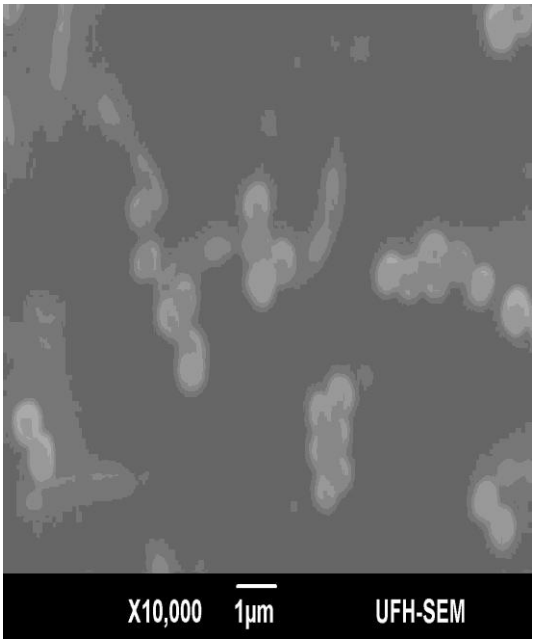
(c)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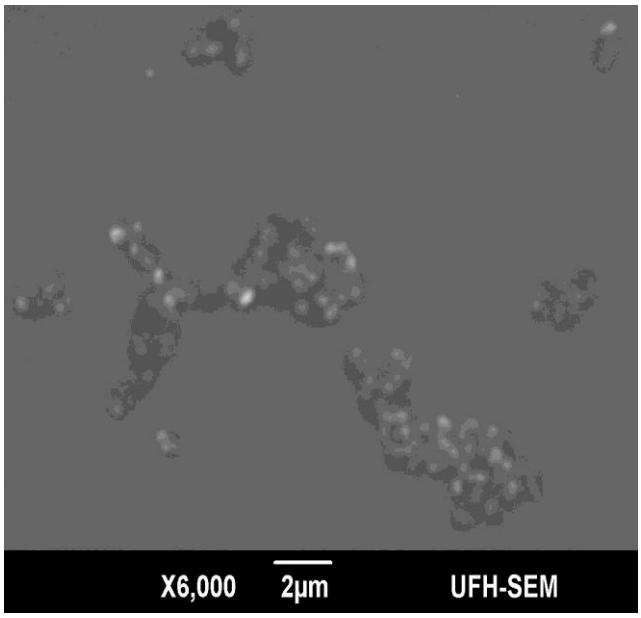
(d)



(e)

Figure 8.1: Scanning electron micrographs of autoaggregates and coaggregates biofilms of *E. cloacae* and its coaggregate partners S. *aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442. (a) weak adherent autoaggreagate; (b) moderate adherent autoaggreagate; (c) strong adherent autoaggreagate; (d) coaggregate of *E. cloacae* and partner *S. aureus* NCTC 6571; (e) coaggregate of *E. cloacae* and partner *P. aeruginosa* ATCC 15442.

8.5 Conclusion

This study demonstrated that *E. cloacae* readily form biofilm on plastic surfaces, which are nowadays frequently used in food-processing environments raising a great concern to the food industry as organisms in biofilm are difficult to eliminate hence creating a reservoir for cross contamination. The study also indicates the suitability of BHI and TSB medium for the cultivation of *E. cloacae* biofilm however, temperature and incubation time significantly affect the biofilm formation by these bacteria.

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CHAPTER NINE

General Discussion, Conclusion and Recommendations

General discussion, conclusion and recommendations

9.1 General discussion

Foodborne diseases remain to be a major challenge in both developed and developing countries despite various control efforts such as HACCP and GAP. Changes in eating habits, mass catering, increased international travel and poor hygiene practices are some of the key contributing factors to this challenge (Tauxe *et al*., 2010; Nyenje and Ndip, 2013). Detection and quantification of bacteria in raw materials, prepared food and food contact surfaces is therefore a key factor in assessing the quality and safety of foods. It also reveals the level of hygiene adopted by food-handlers in the course of preparation of such foods (Nkere *et al*., 2011).

Broadly, there are two quantitative methods (MPN and plate count) of enumerating viable bacterial cells present in a food sample. Since MPN values are only estimates, the plate count method was employed in this study which has been reported to provide high level of sensitivity (Davey, 2011). Mean contamination levels of ≥ 5.0 $\log_{10}$ CFU g^{-1} was reported in all the sample types tested except the pies. Most international food authorities such as, Health Protection Agency (HPA) and New South Wales Food Authority (NSWAF), recommends the standard limit for total bacterial count of fully cooked ready-to-eat foods to be < 5.0 $\log_{10}$ CFU g^{-1} (HPA, 2009; NSWAF, 2009); implying that these foods could be of high risk in transmitting foodborne pathogens to consumers. The high level of contamination in ready-to-eat foods observed had also been reported elsewhere (Ifediora *et al*., 2006; Nkere *et al*., 2011).

Poor environmental sanitation, and poor personal hygiene, particularly among food-handlers mostly accounts for the contamination of these foods while improper storage leads to multiplication of pathogens in food to infective doses. The conditions described above prevail in the study area of the present study and are compounded by inadequate access to running water and lack of appropriate waste-disposal facilities. These factors contributed negatively to poor

personal and environmental hygiene of food vendors from the roadside cafeterias in this locality; exemplified by high contamination level of unhygienic cafeterias (54%) compared to hygienic cafeterias (47%) which had running water and proper waste disposal system.

Worldwide there has been an increasing demand for fresh and minimally processed foods such as fresh fruits and vegetables which are sources of vitamins, minerals, and fibres, as well as for the reduction in disease risks such as heart diseases, diabetes and cancer (Ragaerta *et al*., 2004; Mathews *et al*., 2006; Khiyami *et al*., 2011; Boeing *et al*., 2012; Ijabadeniyi *et al*., 2012). However, vegetables have been recognized as the leading vehicle of illnesses associated with *E. coli* O157:H7 and *Salmonella* foodborne outbreaks (Pezzoli *et al*., 2008; Fynn, 2009; Tauxe *et al*., 2010). Similarly, in this study, vegetables had the highest bacterial count, of 6.3 - 6.8 $\log_{10}$ CFU g^{-1}, suggestive of posing health hazard to the consumers.

In spite of the fact that vegetables may be contaminated from the farm when cultivated in, or irrigated by untreated water containing human or animal manure (Ijabadeniyi *et al*., 2012), fresh vegetables when subjected to peeling/cutting or shredding during minimal processing, releases plant cellular fluids that provides nutritive medium for microbial growth. Thus, several outbreaks of foodborne illnesses have also been found to be associated with cut vegetables (Doyle and Erickson, 2006). Hence, it can be speculated that similar factors might have been the possible source of contamination of the vegetables under study which were also cut and minimally cooked.

Precise and ultimate microorganism identification and pathogen detection is crucial for accurate disease diagnosis and treatment (Jasson *et al*., 2010). The current routine isolation and identification of foodborne pathogens is based on colony pigmentation on a selective/differential media, followed by further characterisation by biochemical and/or serological tests (Betts and Blackburn, 2009). However, these methods are tedious and lengthy underscoring the need for

rapid and early detection of food contaminants, which is crucial for the control of foodborne pathogens (Andrea and Mariani, 2009); besides, some species are morphologically and biochemically related, hence leading to misdiagnosis and wrong treatment. The present study therefore employed both the standard methods and molecular techniques which have been reported to be rapid, specific and sensitive (Velusamy *et al*., 2010).

The study used API 20E, API 20NE and API Listeria biochemical test kits which are rapid, as the different biochemical test are customized on multichamber strips to confirm the presumptive colonies. Despite the fact that some of the major foodborne pathogens such as *Salmonella* were not isolated in this study; the isolation of other Enterobacteriaceae species and *S. aureus* which are environmental pathogens as well as commensal flora of the gut/skin of humans and animals is equally important as it suggests poor general hygiene status of food products in the study area. Of particular interest was *P. mirabilis,* a gut commensal flora of both humans and animals which was isolated from one source; suggestive of poor personal hygiene from a food-handler. Several outbreaks have been associated with poor personal hygiene of infected food-handlers (Wang *et al*., 2010; Bukar *et al*., 2010; Adolf and Aziz, 2012).

The study also recorded a high occurrence of *Listeria* species in meat product especially pies. Although the pies were first microwaved upon ordering, it was noted that they were not heated to high temperatures that could kill pathogens, but rather warmed to a good temperature for eating; this might have contributed to the bacterial contamination levels in pies. More to this, the pies appeared stale. Studies have shown that sufficient heating and prevention of cross-contamination adequately reduce bacterial counts of foodborne pathogens (Blankenship and Craven, 1982; Juneja, 2003; Bergsma *et al*., 2007; de Jong *et al*., 2012). In their study, de Jong *et al*. (2012) observed high bacterial reduction of *C. jejuni, Salmonella* Typhmurium and *E. coli* from chicken breast fillet after cooking at high temperatures (70 °C and 85°C).

The two most prevalent organisms (*L. ivanovii* and *E. cloacae*) were further confirmed using PCR. As previously reported by other researchers (Israil *et al*., 2003; Martinez-Urtaza *et al*., 2004), the current study also observed less satisfactory results with the phenotypic characterization. For example, some *L. ivanovii* isolates that reported 99.9% identity by API Listeria were found to be non *L. ivanovii* but other *Listeria* species (*L. seeligeri*/*L. welshimeri*) when the isolates were confirmed using PCR. Since biochemical tests are based on the metabolic actions of the bacteria; some reports have indicated that, biochemically based systems may not be reliable for environmental isolates because of inadequate information on environmental bacteria in computerized databases (Croci *et al*., 2007). Given that these isolates were from various food samples, it can be speculated that similar factors were responsible for the discrepancies observed. Furthermore, the methods are prone to human errors through general manipulations of the assay based on colour change which is sometimes a challenge when the positive and negative colours are closely related. On the other hand, some species are heterogeneous like *E. cloacae* complex, which have both non-pathogenic and pathogenic sub-species within the complex. Therefore, API 20E will just identify the *E. cloacae* complex not the specific sub-species. All these factors reflect the importance of genotype characterisation of foodborne pathogens where virulence and drug resistance genes can be detected and characterised for better understanding of their pathogenicity and any drug related resistance due to mutation.

Ribosomal 16S rRNA and 23S rRNA locus are generally selected as the target genes for successful ribotyping subgroups of bacteria (Hayden *et al*., 2001; Corinne *et al*., 2008; Ghebremedhin *et al*., 2008). The *groEL* gene, also known as *hsp60*, has been documented as one of the most conservative systems in nature with greater level of interspecies variation making it a good target gene for species classification (Hu *et al*., 2010). In the present study, *hsp60* gene of *E. cloacae* was targeted; which successfully confirmed 30 (96.7%) strains as *E. cloacae*. These

findings are in agreement with other studies that used *groEL* genes as a target gene to analyze several bacterial pathogens for homology, variation and gene distribution (Mondal *et al*., 2008; Bogumil and Dagan, 2010; Hu *et al*., 2010).

Foodborne illnesses are usually mild and self-limiting; however, severe cases may warrant antimicrobial therapy. Antibiotic susceptibility screening is crucial for successful patient management and a useful indicator for epidemiological analysis (Giovanni *et al*., 2002). The present study noted an alarming antimicrobial resistance of *E. cloacae* and *Listeria* species to all β-lactam antibiotics tested and a good number of these strains were also resistant to erythromycin, tetracycline, sulfamethoxazole/trimethoprim and vancomycin. These findings are worrisome as β-lactam antibiotics, are among the first-line treatment for critically ill patients, especially when a Gram-negative infection is suspected, owing to their clinical efficacy and low toxicity (Jain *et al*., 2008; Colomer-Lluch, 2011). They have however been also reported to be effective against Gram-positive organisms (Conter *et al*., 2009).

