DISSERTATION SUBMISSION

Antimicrobial Susceptibility and Resistance of the Bacterial Population in a Seafood Processing Facility

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Declaration

I, Michael Beauzec, declare that the research dissertation that I herewith submit for the master's degree qualification in Microbiology at the University of the Free State is my independent work, and that I have not previously submitted it for qualification at another institution of higher education.

26 November 2024

Student's Signature

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I would like to thank my wife, for support enough to hold a skyscraper and love enough to fill it. I would like to thank my brother, for sharing with me a love of knowledge for the sake of itself. I would like to thank my parents and my sisters and the rest of my family, for being the framework on which I have been built. I would like to thank my supervisor and co-supervisor, for sharing knowledge and guiding me. I would like to thank my employer, for allowing me the opportunity. I would like to thank my wife, again, because once, twice, a million times, will never be enough.

Abstract

Fish and fish products are recommended by international health organisations as a healthy protein option, especially for young children. However, fish is also often associated with foodborne disease outbreaks. The processing of wild caught fish in land-based factories present unique circumstances for bacterial populations to develop and change over time. Bacteria are constantly introduced into the processing environment with the raw material as well as by the staff and factory surroundings. The control of these bacteria can become increasingly difficult due to the development of resistance to disinfectants and the abundance of less effective cleaning agents on the market. Disinfectant resistance and antibiotic resistance have been shown to be linked. The possibility of foodborne pathogens that are also resistant to antibiotics and disinfectants cannot be ruled out. Pathogens that are resistant to cleaning agents may also be resistant to the human immune system which often utilises chemical principles similar to common disinfectants. Resistance genes can also spread across species and even genera. The development of cleaning strategies that can reduce bacterial loads to an acceptable level, whilst also being effective against more resistant bacteria is of crucial importance to the long-term sustainability of the food chain. In the study, a fish processing facility was used to illustrate the ability to isolate the more resistant bacteria in a facility before testing various cleaning agents against these organisms with the concept that most other bacteria would also be susceptible if the most resistant are. The cleaning agents determined to be effective, were used when developing a new cleaning protocol for the site. The cleaning program was implemented at the facility and showed a decreased bacterial load at every point tested. The study shows the application of scientific principles to improve biosecurity measures in an industrial setting, specifically in a seafood processing facility in South Africa.

List of Abbreviations

AMP: Ampicillin

ARG: Antimicrobial Resistance Gene

CHL: Chloramphenicol

DHAP: Dihydroxyacetone phosphate

EFSA: European Food Safety Authority

HACCP: Hazard Analysis and Critical Control Point

KAN: Kanamycin

MDR: Multidrug Resistance

MGO: Methylglyoxal

MIC: Minimum Inhibitory Concentration

NEM: N-ethylmaleimide

PCR: Polymerase Chain Reaction

PDAC: Polydimethyl Ammonium Chloride

QAC: Quaternary Ammonium Compound

RCS: Reactive Chlorine Species

RES: Reactive Electrophile Species

RND: Resistance-Nodulation-Cell Division

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SMR: Small Multidrug Resistance

TAE: Tris-acetate-EDTA

TET: Tetracycline

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

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Introduction to Study

The importance of food safety and the potential for large-scale harm if not addressed, has become an increasingly relevant topic in South Africa following the world's largest foodborne listeriosis outbreak in 2017/2018 from a single food processing establishment. The identification and tracking the source of an outbreak is not sufficient to ensure a safe food supply. It is essential for food handlers to be actively involved in the control of biological hazards and to prevent the spread of harmful bacteria in food products.

The fish processing industry in South Africa is still in a relatively informal position wherein knowledge of bacterial contaminants and their danger is not a widespread concern. Also of little concern is the development and spread of antibiotic and disinfectant-resistance in these bacterial populations. It is not uncommon for a single cleaning agent, especially hypochlorite-based agents, to be used for years at a time and often at sublethal concentrations. This practice actively selects for resistance.

The aim of the study was to determine the effectiveness of disinfectants in reducing the bacterial population to an acceptable level in a typical marine seafood processing factory. The secondary aim was then to select the most disinfectant-resistant of the bacteria collected, and to investigate the presence of resistance in these organisms. These bacteria would be used to test effectiveness of various industrial cleaning agents in a laboratory setting to create a theoretical cleaning plan that would be effective in reducing bacteria and combat disinfectant resistance. Finally, this theoretical cleaning plan would be implemented at a facility and evaluated for effectiveness in reducing bacterial loads in a real-life industrial setting.

Chapter 1 : Literature Review

1.1 Sources of Bacterial Contamination in a Marine Seafood Processing Plant

Bacterial contamination at seafood processors can come from a multitude of sources. The product itself can be contaminated by its environment prior to harvest as they live in complex ecosystems in the ocean. Contamination can also be introduced during harvesting, initial processing and storage on the catching vessel; during transfer of the fish from vessel to shore, and during processing at a land-based factory (Wekell et al., 1994).

The bacterial content of the ocean cannot be controlled without severely disrupting the ecosystem. Improper waste disposal and spillage from land-based operations and seafaring vessels, can cause potentially harmful bacteria to proliferate in fish stocks destined for the plates of consumers. Unhygienic conditions on fishing vessels and extended processing times lead to cross contamination between fish, working surfaces, utensils and food handlers. Seawater is also generally used to rinse fish during onboard processing, becoming an additional potential contamination point. Similarly, improper handling and hygiene practices at land-based factories can lead to cross contamination and proliferation of bacterial pathogens to a level that can be deleterious to the health of consumers.

Common bacterial pathogens associated with marine environments and fishery products include *Vibrio* species, *Listeria monocytogenes*, and *Clostridium botulinum* (Wekell et al., 1994). Estuarine environments are known natural harbours of pathogenic organisms such as members of *Vibrionaceae* but are also often contaminated by domestic and industrial waste as well as runoff from agricultural processes commonly found along rivers. This leads to the accumulation of pathogens more commonly associated with human and animal faeces or waste including *Escherichia coli*, *Salmonella* spp., *Shigella*, *Campylobacter* and *Staphylococcus aureus* (Kvenberg, 1991). These bacteria can also be introduced by food handlers throughout processing and thereafter spread rapidly.

The processing of fish is, by its nature, one where a large volume of water is present, and a constantly wet environment is practically unavoidable. This can lead to the formation of biofilms, and subsequently, water passing through water distribution systems or over other surfaces on which biofilms have formed can act as a vector by which bacteria, including resistant bacteria, can contaminate otherwise hygienic food and food contact surfaces (Shikongo-Nambabi et al., 2010).

1.2 Disinfectants as Bacterial Control Method in Fisheries

To combat this presence of potential pathogens, all seafood processors in South Africa are bound by legislation to implement food safety and quality assurance systems based on Hazard Analysis and Critical Control Point (HACCP) principles. Critical to these HACCP systems is the implementation of systems to reduce, kill, or inhibit the growth of microorganisms. The baseline load of microorganisms in a processing area is controlled by effective cleaning and sanitising methods. Procedures for personal hygiene and the use of clean equipment and water decrease the risk of introducing process-related pathogens. Processing methods such as vacuum sealing and freezing reduces the growth rate of microorganisms in the final product. Since bacteria are constantly introduced into the processing area with the raw materials, the cleaning and disinfecting process is of high importance.

Cleaning in a food processing area usually involves the use of a detergent and physical methods to remove visible dirt made up of lipids, proteins, carbohydrates and some minerals, before the application of a sanitiser in an attempt to remove some residual microorganisms. Detergents are often substituted for detergent-sanitisers, which include a sanitising agent which has the benefit of reducing operation steps. Consequently, chlorine-treated water is used in a rinse step, a detergent-sanitiser is applied and rinsed off, then another sanitiser, and once again a rinse step with treated water. This results in a scenario where the potential exists for multiple exposure to a similar active ingredient (hypochlorites are commonly utilised for the treatment of water and often in high concentrations) with the same method of action (Ray & Bhunia, 2014).

The decision on which chemical agents to use in a food processing facility is usually influenced by multiple factors such as anti-microbial effectiveness, cost effectiveness, ease of use, toxicity, corrosiveness and effect on the quality of food (Ray & Bhunia, 2014). Decision-makers and chemical distributors may not be familiar with concepts of microbial resistance or take into consideration which organisms are more present in the environment in question. Some commonly used chemical agents utilised in fisheries include chlorine-based compounds, iodophors, quaternary ammonium compounds (QACs), and occasionally other oxidising agents such as hydrogen peroxide (H_2O_2) and peracetic acid (CH_3COOOH) (Ray & Bhunia, 2014).

Chlorine-based compounds are often implemented in the cleaning of both water supply and surfaces/equipment of food processing facilities due to their broad-spectrum activity and inexpensive pricing (Ray & Bhunia, 2014). Chlorine compounds used in food processing areas include hypochlorites, chlorine dioxide, liquid chlorine, and inorganic or organic chloramines. Chlorine compounds act by oxidation of the -SH group present in many structural proteins and enzymes (Fukuzaki, 2006). Oxidative disruption of the membrane, nucleic acids, protein synthesis and metabolic activities are possible methods of action suggested (Fukuzaki, 2006; Ray & Bhunia, 2014). The antimicrobial activity of hypochlorites and liquid chlorine comes from the presence of hypochlorous acid (HOCl) when diluted. HOCl can readily pass through the microbial cell membrane by means of passive diffusion since it has a relatively small molecular size and electrical neutrality. Therefore, it attacks the membrane proteins from inside, as well as outside the cell (Fukuzaki, 2006). Chlorine compounds are trusted since they have been used for long with proven success, they have low toxicity to humans and are relatively easy to use. They do have significant drawbacks in that they are corrosive, unstable in the presence of organic matter and at high temperatures and their effectiveness is heavily influenced by pH (Ray and Bhunia, 2014). They can be harmful to food, especially to fish that are very sensitive to lipid oxidation (Bonnell, 1994; Ray & Bhunia, 2014).

Peroxygens, like hydrogen peroxide and peracetic acid, produce highly reactive hydroxyl radicals (Ray & Bhunia, 2014). Due to their highly reactive nature, they are much less easy to use, more corrosive, and they are often more expensive than chlorine compounds. Their mode of action is similar to chlorine compounds in that they cause oxidative damage to cellular membrane proteins, membrane lipids and other essential cellular components (Ray and Bhunia, 2014). An additional benefit to the use of H_2O_2 and CH_3COOH is the decomposition products, namely oxygen and water, being relatively inert and environmentally friendly (Ray and Bhunia, 2014).

Iodophores are less commonly used due to their higher cost, reduced efficacy against spores and viruses compared to hypochlorites, and propensity to affect product flavour. Iodophors are a combination of iodine and surface-active compounds like alkylphenoxypolyglycol, which makes them more water-soluble (Ray and Bhunia, 2014). The antimicrobial activity of iodophors comes from oxidation of the -SH group of many important proteins and enzymes by hypoiodous acid (HIO) and elemental Iodine (I_2) (Ray and Bhunia, 2014).