Worthy of note is the fact that *L. ivanovii* and *E. cloacae* were largely associated with meat products; it can be deduced that maintenance of good hygiene practices in meat processing industries may reduce the chances of contamination by these organisms; hence reducing the risk of antimicrobial resistant transmission. These findings also support the evidence that the use of antibiotics in animal husbandry expedites the development of antibiotic resistance in human medicine as these organisms are commonly found in animals.

Most of the bacteria that are resistant to a wide range of penicillins and cephalosporins produce β-lactamase, enzymes that have the ability to cleave the β-lactam ring of the β-lactam antibiotics, rendering them inactive. Members of the Enterobacteriaceae family that are resistant to the third generation of cephalosporins are now the main problem in clinical practice. They produce ESBLs, derivatives of the broad spectrum β-lactamases (*TEM-1* and *SHV-1*) (Rubtsov *et al*.,

2010). In view of the fact that most ESBL genes are plasmid mediated, resistant pathogens are rapidly spreading globally (Pitout *et al*., 2008); subsequently, creating a problem in the choice of an adequate method of treatment, which leads to inefficient therapy, and lengthening or increasing the cost of therapy (Rubtsov *et al*., 2010). Thus, efficient laboratory diagnosis of ESBL production by Gram-negative microorganisms in routine practices is a warrant. However, most tests available on the market for the detection and confirmation of ESBL production are phenotypic methods hence, cannot give additional information regarding any enzyme changes; hence, the present study used molecular techniques to detect and characterize β-lactames genes in an effort to determine the different variants and their mutations. In this study, both *E. cloacae* and *Listeria* strains contained the $bla_{\text{TEM-1}}$ gene, and other strains had mutant $bla_{\text{TEM-1}}$ gene; implying that these mutant organisms might have the ability to produce ESBLs which hydrolyzes penicillins and cephalosporins. These findings justify the marked penicillin resistance demonstrated by these organisms; but also attributes to the fact that the food chain might be the source of antibiotic resistance gene in human. The findings of this study are in agreement with other studies (Schwaber *et al*., 2007; Colomer-Lluch *et al*., 2011).

Discovering virulence factors is important in understanding bacterial pathogenesis and their interactions with the host, which may also serve as novel targets in drug and vaccine development (Muley *et al*., 2011). In essence, the ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of infection (Beceiro *et al*., 2013). Bacterial species can be effectively grouped into human pathogens and non-pathogens based on the presence or absence of virulence genes. The acquisition of these virulent genes may represent a survival advantage to the microorganism. In this study, some strains of *E. cloacae* harboured some of these virulence associated genes implying that in susceptible host these strains might cause an infection. It is conceivable that virulence genetic determinants, if located on the same genetic platform as antimicrobial

resistance genes (plasmids, transposons, integrons) may be co-mobilized under antimicrobial selective pressure; therefore, diagnostic methods that focus to detect virulence markers may help to resolve the increasing problem of the association between virulence and resistance, which is becoming more beneficial for pathogenic bacteria (Beceiro *et al*., 2013).

Biofilm formation by prokaryotic cells is one of the survival strategies to harsh environmental condition. Due to this capability to endure undesirable environmental conditions, biofilm formation may convey a selective advantage to certain pathogens. In their study, Hall-Stoodley and Stoodley (2005) found that *Vibrio cholerae* biofilms on crustacean shells, aquatic insects and plants result in the spreading of sufficient numbers of cells for an infective dose, which is not normally found for planktonic cells in a bulk fluid. Similarly, in this study, *L. ivanovii* and *E. cloacae* which were reported to be endemic in the study area (Odjadjare *et al*., 2010; Nyenje *et al*., 2012) demonstrated the ability to exist as biofilm structures. Therefore, it could be suggested that these organisms can form biofilms in food preparations area/kitchen utensils hence; creating a pool of bacteria that are constantly spreading to the environment. Previous studies have demonstrated the ability of both Gram-positive and Gram-negative microorganisms to exit in multispecies biofilm (Basson *et al*., 2007; Habimana *et al*., 2010; van der Veen and Abee, 2011). Likewise, strains from this study coaggregated well with both Gram-negative and Gram-positive reference strains tested.

Although virulence genes were not detected from *L. ivanovii* strains, 90% of the strains demonstrated the ability to form biofilms which is one of the survival mechanisms of bacteria. It is believed that organisms in biofilm produce certain proteins to adapt to harsh conditions (Davey and O'toole, 2000). Therefore, these *L. ivanovii* strains might equally be considered pathogenic.

9.2 General conclusion

From the results of this study, the following conclusions can be drawn:

1. Most of the ready-to-eat food samples examined in this study did not meet bacteriological quality standards, evidenced by the presence of bacteria species such as *Listeria* species (22%), *E. cloacae* (18%), *A. hydrophila* (12%), *K. oxytoca* (8%), *P. mirabilis* (6.3%) and *S. aureus* (3.2%); furthermore, all samples tested had a mean bacterial count levels of ≥ 5.0 $\log_{10}$ CFU g^{-1} except the pies; therefore posing potential risks to consumers.
2. The bacterial counts of vegetables, rice, potatoes, beef and chicken ste were statistically significant when compared with pies ($p< 0.5$) similarliry, statistical significant difference ($p< 0.5$) in bacterial load of beef and rice from hygienic and unhygienic cafeterias was also observed.
3. Marked susceptibility of *L. ivanovii* isolates was observed against chloramphenicol, ciprofloxacin, streptomycin and sulfamethoxazole/trimethoprim (100% each), while *E. cloacae* strains were susceptible to nalidixic acid (97%), streptomycin (94%), gentamicin and chloramphenicol (91%) each; hence these drugs could be considered in the treatment of infections caused by these organisms in the study area.
4. Multidrug resistance was a common trend observed in a good number of strains and nineteen antibiotic resistant patterns were obtained; the presence of these multi-resistant strains in these foods could be important vehicles transmitting multi-resistant bacteria and resistance genes to humans on a daily basis.
5. The study also reported a high efficiency of the M.I.C. Evaluator strip method evidenced by 100% categorical agreement to ciprofloxacin by M.I.C. Evaluator strip method as compared to 90% categorical agreement registered by broth microdilution method.

6. β-lactamase TEM-1 was prevalent in 100% of *E. cloacae* and 96% of *Listeria* species*;* implying that these organisms could bear deleterious effect on human health once transmitted via the food chain.
7. All *E. cloacae* strains (100%) and 88% of *L. ivanovii* isolates demonstrated adherence characteristics (biofilms) to a surface at 25°C; it entails that under favourable conditions, these organisms may stick to kitchen utensils and other environments leading to cross-contamination of food processed in these areas.
8. Non adherent strains were able to autoaggregate and coaggregate implying that preventing primary adhesion would prevent biofilm formation in these strains.

9.3 Recommendations

1. Future studies are required to screen the personal hygiene standards of the food-handlers, and the food contact surfaces to establish the possible source of contamination.
2. There is need for improved and more efficient monitoring of the microbial quality of foods in the study area.
3. Future studies are necessary to determine the gene(s) expressed by the species that formed biofilm, to throw more light on their pathogenic potential in our environment; as this could also help to formulate effective control strategies.
4. Standardisation and further analysis regarding the suitability of the M.I.C. Evaluator for testing of a wide range of organisms and antimicrobials is warranted; in a bid to establish their suitability for routine antimicrobial susceptibility testing
5. There is also need for further studies using animal model/cell lines to test the pathogenic potential of the strains.

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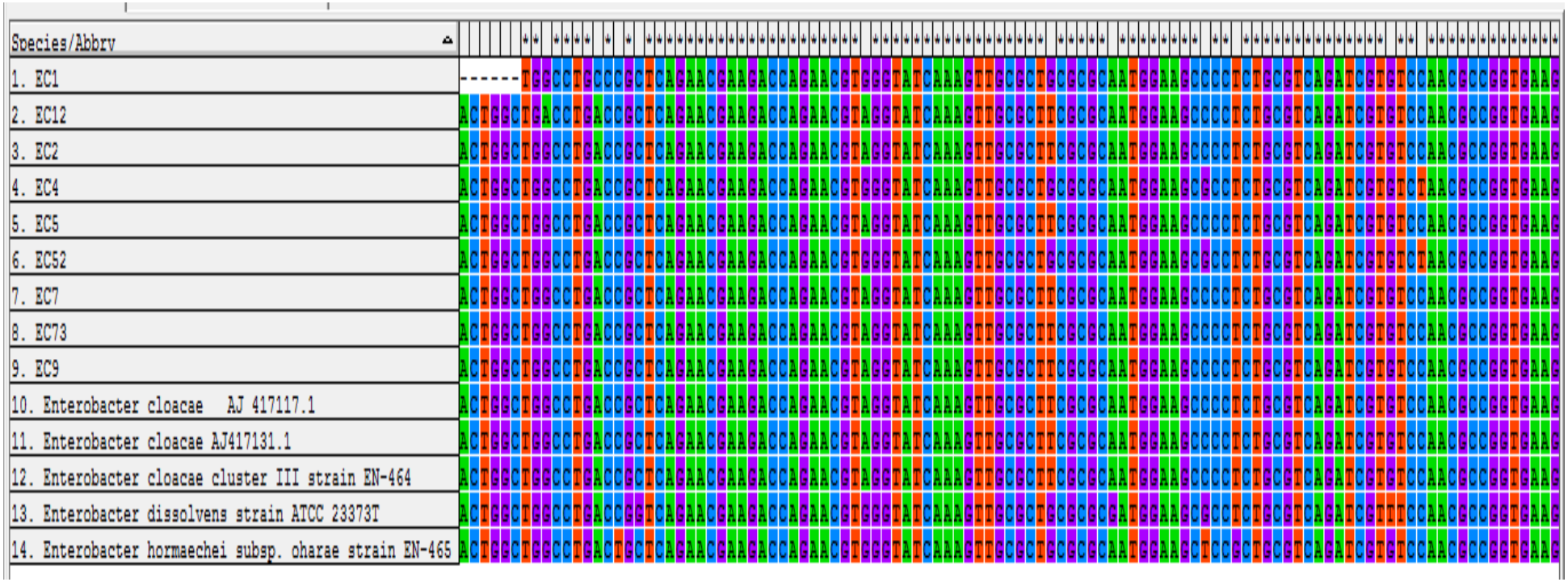
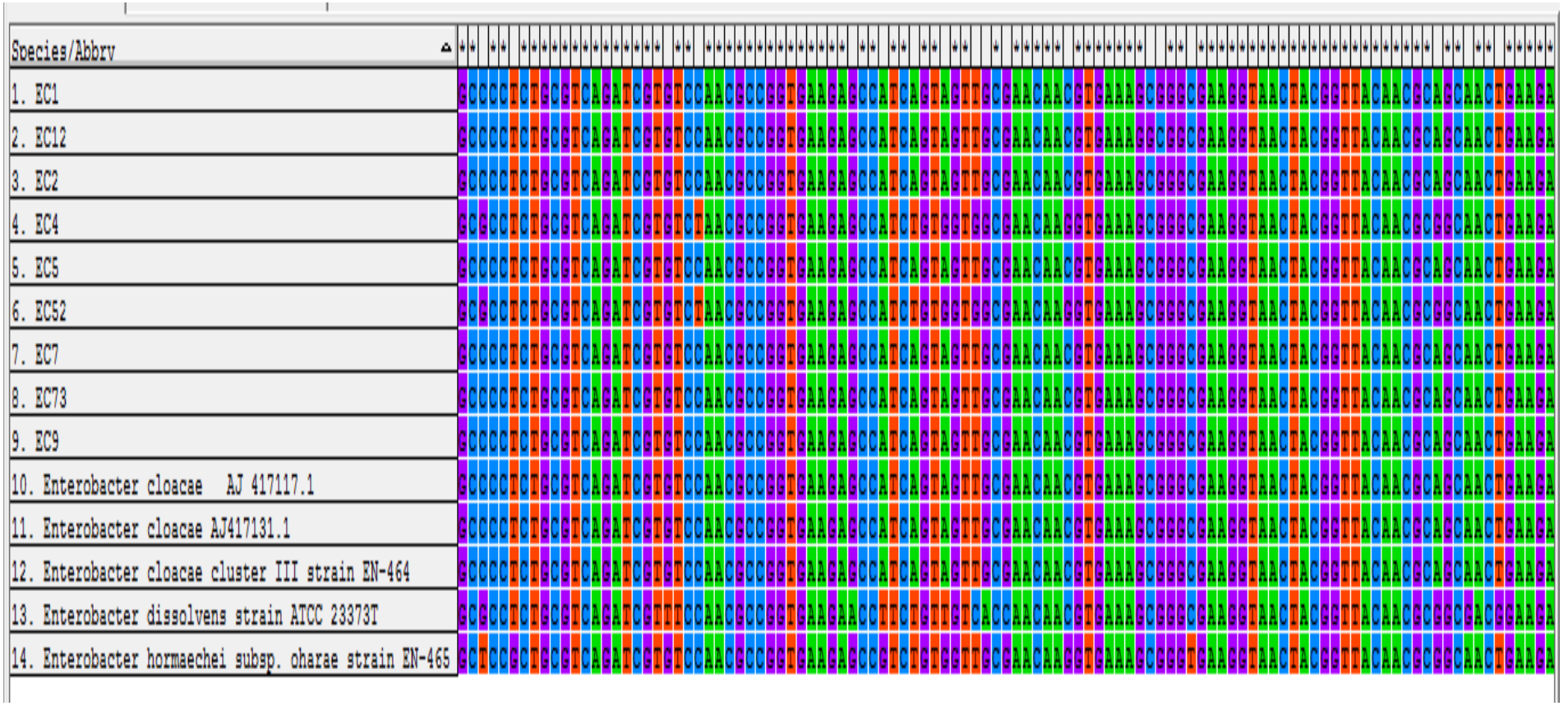
APPENDICES

Appendix 1: Sequence alignments of *hsp60*, *iap60*, *ucaA* and *bla*-$_{TEM}$ genes.

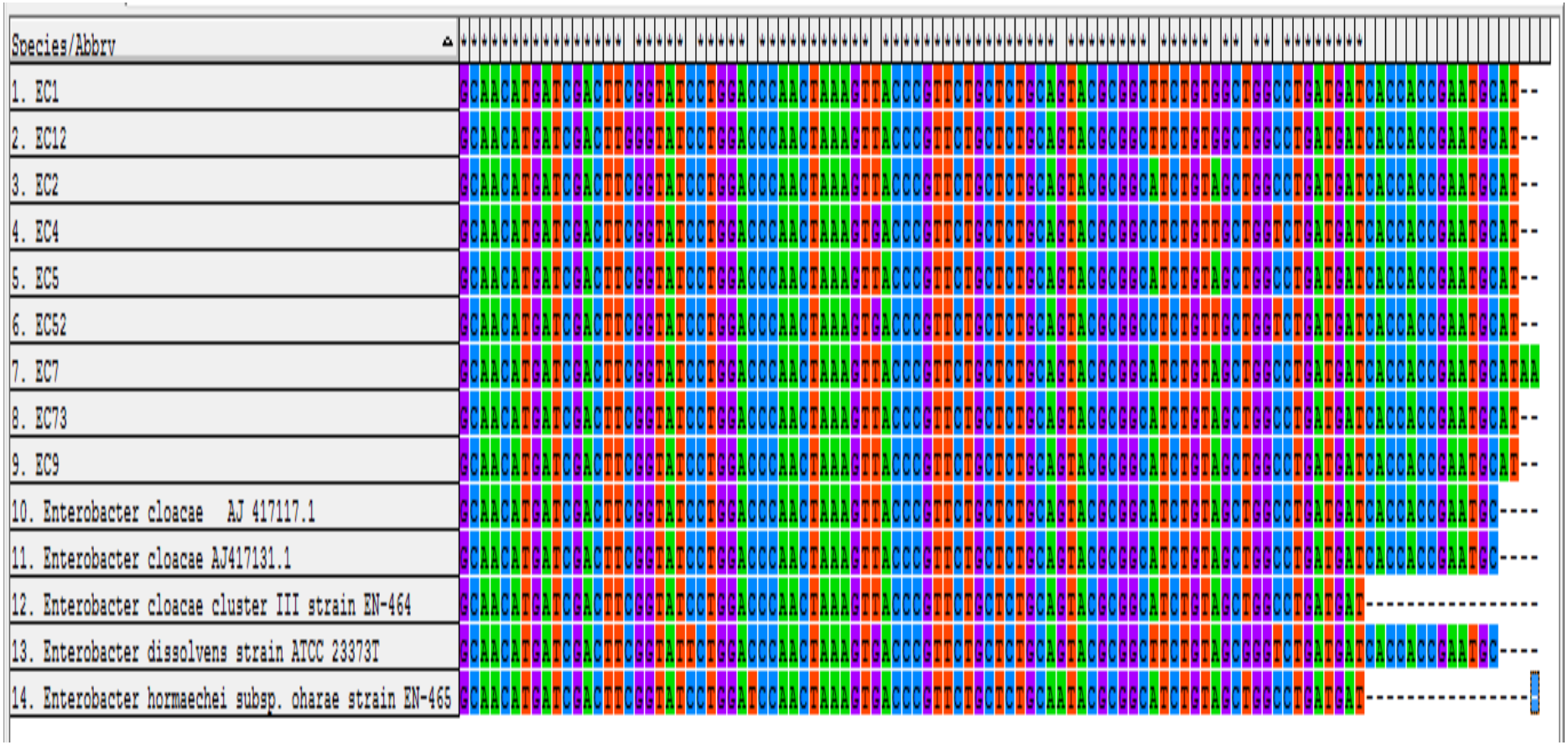
1.1a: Sequence alignments of *hsp60*



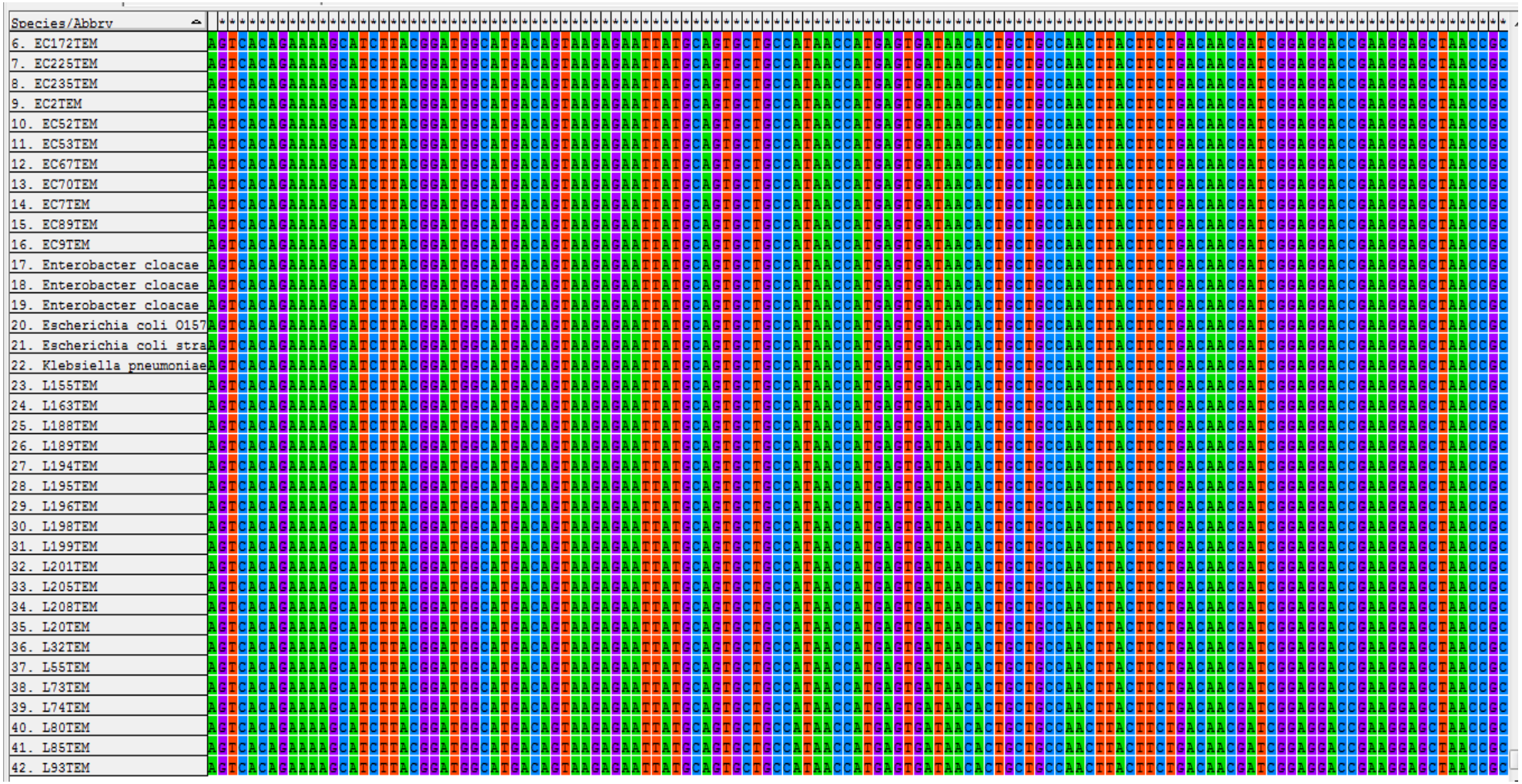
1.1b: Sequence alignments of *hsp60*



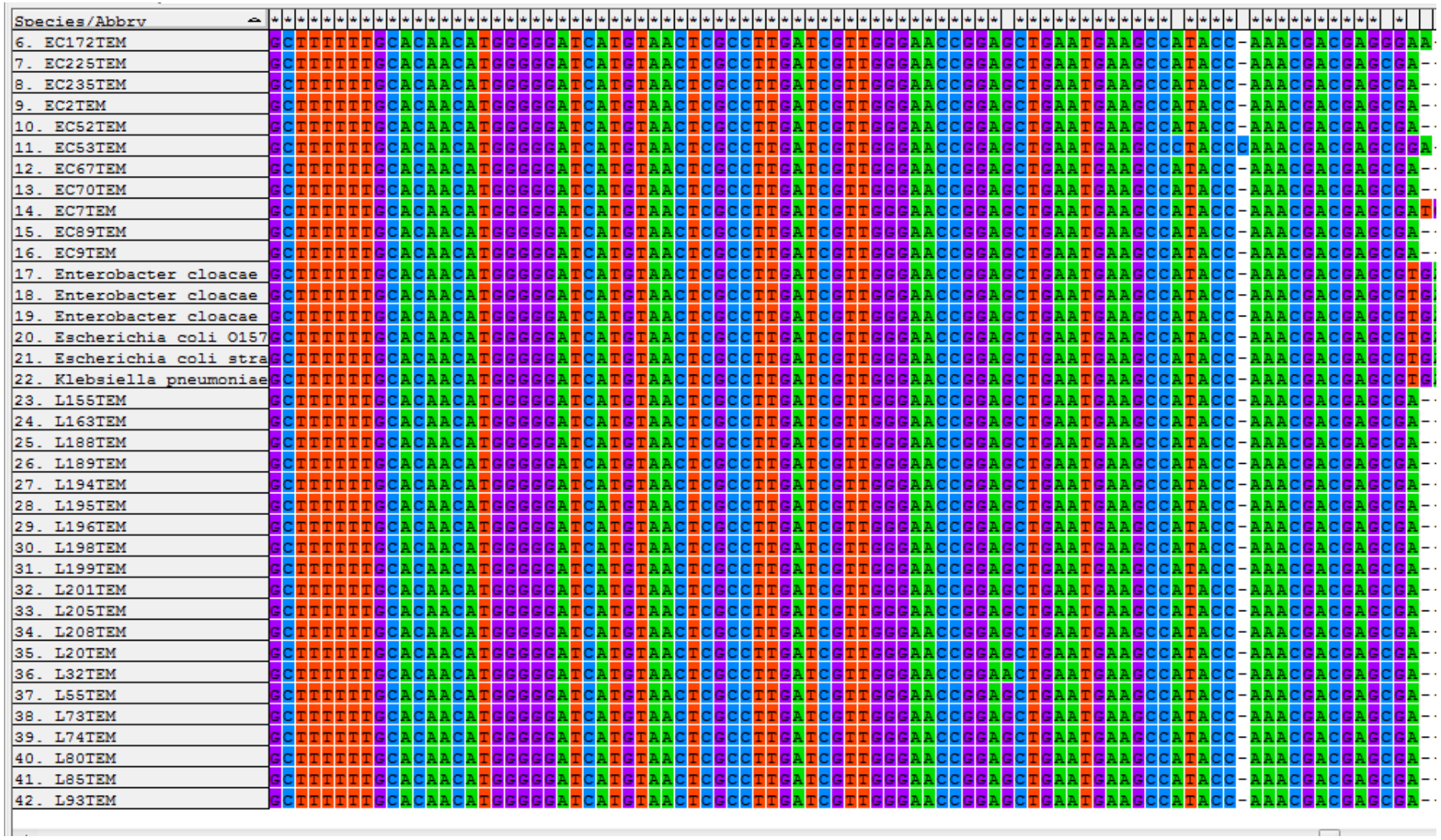