Quaternary ammonium compounds (QACs) are synthesised by the reaction of tertiary amines with benzyl chloride or alkyl halides (Ray & Bhunia, 2014). They are generally used as sanitisers but can also be utilised as detergent-sanitisers. QACs are bacteriostatic in low concentrations such as when a film residue is left on surfaces (Ray and Bhunia, 2014). These agents are most effective against Gram-positive bacteria and less effective against most Gram-negatives, spores, fungi, and viruses (Ray and Bhunia, 2014). Bactericidal action is caused by a disruption to the cellular membrane, increasing membrane permeability and causing leaking of the cytoplasm out of the cell (Bragg *et al.*, 2014). At low concentrations, they disrupt the interaction between lipids and proteins in membrane structures, and at high concentrations, they cause the cytoplasm to coagulate, likely due to denaturation of proteins (Ray and Bhunia, 2014; Al-Adham *et al.*, 2013). Organic matter does not impact their effectiveness as much as with other active ingredients (Ray and Bhunia, 2014). QACs are stable, noncorrosive, nontoxic, nonirritating, and show the aforementioned bacteriostatic effect (Ray and

Bhunia, 2014). This bacteriostatic film must be rinsed off prior to equipment use, because it can cause chemical contamination of the product if not done correctly (Ray and Bhunia, 2014). Other significant disadvantages include high prices, incompatibility with anionic detergents, and, importantly, a reduced effectiveness against Gram-negative bacteria (Al-Adham *et al.*, 2013; Ray and Bhunia, 2014).

1.3 Importance of Fish and Fish Products as Safe Food

From 2011 to 2014, fish was the most common food category implicated in foodborne disease outbreaks in the United States of America (Barrett *et al.*, 2017). Despite this, health advisory bodies including the American Heart Association and the European Food Safety Authority (EFSA) advise regular consumption of fish products (American Health Association, 2021; EFSA, 2015). Although the most common outbreaks were due to scombrotoxin, most hospitalizations were caused by *Salmonella* (Barrett *et al.*, 2017). *Salmonella* contamination of seafood products is not considered a product-related hazard, but rather a process-related hazard introduced during processing and preparation.

The global food industry is experiencing an increased demand for minimally processed products with less preservatives (Moodie *et al.*, 2013). This can lead to an increased demand in fish products as a source of proteins and fats with very little preservatives or additives. With the high occurrence of food disease outbreaks caused by fish, the importance of control of the presence and growth of microorganisms during harvesting and processing can therefore not be understated (Barrett *et al.*, 2017).

Adding to the difficulty of ensuring a safe final product is the ongoing and increasing crisis of antibiotic and disinfectant resistance. In addition, hypochlorite-resistant *Salmonella* or *E. coli,* for example, would also likely be less sensitive to the action of neutrophils of the human immune system, which produces HOCl as an important oxidant (Kettle & Winterbourn, 1997). Hypochlorous acid is also increasingly used for topical wound care (Gold *et al.*, 2020). Although many alternatives to antibiotics have been identified, the most viable currently is better use of disinfectants and improved biosecurity (Bragg *et* *al.*, 2014). Disinfectants are abundant and often overused or misused throughout food processing, agricultural, health care, pharmaceutical, and cosmetics industries in an attempt to comply with health standards and prevent unwanted bacterial growth (Roca *et al.*, 2015). Resistant bacteria will emerge regardless of the presence of antibacterials, but the presence of these agents imposes selective pressure that will promote the proliferation of resistant cells (Madigan *et al.*, 2019). An increase in resistant bacteria due to a lack of good biosecurity can lead to a major increase in food-borne disease outbreaks (Bragg *et al.*, 2014).

1.4 Mechanisms of Disinfectant Resistance

Resistance to disinfectants by microorganisms is facilitated by intrinsic resistance and acquired resistance (Poole, 2002). Intrinsic resistance refers to factors that are inherent to the bacteria. This includes the outer membrane of Gram-negative bacteria or the outer cell wall of *Mycobacterium* species. The formation of biofilms is also an intrinsic resistance mechanism as it decreases the sensitivity of certain microorganisms to disinfectants (Maillard, 2013). Acquired resistance occurs when the bacteria gain a resistance mechanism, either by mutation (often due to exposure to antimicrobials), or by expression of resistance genes acquired from other resistant bacteria via horizontal gene transfer (Poole, 2002).

According to (Davison, 1999), Bacteria can obtain genetic information by three mechanisms: transformation, conjugation and transduction. Transformation involves the uptake of free DNA from the environment into the cell and can be induced by many chemical or physical processes such as chemical treatment, temperature alteration or electrical stimulation. Environmental DNA can be stabilised in soils, making DNA more available to bacteria in these environments compared to aquatic environments where the available DNA would be diluted and more readily accessible to DNase. In biofilms, the rupture of only a few cells would liberate large amounts of DNA which could then be readily taken up by other cells within the biofilm. Transduction is the process wherein bacteriophages incorporate bacterial genes and transfer this genetic information to another bacterium. Both chromosomal and plasmid DNA can be transferred by this transduction . Most bacteriophages have a limited host range, and bacteria can become resistant to phage adsorption, but transduction is still viable since phages are very common and the added protection of the protein coat ensures a more stable transport form than free DNA in the environment (Davison, 1999). The apparent most common form of horizontal gene transfer between bacteria is via the process of conjugation. Many different forms of conjugation have been identified. A self-transmissible conjugative plasmid may be transferred from one bacterium to another. A non-self-transmissible plasmid containing an origin of conjugal transfer can be transferred by the action of a different conjugative plasmid in a process called mobilization. In co-integration two separate circular plasmids fuse to become a single plasmid. Consequently, a non-self-transmissible, nonmobilisable plasmid can be transferred due to its co-integrated partner being self-transmissible. The fusion of plasmids is facilitated by the presence of transposons or insertion elements. In a specialised form of co-integration, chromosomal gene transfer can also occur (Davison, 1999).

Plasmids encoding for resistance to disinfectants in *Enterococcus* species and *Staphylococcus* species have been shown to be transferrable by means of conjugation (Braga *et al.*, 2011; Wassenaar *et al.*, 2015). Less is known about the transferability of disinfectant resistance genes via transformation or transduction, but disinfectant resistance genes are transferable via the same general mechanisms of horizontal gene transfer that all genes are (Ochman *et al.*, 2000). It has been shown that a link exists between antibiotic resistance gene transfer and disinfectant resistance gene transfer (Kim *et al.*, 2018). A bacterial population's decreased sensitivity to a disinfectant is primarily achieved by the acquisition of disinfectant resistance genes (Ochman *et al.*, 2000).

1.5 QAC Resistance Transferability

QAC resistance has been shown to be acquirable by *Staphylococcus aureus* with QAC resistance genes, or *qac* genes, generally being plasmid-borne and responsible for encoding efflux proteins that expel hydrophobic compounds from the cell (Hegstad *et al.*, 2010). Some identified QAC resistance genes in

Staphylococcus spp. are *qacA,B, G, H, J* and *smr* (Wassenaar *et al.*, 2015). The genes *qacA* and *qacB* are found on plasmids exceeding three kilobases in length and encode transmembrane efflux proteins forming part of the major facilitator superfamily (Heir *et al.*, 1998; Wassenaar *et al.*, 2015). The genes *qacG, H,* and *J,* as well as *smr,* encode proteins that form part of the small multidrug resistance family and are generally found on smaller plasmids (Heir *et al.*, 1998; Wassenaar *et al.*, 2015). These plasmid-borne *qac* genes encoding efflux pumps have been detected in *Staphylococcus* species isolated from various settings including hospitals and other healthcare settings, livestock, humans, pets, and the food industry (Hegstad *et al.*, 2010; Heir *et al.*, 1995 a, b; Singh Sidhu *et al.*, 2002; Sidhu *et al.*, 2007; Correa *et al.*, 2008 a, b; Bjorland *et al.*, 2005). Efflux pumps are membrane embedded proteins that expel a substrate from the interior of the cell, actively lowering the concentration of the substrate such as a disinfectant, toxin, or dye, inside the cell (Buffet-Bataillon *et al.*, 2012).

1.6 Bacterial Response to Hypochlorite

Oxidising compounds, such as sodium hypochlorite (NaOCl) or hydrogen peroxide-based disinfectants, are often preferred over QACs due to their lower price and ease of use. They have also been shown to be more bactericidal against *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms compared to QACs (Lineback *et al.*, 2018). Chlorine exposure has been linked to an increase in antibacterial resistance in water treatment plants with post-treatment bacterial populations having higher concentrations of antibiotic resistance genes (ARGs), as well as integrons, insertion sequences and plasmids involved in the horizontal transfer of these genes (Shi *et al.*, 2013). A correlation between antibiotic resistance and chlorine resistance has been observed (Khan *et al.*, 2016). Chlorine-tolerant bacteria have been shown to be more likely to be antibiotic resistant, and antibiotic resistant bacteria have been shown to survive longer than antibiotic-sensitive bacteria when exposed to free chlorine (Khan *et al.*, 2016). In *Pseudomonas* species*,* sodium hypochlorite treatment at 100 mg/L (recommended concentration for treatment is 200 mg/L) has been shown to increase the minimum inhibitory concentration (MIC) against NaOCl itself, ampicillin (AMP), tetracycline (TET), chloramphenicol (CHL), and kanamycin (KAN) (Tong *et al.*, 2021).

Gram-negative bacteria respond to hypochlorite-induced stress by means of chaperone holdases, transcriptional regulators and biofilm formation (Nizer *et al.*, 2020). The damage caused by oxidative stress is a very rapid process due to the very high reactivity of hypochlorous acid (HOCl) with amino acids. The transcriptional regulation response is slow compared to this reaction, which is why chaperone holdases are important in the immediate protection response to HOCl (Goemans and Collet, 2019). Chaperones are proteins that are responsible for the protection of other proteins by binding to these proteins and preventing misfolding and aggregation (Buchner, 2019). Three important ATP-independent chaperones, also called holdases, that are sensitive to HOCl specifically, have been identified in Gram-negative bacteria. These are Hsp33 (heat shock protein), RidA, and CnoX (Goemans and Collet, 2019). Hsp33 is activated in response to the unfolding of proteins by the action of HOCl, but can also, to a lesser extent, be activated by alternative oxidising agents such as H_2O_2, and nitric oxide (NO) or heat stress-induced protein unfolding (Wholey and Jakob, 2012). Upon exposure to oxidising agents, cysteine residues are oxidised to release a zinc ion which causes the formation of two intramolecular disulfide bonds within the molecule. These modifications cause the formation of the dimeric activated form of Hsp33 which binds to unfolded proteins and prevents their aggregation. As oxidative stress reduces, Hsp33 release the protein to be refolded and Hsp33 returns to its reduced form (Goemans and Collet, 2019; Nizer *et al.*, 2020).