1.1c: Sequence alignments of *hsp60*



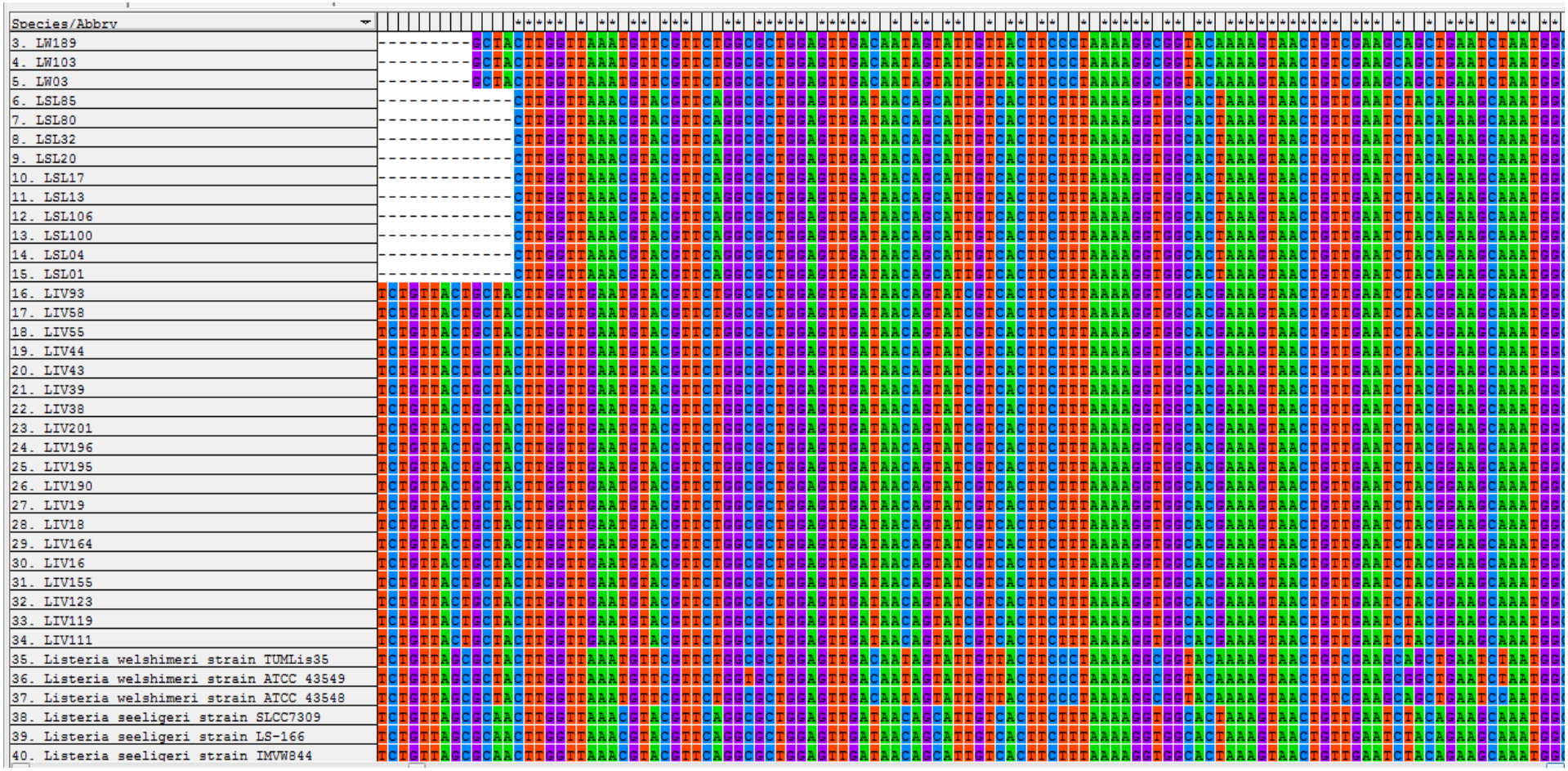
1.2a: Sequence alignments of *bla*-$_{\text{TEM-1}}$



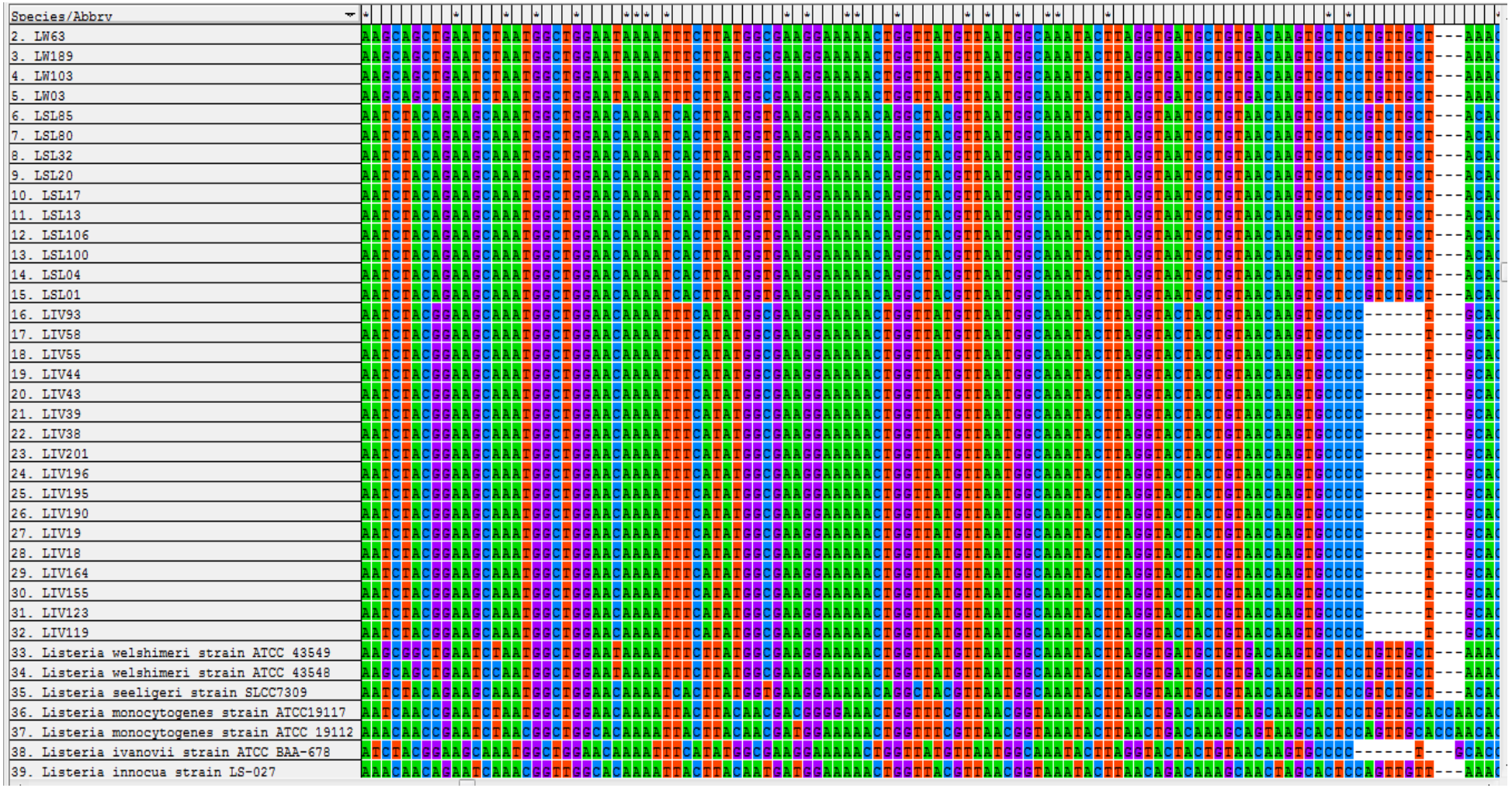
1.2b: Sequence alignments of *bla*-$_{\text{TEM-1}}$



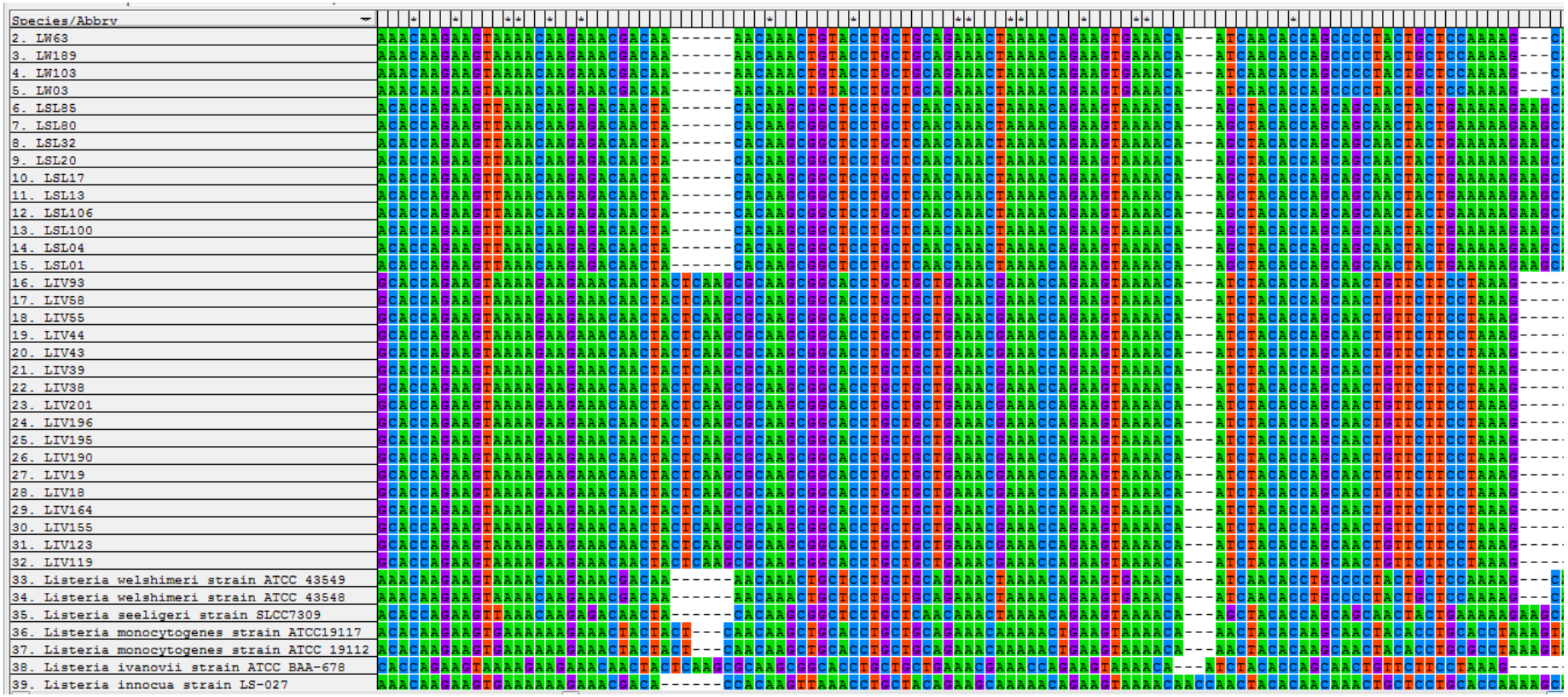
1.3a: Sequence alignments of Siwi2 (*iap 60* gene)