RidA is an enamine/imine deaminase involved in the natural formation of branched-chain amino acids but loses this deaminase activity when chlorinated due to HOCl stress on the cell (Goemans and Collet, 2019). Under these conditions RidA becomes an important chaperone. It is activated by the chlorination of positively charged amino acids which increases its hydrophobicity and allows it to bind to proteins (Müller *et al.*, 2014).

CnoX acts as protection against both aggregation and oxidation, being referred to as a chaperodoxin (Goemans *et al.*, 2018). CnoX is comprised of a C-terminal tetracopeptide (TPR) domain responsible for holdase activity, as well as an N-terminal thioredoxin (Trx) domain responsible for protection from oxidation (Goemans *et al.*, 2018). HOCl activates the holdase activity of CnoX by chlorination of amino acid residues in the TPR domain, increasing hydrophobicity and inducing binding to unfolded proteins (Goemans *et al.*, 2018). The chlorination of Cys63 of the Trx domain causes this domain to bind to the proteins by disulphide bonds, protecting the proteins from oxidation (Goemans *et al.*, 2018). CnoX is sensitive to multiple substances, but its holdase activity is only induced by HOCl (Goemans *et al.*, 2018; Nizer *et al.*, 2020).

Holdases allow the initial survival of the cell and provide the needed time for the transcriptional reaction to take place. Transcriptional factors are proteins that bind to DNA promoter sequences to regulate the transcription of several genes by stimulating (upregulation) or repressing (downregulation) gene expression (Browning and Busby, 2004). Transcriptional factors are activated by different molecules and pathways, including oxidising agents such as ROS and RCS (Nizer *et al.*, 2020). Sensor proteins that respond to RCS, often also respond to ROS, but some transcriptional regulators that respond specifically to HOCl have been identified. These include HypT, RclR, and NemR in *E. coli* (Baek *et al.*, 2020).

HypT (hypochlorite-responsive transcription factor), formerly known as YjiE, is specific to HOCl and belongs to the family LysR-type transcriptional regulator (Gebendorfer *et al.*, 2012). It can activate or repress gene expression and regulates genes involved in adherence, efflux pumps, antibiotic resistance, biofilm formation, and the oxidative stress response (Nizer *et al.*, 2020). HypT upregulates genes involved in the biosynthesis of methionine, sulphur and cysteine, and downregulates genes associated with iron acquisition (Gebendorfer *et al.*, 2012). The main targets of HOCl are compounds containing sulphur, including methionine and cysteine, so the upregulation of genes involved in biosynthesis of these compounds would counteract some of the effects of HOCl exposure

(Gebendorfer *et al.*, 2012; Nizer *et al.*, 2020). Although iron is essential in many metabolic pathways, it is also capable of being toxic to cells by producing reactive oxygen species (ROS) through the Fenton reaction, therefore, reducing its acquisition would decrease this stress on the cell (Cornelis *et al.*, 2011; Nizer *et al.*, 2020). HypT is activated by the oxidation of Met123, Met206, and Met230 to methionine sulfoxide (Drazic *et al.*, 2013). HOCl alters the regulatory domain (RD) of the oxidised HypT, causing a counter-rotation of the DNA-binding domain of HypT. HypT forms a DNA loop, which represses the promoter *fhuA* (Jo *et al.*, 2019).

RclR is a conserved transcriptional regulator in Gram-negative bacteria (Parker *et al.*, 2013). RclR responds to stress caused by HOCl and *N*-chlorotaurine, both of which are important oxidants in the human immune response (Nagl *et al.*, 2018; Kettle & Winterbourn, 1997). The transcription of *rclB* is increased by exposure to HOCl and *N*-chlorotaurine, but is not increased by the exposure to ROS, reactive electrophile species (RES), or reactive nitrogen species (RNS) (Parker *et al.*, 2013). RclR is activated by the oxidation of Cys-21 and Cys-89, which, when oxidised, form a disulphide bond that stabilises the molecule (Baek *et al.*, 2020). RclR activation in turn induces the transcription of the genes *rclA, rclB,* and *rclC,* which encode a flavoprotein disulphide reductase, an uncharacterised periplasmic protein, and a transmembrane quinone-binding protein, respectively (Baek *et al.*, 2020). *Escherichia coli* mutants lacking any of the genes *rclA, rclB, rclC,* or *rclR* are more sensitive to HOCl (Parker *et al.*, 2013).

Another transcriptional regulator involved in the HOCl stress response is a repressor called NemR, or, *N*-ethylmaleimide (NEM)-specific transcription regulator (Gray *et al.*, 2013). It is responsible for the regulation of the expression of *N*-ethylmaleimide reductase (NemA) and glycoxylase (GloA) (Feige, 2018). NemR is activated in response to RCS, but also to RES (such as quinones, glycoxals and NEM) and methylglyoxal (MGO) (Lee *et al.*, 2013; Baek *et al.*, 2020). MGO formation is associated with the depletion of phosphate pools which can be caused by the accumulation of dihydroxyacetone phosphate (DHAP), which is an effect associated with HOCl exposure (Gray *et al.*, 2014). Under nonstress conditions NemR is bound to the DNA and represses transcription, but when activated Cys116 is oxidised and NemR dissociates from the DNA inducing expression of the genes *nemA* and *gloA* (Feige, 2018).

Exposure to NaOCl causes an oxidative stress response in bacteria (Jin *et al.*, 2020; Hou *et al.*, 2019). Antioxidant genes encoding peroxiredoxin reductase (*ahpC)*, superoxide dismutase (C4Q27_06410) and glutathione peroxidase (C4Q27_19360 and C4Q27_18270) are upregulated along with C4Q27_05020, C4Q27_22200 and *msrA,* which encode oxidative damage protectant, alkyl hydroperoxide reductase and peptide-methionine(S)-S-oxide reductase (Msr), respectively (Tong *et al.*, 2021). Also upregulated in response to chlorine stress are genes involved in the SOS response to DNA damage such as *recN,* and DNA-binding transcription regulators *soxG* and *soxR* (Tong *et al.*, 2021). Peroxidases are enzymes responsible for the detoxification of hydrogen peroxide and are important in cellular defence against reactive chlorine species (Imlay, 2008). The overexpression of these genes can help to explain the increased resistance to NaOCl by combatting the effects of oxidation on multiple targets inside the cell.

NaOCl exposure also affects the transcription of genes involved in the regulation of cell membrane permeability (Tong *et al.*, 2021). Genes encoding osmotic stress response (C4Q27_11295 and C4Q27_14680) and mechanosensitive channels (C4Q27_04410) were upregulated when *Pseudomonas* species were exposed to HOCl, whereas genes encoding outer membrane lipoproteins, outer membrane porins and outer membrane assembly proteins (C4Q27_01545, C4Q27_23860, and *bam*) were downregulated under identical circumstances (Tong *et al.*, 2021).

The multidrug efflux pump MuxABC-OpmB belongs to the Resistance Nodulation Cell Division (RND) family and mediates multiple antibiotic resistance (Mima *et al.*, 2009). The genes *muxA* and *muxB* form part of the genes encoding this efflux pump and have been shown to be upregulated after exposure to

HOCl (Tong *et al.*, 2021). Other genes associated with efflux pumps were also upregulated in the same experiment.

1.7 Hypochlorite Exposure and Antimicrobial Resistance

The response to hypochlorite in Gram-negative bacteria is multifaceted and has been shown to increase both resistance to HOCl and to facilitate multidrug resistance (Hou *et al.*, 2019). For example, MexEf-OprN mutants of *Pseudomonas* species were more sensitive to HOCl, indicating that this multidrug efflux pump confers resistance to HOCl in addition to antimicrobials (Farrant *et al.*, 2020; Köhler *et al.*, 1997). NaOCl can also increase the rate of ARG (antimicrobial resistance gene) horizontal transfer (Zhang *et al.*, 2017). Oxidation events within the cell, caused by NaOCl, increase membrane permeability and induce the expression of conjugation-related genes. This results in an increase in the conjugative transfer of plasmids and could result in the dissemination of resistance in populations found in environments where NaOCl is regularly present in sub-inhibitory concentrations (Zhang *et al.*, 2017).

1.8 Research Importance

The continued use of hypochlorite-based antimicrobials has been taken for granted. Although much has been studied regarding the bacterial response to ROS, the body of research on the adaptive response to RCS and resistance is limited (Nizer *et al.*, 2020; Gray *et al.*, 2013). Fish and fishery products are often recommended for growing children and as a healthy alternative protein to traditional meat products (American Health Association, 2021; EFSA, 2015). Potential diseases caused by contaminated fishery products may be more harmful to less developed immune systems if the infecting agent has increased antimicrobial and disinfectant resistance (Gray *et al.*, 2013; Nizer *et al.*, 2020). It is therefore important to study the makeup of bacterial populations in marine seafood processing establishments, the sensitivity of these bacteria to the most common cleaning agents used in the industry and the presence of resistance. Additionally, HOCl products are abundant throughout food processing, water treatment and some clinical applications (Zhang *et al.*, 2017; Gold *et al.*, 2020; Jin *et al.*, 2020). The consequences of sub-optimal use would be of importance to all these applications.

Chapter 2 : Collection, Selection, Identification of Bacteria and their Susceptibility to Disinfectants

2.1 Introduction

Seafood processors face the challenge of an environment to which bacteria are constantly introduced due to the natural presence of bacteria on and in the raw materials. This is especially true for wild-caught fish processors as the ocean's bacterial content cannot be controlled (Wekell *et al.*, 1994). Estuarine and other coastal environments are often contaminated with waste runoff from human infrastructure which spreads common pathogens associated with human and animal faeces such as *Escherichia coli* and *Salmonella* spp. (Kvenberg, 1991). These bacteria associated with bad human hygiene can also be introduced into the environment by the staff working in the facility and improper handling practices (Wekell *et al.*, 1994). The processing of fish uses large volumes of water which can be itself contaminated, especially when treated seawater is used in processing. The wet conditions can create an environment in which biofilms readily develop (Shikongo-Nambabi *et al.*, 2010).

Regular cleaning, using disinfectants, is used to control bacterial growth in seafood processing facilities. The cleaning process generally involves rinsing with water, using detergents and physical scrubbing to remove most visible dirt, and finally applying a sanitiser to remove residual microorganisms (Ray and Bhunia, 2014).

The bacterial populations of these facilities in South Africa are not well studied. Previous studies in neighbouring Namibia, which shares a coastline and many fishing and fish processing practices with South Africa, have provided valuable information regarding the bacterial loads and potential control using H_2O_2 and ozone (Shikongo-Nambabi *et al.*, 2011, 2010). The studies focussed on environments where treated seawater was used for all processing and cleaning needs instead of a more regulated and consistent water supply such as potable water provided by municipal supply.