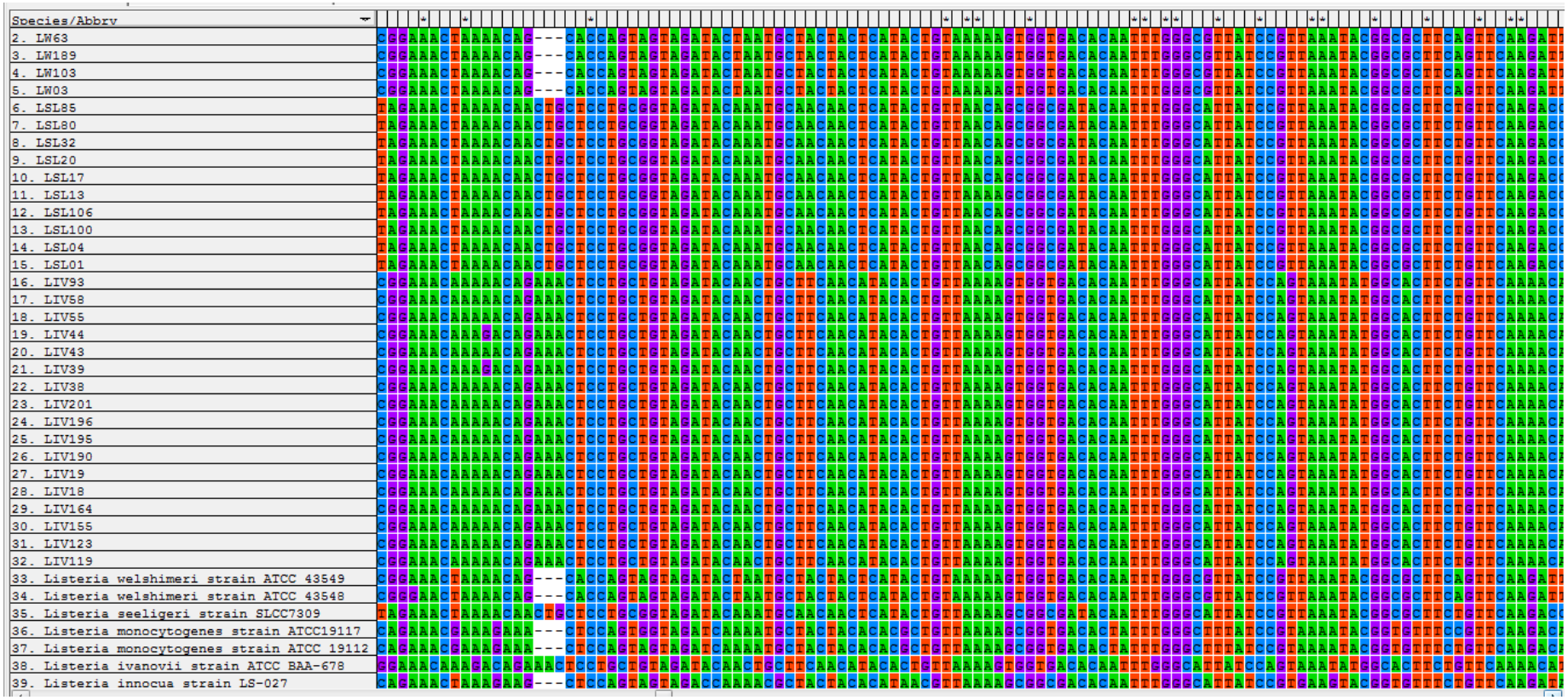
1.3b: Sequence aligments of Siwi2 (*iap 60* gene)



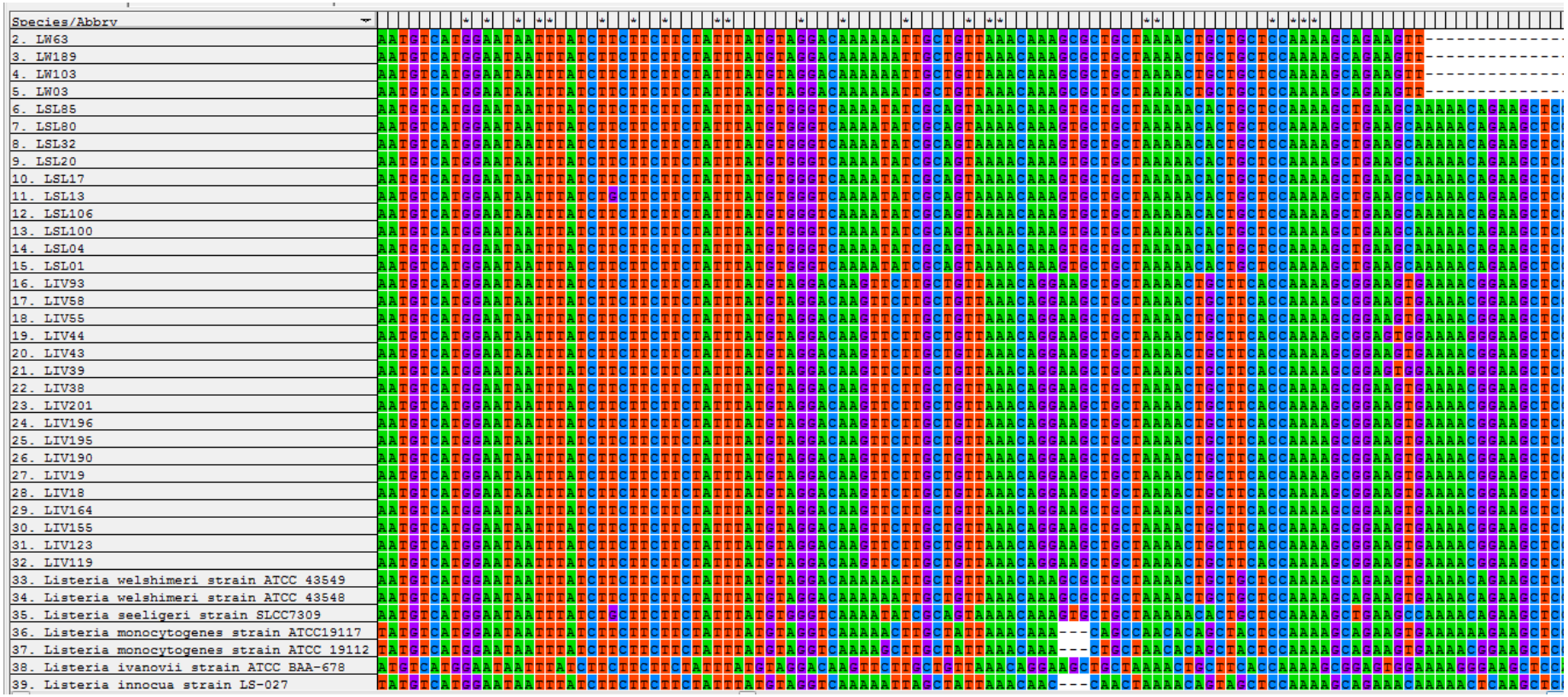
1.3 c: Sequence aligments of Siwi2 (*iap 60* gene)



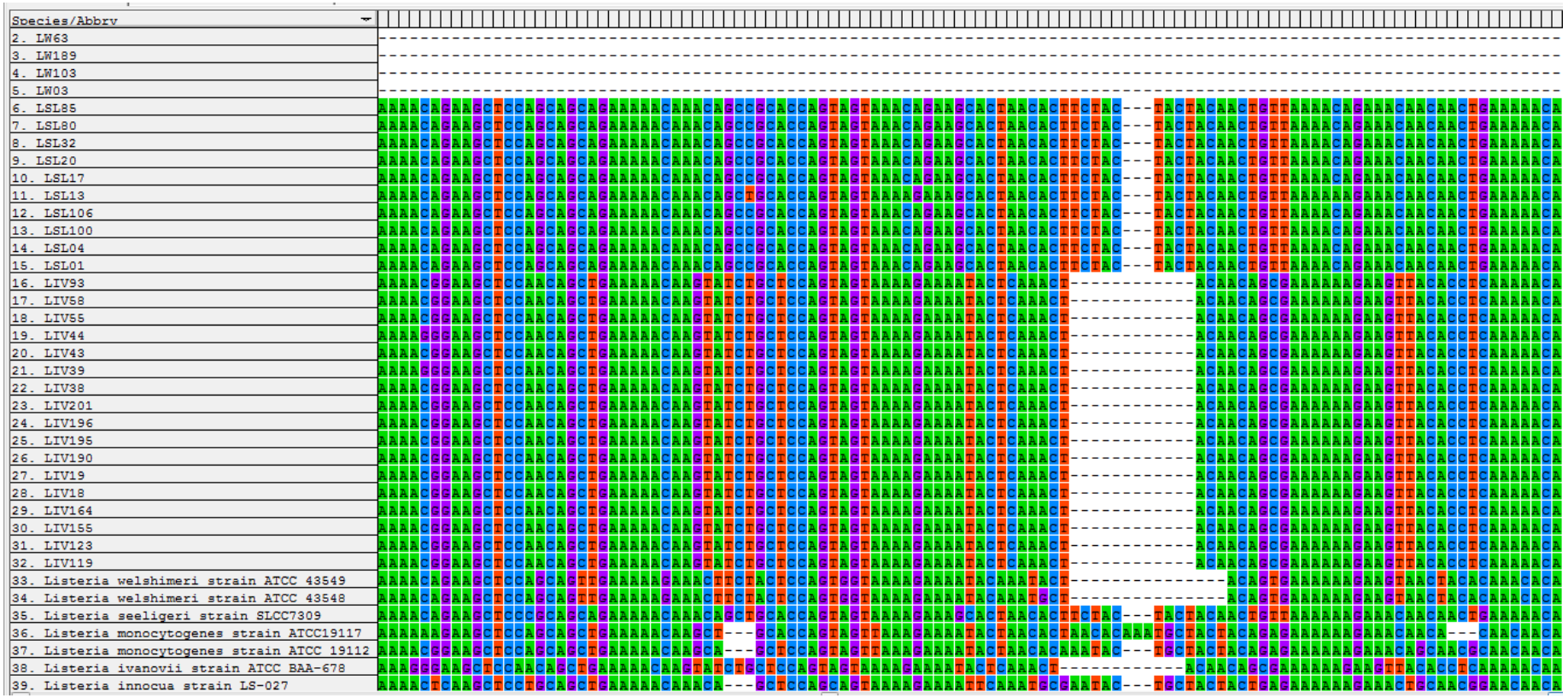
1.3d: Sequence aligments of Siwi2 (*iap 60* gene)