For industrial cleaning applications, it is recommended that a variety of cleaning agents with different active ingredients be used, preferably in a rotational manner. This is done to assist in the prevention of resistance and to target different bacterial defence mechanisms (Ray and Bhunia, 2014). Bacteria can, however, have resistance to multiple agents simultaneously. It is important to study the sensitivity of bacteria that have shown signs of resistance to disinfectants in order to develop cleaning plans that are effective in reducing bacterial loads in the food processing setting. Bacteria that were isolated by means of selection, could be used as an example of bacteria isolated from a fish processing environment that also show a lower sensitivity to certain disinfectants. Agents that are effective in eliminating these bacteria at or below recommended usage levels could be considered to form part of a new cleaning plan. Cleaning agents that do not reduce these bacteria effectively could reasonably be disregarded. It is not beneficial to the processor to use agents that are not effective in reducing resistant bacteria, even if other bacteria are reduced, since resistance is transferrable, and resistant populations will likely only increase (Mc Carlie *et al.*, 2020).

Before implementation into an industrial environment, which can be costly and time consuming, it is crucial to have a strong theoretical, scientific base from which to work. The aim of this chapter was to select for the most disinfectant-resistant bacteria collected from an industrial seafood facility, identify these, and determine the susceptibility of these bacteria to industrial disinfectants in a controlled, laboratory setting.

2.2 Materials and Methodology

Sample collection

Sampling was done at a seafood processing facility located on the quayside of the Port Elizabeth Harbour in Gqeberha, South Africa (33°57'58.9"S 25°37'59.9"E). Tryptic soy agar (TSA) contact plates were used. Each plate was placed on the surface being sampled, immediately lifted and the lid replaced and incubated at room temperature for 48 hours. Sampling was done on a Saturday morning after a week (Monday to Friday) of production with regular cleaning protocols followed. A total of twenty plates were used to create a representative sample of the majority of the facility. More samples were allocated to food-contact areas than non-food-contact areas.

Three plates representing different locations of the facility were selected for further isolations, including a pooled minimum inhibitory concentration procedure, which would select for more resistant bacteria in the population. The plates selected are indicated in Table 2.1.

Table 2.1: Seafood processing facility bacterial sample origins

Sample Name	**Sampling Area**
PLATE A	Product Conveyor Belt Production Line 3
PLATE B	Product Conveyor Belt Production Line 2
PLATE C	Raw Material Storage Area Wall

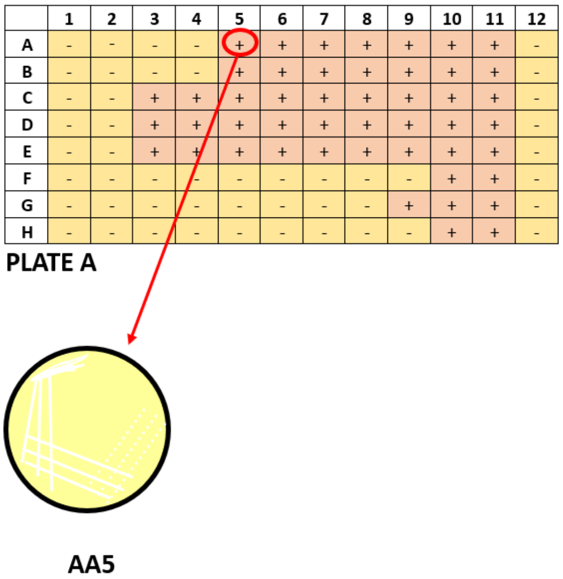
Selection by antimicrobial pressure

All colonies on each of the three plates were collected using a sterile swab and were used to inoculate a 10 ml tube of trypticase soy broth medium for use in a broth microdilution MIC screening.

Dilutions of different antimicrobials were prepared using sterile water. The prepared solutions had 1% concentrations of cleaning agents. Active ingredients for the cleaning agents were: Agent 1 – 120 g/L polydimethyl ammonium chloride (PDAC), Agent 2 – 70 g/L gluteraldehyde and 60 g/L generic QAC compounds, Agent 3 – organic chlorine dissolved in water to a concentration of 1000 ppm available chlorine.

A volume of 100 µL sterile water was added to columns 2-12, rows A-H of a 96-well microtiter plate. Thereafter, 200 µL of the antibacterial dilutions was added to column 1. Agent 1 was added to rows A and B, Agent 2 was added to rows C-E, and Agent 3 was added to rows F-H. A serial dilution was made by transferring 100 µL from column 1 to 2 and mixed, diluting the agent by half. This was continued sequentially up to column 10. Columns 11 and 12 were used for the positive and negative controls, respectively. Thereafter, 10 µL of the suspended bacterial culture (Plate A) was added to each well of columns 1-11 and left for 20 minutes contact time. A separate microtiter plate was prepared with 90 µL Tryptic Soy Broth (TSB) growth media in all wells. After the 20 minutes contact time, 10 µL was transferred between corresponding wells from the disinfectant/water plate to the media plate. The plate was covered in parafilm and incubated at 28°C overnight. The procedure was repeated for cultures B and C. After overnight incubation, the plates were analysed for growth.

After incubation, the MICs were determined as the lowest concentration at which no growth was seen. The highest concentration wells where growth was still observed, i.e., adjacent to the MIC-determining well, were plated onto TSA plates to obtain pure cultures. These new cultures were labelled using the corresponding sample name and the well from which it was isolated. Effectively selecting for more resistant bacteria within the populations isolated from the processing plant.