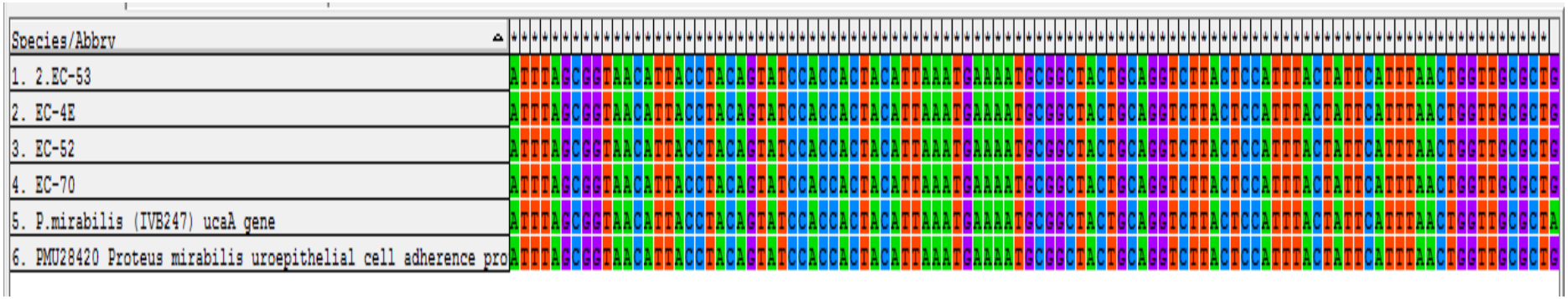
1.3e: Sequence aligments of Siwi2 (*iap 60* gene)



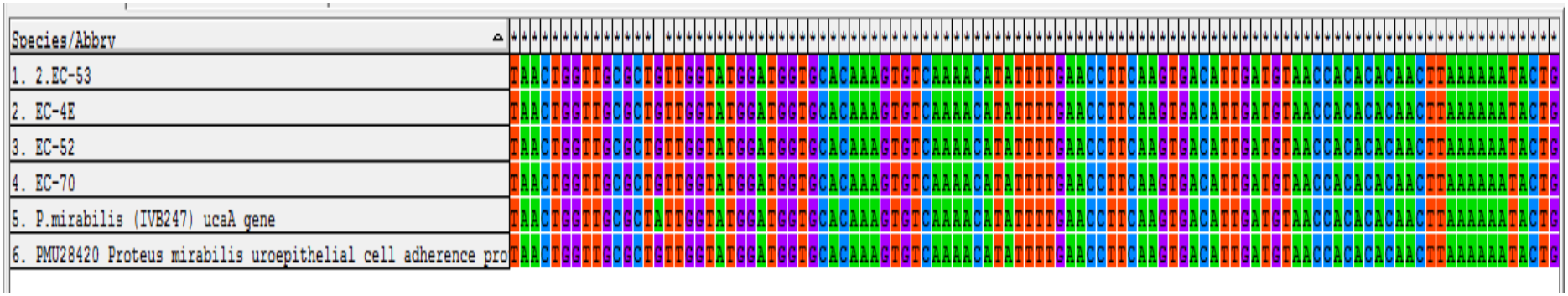
1.3f: Sequence aligments of Siwi2 (*iap 60* gene)



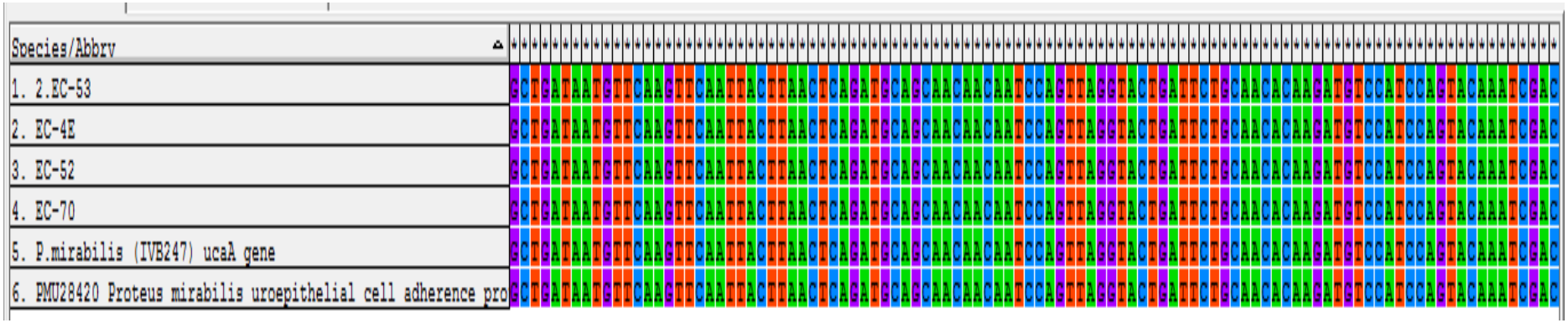
1.4a: Sequence aligments of *ucaA* gene



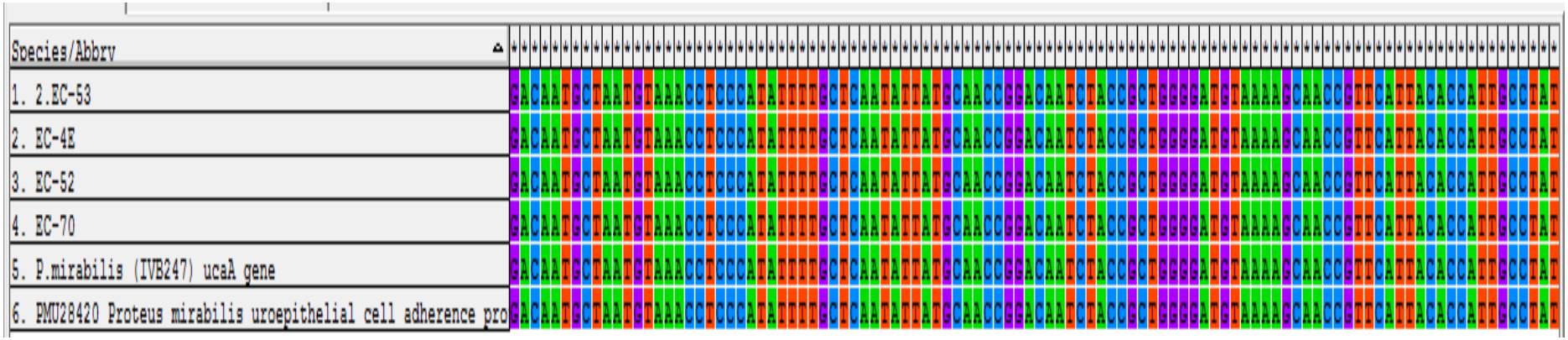
1.4b: Sequence aligments of *ucaA* gene



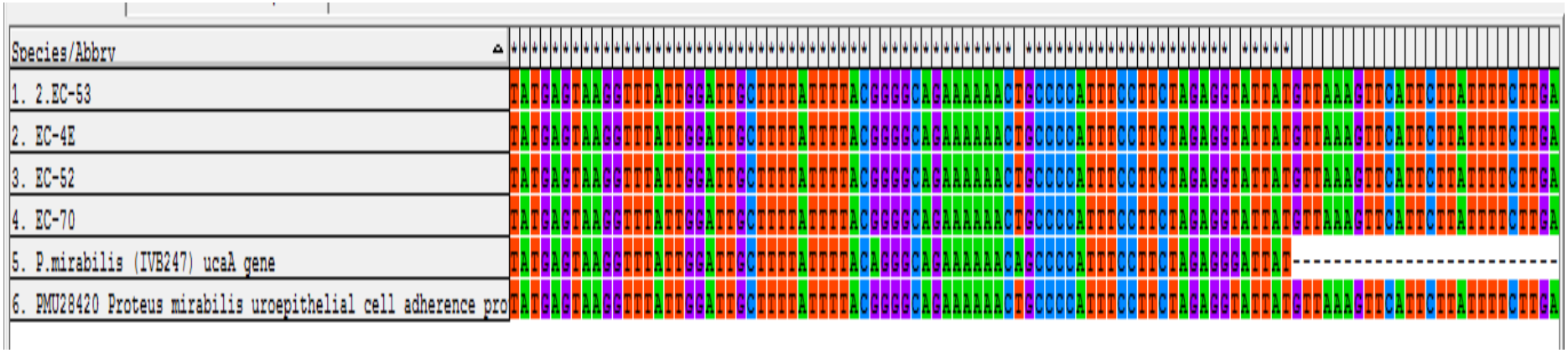
1.4c: Sequence aligments of *ucaA* gene



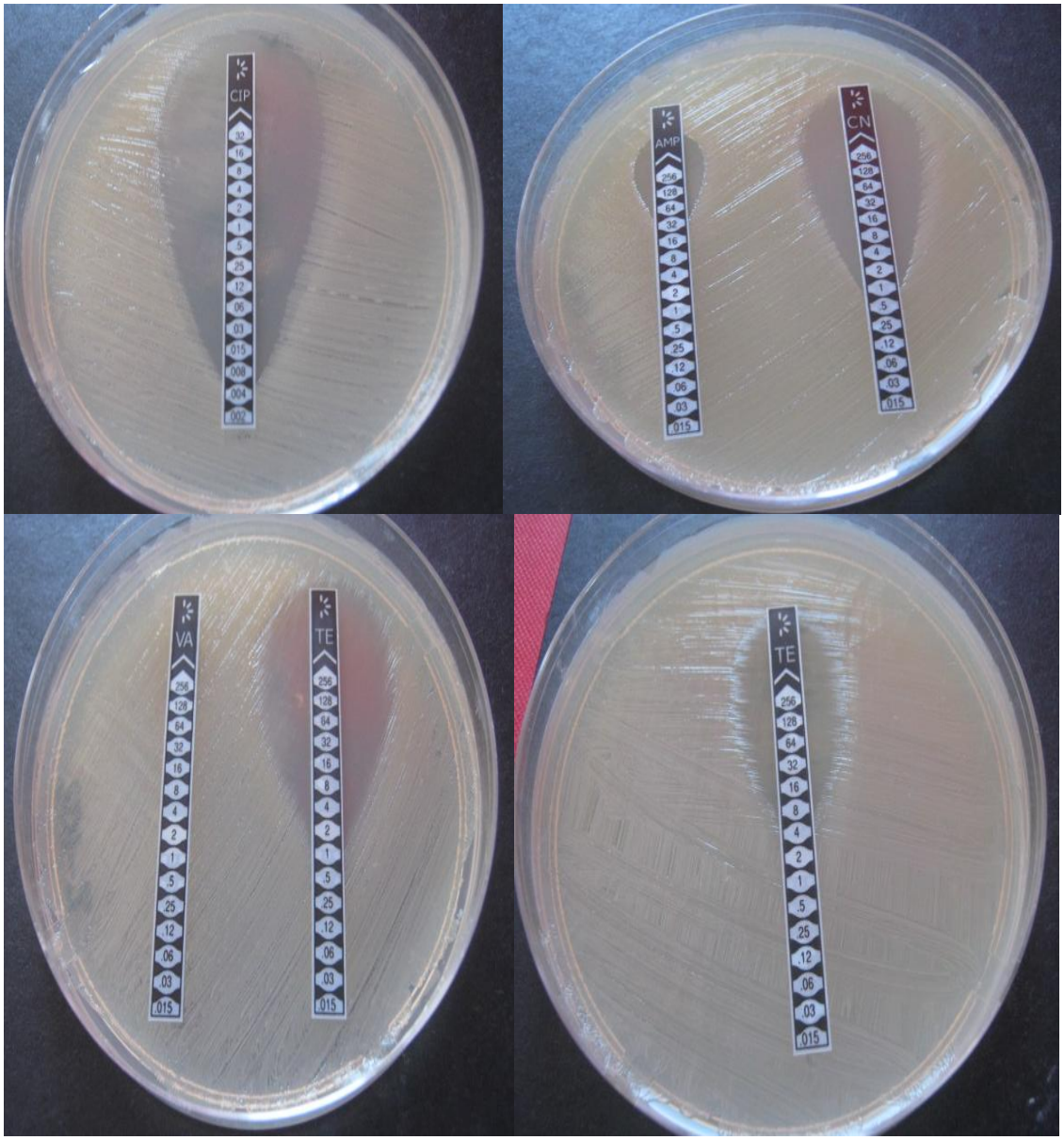
1.4d: Sequence aligments of *ucaA* gene



1.4e: Sequence aligments of *ucaA* gene

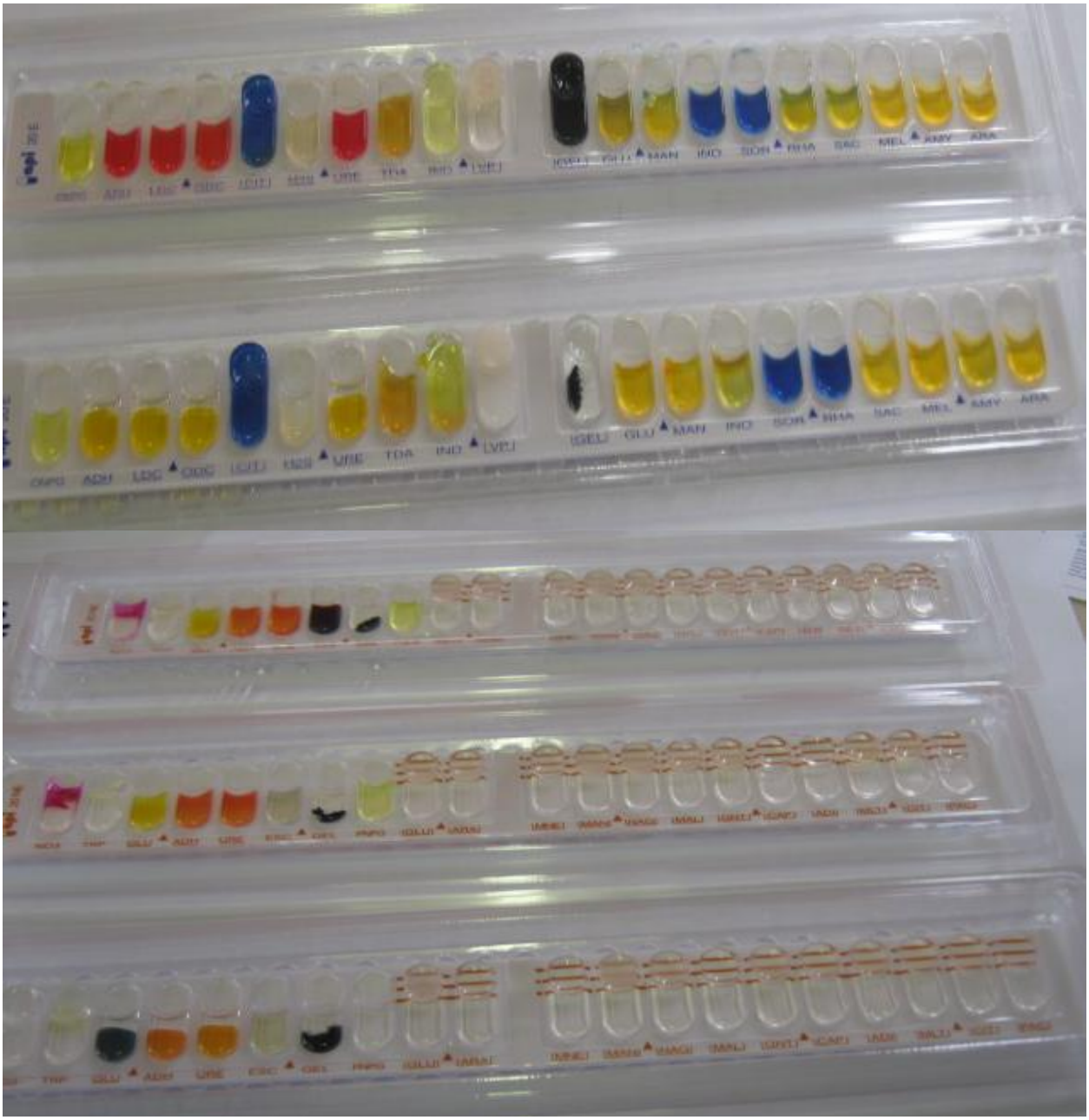


Appendix 2: Plates presenting M.I .C. Evaluator zones of inhibitions

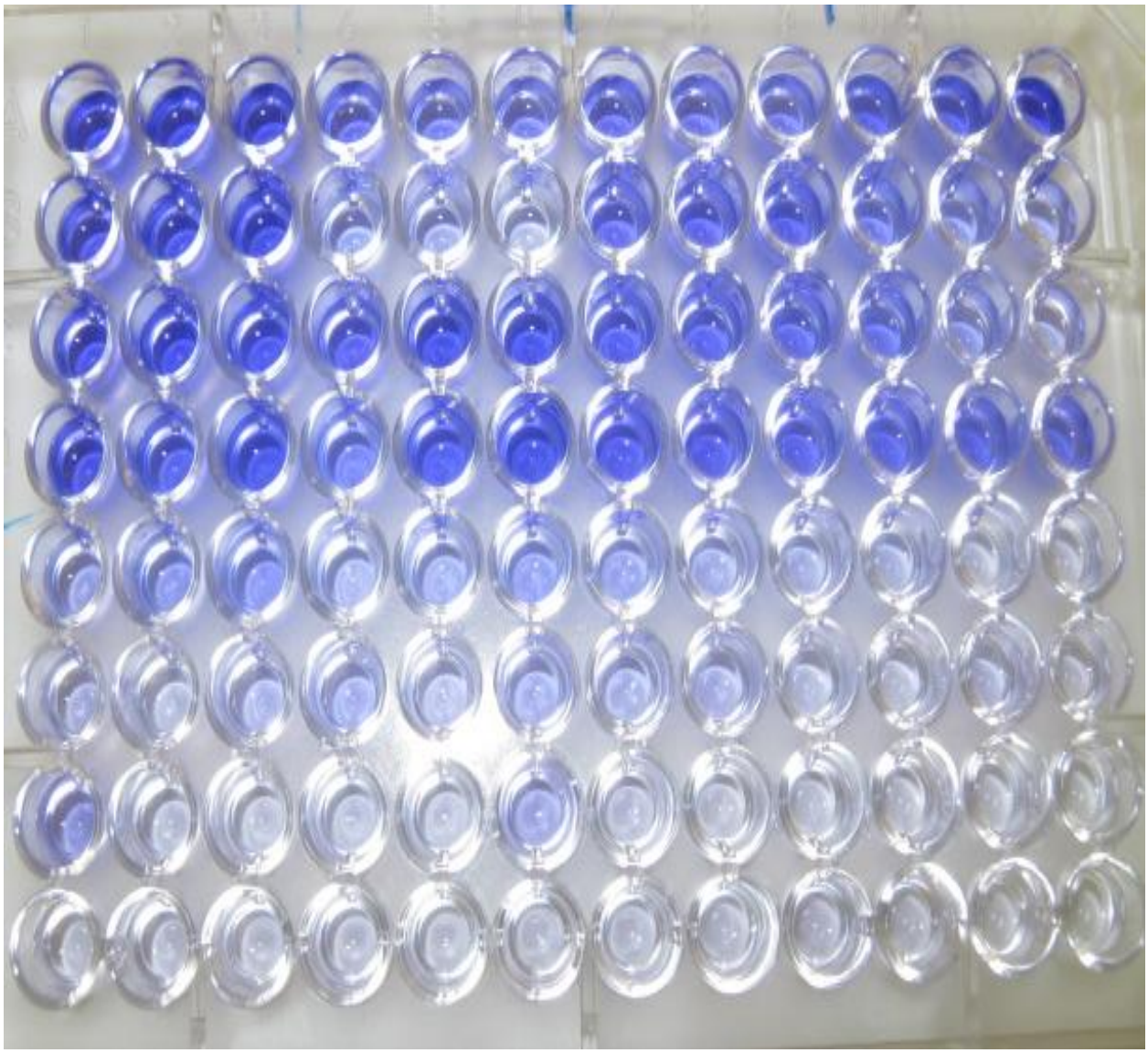


CIP, ciplofloxacin strip; AMP, ampicillin strip; CN, gentamicin strip; VA, vancomycin strip; TE, tetracycline strip. The MIC was read where inhibition of growth intersected the strip and interpretation was done using CLSI breakpoints.

Appendix 3: Portrait depecting an API reaction tray



Appendix 4: A microtiter plate illustrating biofilm formation



Appendix 5: The accession numbers from the GenBank

5.1 The accession numbers of *Enterobacter cloacae's hsp 60* gene sequences deposited with the GenBank

Strain number	Accession number
EC1	KF 768566
EC2	KF 768567
EC4	KF 768568
EC5B	KF 768569
EC9	KF 768570
EC12	KF 768571
EC52	KF 768572
EC73	KF 768573

5.2 The accession numbers of *Enterobacter cloacae ucaA* gene sequences deposited with the GenBank

Strain number	Accession number
EC70	KF 768595
EC53	KF 768596
EC52	KF 768597
EC4	KF 768598

5.3 The accession numbers of *Enterobacter cloacae's* bla_{TEM-1} gene sequences deposited with the GenBank

Strain number	Accession number
EC2	KF 768574
EC7	KF 768575
EC9	KF 768576
EC12	KF 768577
EC52	KF 768578
EC53	KF 768579
EC67	KF 768580
EC70	KF 768581
EC89	KF 768582
EC106	KF 768583
EC112	KF 768584
EC114	KF 768585
EC171	KF 768586
EC172	KF 768587
EC177	KF 768588
EC194	KF 768589
EC198	KF 768590
EC207	KF 768591
EC217	KF 768592
EC225	KF 768593
EC235	KF 768594

5.4 The accession numbers of *Listeria* species bla_{TEM-1} gene sequences deposited with the GenBank

Strain number	Accession number
L03	KF 768599
L08	KF 768600
L13	KF 768601
L17	KF 768602
L18	KF 768603
L20	KF 768604
L32	KF 768605
L37	KF 768606
L38	KF 768607
L39	KF 768608
L41	KF 768609
L44	KF 768610
L55	KF 768611
L63	KF 768612
L73	KF 768613
L74	KF 768614
L80	KF 768615
L85	KF 768616
L93	KF 768617
L99	KF 768618
L100	KF 768619
L103	KF 768620
L111	KF 768621
L119	KF 768622

L123	KF 768623
L155	KF 768624
L188	KF 768625
L189	KF 768626
L194	KF 768627
L195	KF 768628
L196	KF 768629
L198	KF 768630
L199	KF 768631
L205	KF 768632
L208	KF 768633

5.5 The accession numbers of *Listeria* species *iap60* gene sequences deposited with the GenBank

Strain number	Accession number
Liv19	KF 800796
Liv16	KF 800797
Liv18	KF 800798
Liv38	KF 800799
Liv39	KF 800800
Liv155	KF 800801
Liv164	KF 800802
Liv190	KF 800803
Liv195	KF 800804
Liv196	KF 800805
Liv201	KF 800806
Liv43	KF 800807
Liv44	KF 800808
Liv55	KF 800809
Liv58	KF 800810
Liv93	KF 800811
Liv111	KF 800812
Liv119	KF 800813
Liv123	KF 800814
LSL01	KF 800815
LSL100	KF 800816
LSL13	KF 800817
LSL17	KF 800818
LSL20	KF 800819

LSL32	KF 800820
LSL106	KF 800821
LSL85	KF 800822
LSL80	KF 800823
LSL04	KF 800824
LW63	KF 800825
LW74	KF 800826
LW189	KF 800827
LW03	KF 800828
LW103	KF 800829

Appendix 6: Publications

6.1 Published articles from thesis

1. **Nyenje, M. E.,** Odjadjare, C. O., Tanih, N. F., Green, E., Ndip, R. N. (2012). Foodborne pathogens recovered from ready-to-eat foods from roadside cafeterias and retail outlets in Alice, Eastern Cape Province, South Africa: public health implications. *International Journal of Environmental Research and Public Health*, **9**: 2608-2619.

2. **Nyenje, M. E**., Tanih, N. F., Green, E., Ndip, R. N. (2012). Current status on antibiogram of *Listeria ivanovii* and *Enterobacter cloacae* isolated from ready-to-eat foods in Alice, South Africa: a cause for concern. *International Journal of Environmental Research and Public Health.* **9**: 3101 - 3114.

3. **Nyenje, M. E**., Nicoline F. Tanih, N. F., Roland N. Ndip, R. N. (2012). A comparative study of M.I.C. Evaluator test with the broth microdilution method for antimicrobial susceptibility testing of *Enterobacter cloacae* isolated from cooked food samples. *Pakistan Journal of Pharmaceutical Sciences* (in press).

4. **Nyenje, M. E**., Green, E., Ndip, R.N. (2012). Biofilm formation and adherence characteristics of *Listeria ivanovii* strains isolated from ready-to-eat foods in Alice, South Africa. *Scientific World Journal*, 2012:873909. doi: 10.1100/2012/873909.

5. **Nyenje, M. E**., Ndip, R. N. (2013). The challenges of foodborne pathogens and antimicrobial chemotherapy: A global perspective. *African Journal of Microbiology Research*, **7**: 1158 – 1172.

6. **Nyenje, M. E**., Green, E., Ndip, R.N. (2013). Evaluation of different growth media and temperature on the suitability of biofilm formation by *Enterobacter cloacae* strains isolated from food samples in South Africa. *Molecules*, **18:** doi: 9582-9593.

6.2 Manuscript submitted for review

Nyenje, M. E., Ndip, R. N. (2013). An overview of methods applied in the detection and characterization of bacterial foodborne pathogens.

6.3 Manuscript in preparation

1. Molecular characterisation of *Listeria ivanovii* and *Enterobacter cloacae* strains isolated from food samples in Alice, South Africa.

2. The distribution of β-lactamase resistant gene among *Listeria ivanovii* and *Enterobacter cloacae* strains isolated from food samples in Alice, South Africa.

6.4 Below are first pages only of published articles

Int. J. Environ. Res. Public Health **2012**, *9*, 2608-2619; doi:10.3390/ijerph9082608

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Article

Foodborne Pathogens Recovered from Ready-to-Eat Foods from Roadside Cafeterias and Retail Outlets in Alice, Eastern Cape Province, South Africa: Public Health Implications

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Abstract: This study assessed the microbiological quality of various ready-to-eat foods sold in Alice, South Africa. Microbiological analysis was conducted on 252 samples which included vegetables, potatoes, rice, pies, beef and chicken stew. The isolates were identified using biochemical tests and the API 20E, API 20NE and API Listeria kits; results were analyzed using the one-way-ANOVA test. Bacterial growth was present in all the food types tested; high levels of total aerobic count were observed in vegetables, 6.8 ± 0.07 followed by rice, 6.7 ± 1.7 while pies had the lowest count (2.58 ± 0.24). Organisms isolated included: *Listeria* spp. (22%), *Enterobacter* spp. (18%), *Aeromonas hydrophila* (12%), *Klebsiella oxytoca* (8%), *Proteus mirabilis* (6.3%), *Staphylococcus aureus* (3.2%) and *Pseudomonas luteola* (2.4%). Interestingly, *Salmonella* spp. and *Escherichia coli* were not isolated in any of the samples. There was a statistically significant difference ($p < 0.05$) in the prevalence of foodborne pathogens from hygienic and unhygienic cafeterias. The results indicated that most of the ready-to-eat food samples examined in this study did not meet bacteriological quality standards, therefore posing

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Article

Current Status of Antibiograms of *Listeria ivanovii* and *Enterobacter cloacae* Isolated from Ready-To-Eat Foods in Alice, South Africa

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Received: 23 July 2012; in revised form: 16 August 2012 / Accepted: 22 August 2012 / Published: 29 August 2012

Abstract: This study assessed the antimicrobial susceptibility of 51 *Listeria ivanovii* and 33 *Enterobacter cloacae* strains isolated from various ready-to-eat foods sold in Alice, South Africa. Isolates were identified using standard microbiological tests and further confirmed using API 20E and API Listeria kits. The disc diffusion technique was used to screen for antimicrobial susceptibility against 15 antimicrobials; minimum inhibitory concentration of five antibiotics was determined by the broth dilution method. All the strains of *E. cloacae* (100%) and 96% of *L. ivanovii* isolates were resistant to at least four or more of the antibiotics; nineteen antibiotypes were obtained based on the antibiotics used in the study. Antibiotype A5: A^R PG^R VA^R E^R AP^R was predominant in both *L. ivanovii* (23.5%) and *E. cloacae* (57.5%) isolates. Marked susceptibility of *Listeria ivanovii* was observed against chloramphenicol, ciprofloxacin, streptomycin and trimethoprim/sulfamethoxazole (100%) each while *E. cloacae* registered 100% susceptibility to ciprofloxacin only. Various percentages of susceptibility was reported to chloramphenicol and gentamicin (91%) each, nalidixic acid (97%) and streptomycin (94%). The MIC_{90} ranged from 0.004–7.5 µg/mL with *E. cloacae* being the most susceptible organism. The study demonstrated the presence of multi-resistant strains of

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African Journal of Microbiology Research

Review

The challenges of foodborne pathogens and antimicrobial chemotherapy: A global perspective

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Accepted 8 March, 2013

Foodborne diseases remain a major cause of morbidity and mortality in the general population, particularly in vulnerable groups. These diseases emanate from either the toxin of the "disease-causing" microbe, or by the human body's reactions to the microbe. More than 250 different types of viruses, bacteria, parasites, toxins, metals, and prions are associated with foodborne diseases in humans, which are mostly manifested as acute gastroenteritis that could be self-limiting. However, in severe cases, antimicrobial therapy is prerequisite. Antimicrobial resistance is a major challenge in the management of severe foodborne illness; since antimicrobial use in animals selects for resistant foodborne pathogens that may be transmitted to humans as food contaminants. This article presents a comprehensive review on the very important and selected foodborne pathogens and associated illnesses, as well as treatment and control measures.

Key words: Foodborne diseases, viruses, parasites, bacteria, fungi, antimicrobial therapy.

INTRODUCTION

The battle against foodborne diseases is facing new challenges due to the globalization of the food market, climate change and changing patterns of human consumption as fresh and minimally processed foods are currently preferred (Schelin et al., 2011). As food is biological in nature, it is capable of supporting the growth of microorganisms and foodborne diseases result from the ingestion of contaminated foods and food products (Guyader and Atmar, 2008). More than 250 different types of viruses, bacteria, parasites, toxins, metals, and prions are associated with foodborne diseases in humans (Schmidt et al., 2009). Although viruses are more responsible for more than 50% of all foodborne illnesses; generally hospitalizations and deaths associated with foodborne infections are due to bacterial agents. The infections range from mild gastroenteritis to life-threatening neurologic, hepatic, and renal syndromes caused by either toxin from the "disease-causing" microbe, or by the human body's reaction to the microbe itself (Tepitski et al., 2009).

Food poisoning is divided into three types: 'infection', 'intoxication', and 'intermediate' (Schmidt et al., 2009). 'Infection' is caused by the oral ingestion of viable microorganisms in adequate amounts to build up infection and the commencement of symptoms is normally delayed, reflecting the time required for an infection to develop. Examples of food-poisoning that cause infection are enteric viruses, *Salmonella*, *Campylobacter* and *Vibrio* species. 'Intoxication' on the other hand, is caused by the ingestion of toxins that have been pre-formed in the food. Therefore, there is no necessity for live organisms to be present and the onset of the symptoms is rapid. Examples are *Bacillus cereus* and *Staphylococcus aureus*. The 'intermediate' food

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Research Article

Biofilm Formation and Adherence Characteristics of *Listeria ivanovii* Strains Isolated from Ready-to-Eat Foods in Alice, South Africa

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The present study was carried out to investigate the potential of *Listeria ivanovii* isolates to exist as biofilm structures. The ability of *Listeria ivanovii* isolates to adhere to a surface was determined using a microtiter plate adherence assay whereas the role of cell surface properties in biofilm formation was assessed using the coaggregation and autoaggregation assays. Seven reference bacterial strains were used for the coaggregation assay. The degree of coaggregation and autoaggregation was determined. The architecture of the biofilms was examined under SEM. A total of 44 (88%) strains adhered to the wells of the microtiter plate while 6 (12%) did not adhere. The coaggregation index ranged from 12 to 77% while the autoaggregation index varied from 11 to 55%. The partner strains of *S. aureus*, *S. pyogenes*, *P. shigelloides*, and *S. sonnei* displayed coaggregation indices of 75% each, while *S. Typhimurium*, *A. hydrophila*, and *P. aeruginosa* registered coaggregation indices of 67%, 58%, and 50%, respectively. The ability of *L. ivanovii* isolates to form single and multispecies biofilms at 25°C is of great concern to the food industry where these organisms may adhere to kitchen utensils and other environments leading to cross-contamination of food processed in these areas.

1. Introduction

In nature, bacterial cells are most frequently found in close association with surfaces and interfaces, in the form of multicellular aggregates embedded in an extracellular matrix generally referred to as biofilms [1]. Biofilms are usually heterogeneous; in that they contain more than one type of bacterial species, but they can be homogeneous in cases such as infections and medical implants [2]. Microbial biofilms pose a challenge in clinical and industrial setting especially in food processing environments where they act as a potential source of microbial contamination of foods that may lead to spoilage and transmission of foodborne pathogens [3, 4]. They can also compromise the cleanliness of food contact surfaces and environmental surfaces by spreading detached individual microorganisms into the surrounding environment [5].

Environmental conditions in food production areas including the presence of moisture, nutrients, and inocula of microorganisms from the raw materials might favour the formation of biofilm. Furthermore, when food processing equipments are not easily cleaned due to its design and food particles not completely removed, the particles aid in the formation of biofilms by providing a coat that not only provides the biofilm with nutrients but also a surface to which it can easily stick on [6]. Once biofilms have formed on food processing surfaces, they are hard to eliminate often resulting in persistence and endemic population.

Biofilms offer their member cells several benefits, including channeling nutrients to the cells and protecting them against harsh environments. In particular, it has been noted that cells within biofilms are more resistance to antibiotics, disinfectants, and to host immune system clearance than their planktonic counterparts [3, 7]. Several mechanisms

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Article

Evaluation of the Effect of Different Growth Media and Temperature on the Suitability of Biofilm Formation by *Enterobacter cloacae* Strains Isolated from Food Samples in South Africa

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Abstract: This study evaluated the effects of growth medium, temperature, and incubation time on biofilm formation by *Enterobacter cloacae* strains. The ability to adhere to a surface was demonstrated using a microtiter plate adherence assay whereas the role of cell surface properties in biofilm formation was assessed using the coaggregation and autoaggregation assays. The architecture of the biofilms was examined under scanning electron microscope (SEM). All the strains adhered to the well of the microtiter plate when incubated for 48 h, irrespective of the growth medium and incubation temperature. It was also noted that 90% and 73% of strains prepared from nutrient broth and cultured in brain heart infusion (BHI) broth and tryptic soy broth (TSB), respectively, were able to form biofilms, in contrast to 73% and 60% strains from nutrient agar and cultured in BHI and TSB respectively grown under similar conditions. However, no statistically significant difference was observed when the two methods were compared. The coaggregation index ranged from 12% to 74%, with the best coaggregate activity observed when partnered with *Streptococcus pyogenes* (54%–74%). The study indicates the suitability of BHI and TSB medium for the cultivation of *E. cloacae* biofilms, however, temperature and incubation time significantly affect biofilm formation by these bacteria.