	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	+	+	+	+	+	+	+	-
B	-	-	-	-	+	+	+	+	+	+	+	-
C	-	-	+	+	+	+	+	+	+	+	+	-
D	-	-	+	+	+	+	+	+	+	+	+	-
E	-	-	+	+	+	+	+	+	+	+	+	-
F	-	-	-	-	-	-	-	-	-	+	+	-
G	-	-	-	-	-	-	-	-	+	+	+	-
H	-	-	-	-	-	-	-	-	-	+	+	-

Figure 2.1: Selection of most resistant bacterial strains from pooled minimum inhibitory concentration

A total of six new isolates were obtained: AA5, AB5, AC3, AD3, CF10, and CG10. For ease of reference, renamed to EF1-EF6 (Table 2.2).

Table 2.2: Reference codes used for bacterial strains selected by pooled minimum inhibitory concentration

Isolate	**New Reference**
AA5	EF1
AB5	EF2
AC3	EF3
AD3	EF4
CF10	EF5
CG10	EF6

Identification

Cultures were screened with a simple Gram-stain and all six were determined to be Gram-negative rods. Preliminary identification was then done using a bioMérieux API® 20 E V5.0 identification strip for each sample. Each strip/incubation box (consisting of a tray and a lid) was prepared by adding distilled water to the honeycomb wells of the tray to establish a humid environment. The inoculum was prepared by inoculating 5 ml of sterile water with a single well-isolated colony. A total of 20 tests are included in the strip (Annex A).

The tube and capule of the CIT, VP, and GEL tests were filled with inoculum. For all other tests, only the tube was filled. An anaerobic environment was created by filling the ADH, LDC, ODC, H2S, and URE capules with mineral oil. Strips were incubated at 36°C overnight. All results were recorded on a results sheet, with the TDA, VP, and IND tests requiring “reveal” steps where reagents are added. For the TDA test a drop of TDA reagent was added and the colour was immediately recorded. For the VP test 1 drop each of VP 1 and VP 2 reagents were added and reading was made after 10 minutes. For the IND test, 1 drop of JAMES reagent was added and the colour was immediately recorded. Positive and negative results were recorded on a provided results sheet where tests were scored 1, 2, or 4 and grouped into groups of 3. The numbers in each group of three were added together to create a 7-digit profile number. The 7-digit profile number was entered into the APIWEB™ online system which provided identification from the API Database.

Sensitivity to Disinfectants

Isolates EF2 and EF3, isolated by means of selective pressure (see Chapter 2: Selection by antimicrobial pressure) were selected as two testing samples as all of the isolated bacteria appeared to be similar, based on the API identification. These two strains and the ATCC13880 *Serratia marcescens* type strain, sourced from the University of the Free State's Department of Microbiology and Biochemistry, were used in MIC tests done using six cleaning agents used currently in the food processing industry. The ATCC13880 *Serratia marcescens* type strain was used to compare to the isolated bacteria from the factory. The type strain was expected to show higher sensitivity and less resistance due to less environmental exposure to conditions conducive to resistance development. The cleaning agents were selected based on different active ingredients, recommended concentrations and potential historical exposure in the environment where the strains were isolated. The chemicals Multiklene, Resolvit, Alkafoam Cl, and Topax 66 had previously been used in this facility, whereas Aerofoam PA, Argonox, and Virukill had not.

Tryptic Soy Broth (TSB) media was inoculated with a single colony from EF2, EF3 and ATCC13880 *Serratia marcescens* and incubated overnight at 37°C to create broth cultures. These cultures were all standardized to O.D 0.8 at 600nm using sterile water.

Sterile water was used to make 20% dilutions of all the antimicrobials to be tested. Thereafter, 100 µL of sterile water was added to Columns 1-12, rows A-H of a 96-well microtiter plate. After this, 100 µL of the diluted disinfectant was added to column 1 and mixed. Two antimicrobials were tested per microtiter plate. One agent was added to row A-D, and another was added to rows E-H. Serial dilutions were made by transferring 100 µL from column 1 to 2 and mixing, diluting the agent by half. This was continued sequentially up to column 10. Columns 11 and 12 were used for the positive and negative controls, respectively. A 10 µL suspension of the standardized bacterial culture (EF2) was added to each well of columns 1-11 and left for 20 minutes contact time. A separate microtiter plate was prepared with 90 µL sterile TSB growth media in all wells. After the 20 minutes contact time, 10 µL was transferred between corresponding wells from the disinfectant/water plate to the media plate. The plate was covered with parafilm and incubated at 37°C overnight. The procedure was repeated for EF3 and ATCC13880 *Serratia marcescens*. Plates were analysed after overnight incubation.

2.3 Results

Selection

The highest MIC for all three chemicals were determined for Sample A (Table 2.3). A 0.125% dilution (or 15g/L) of polydimethyl ammonium chloride was the MIC for Agent 1 on Sample A. For Agent B the MIC was a mere 0.5% or 35 g/L glutaraldehyde. Agent C, chlorine, had a MIC of 4 ppm against Sample A.

Table 2.3: Minimum inhibitory concentration (MIC) of three cleaning agents against three pooled microbial samples

	Agent 1 (%)	Agent 2 (%)	Agent 3 (%)
Sample A	0.125	0.5	0.005
Sample B	<0.002	0.008	<0.002
Sample C	0.008	0.016	0.005

After MICs were determined, the highest concentration wells where growth was still observed, i.e., adjacent to the MIC-determining well, were plated onto TSA plates to obtain pure cultures. New cultures were renamed as per Table 2.2.

Identification

Cultures selected by pooled MICs were identified using bioMérieux API® 20 E V5.0 identification strips (Figure 2.2). Table 2.3 was used to create a 7-digit profile for each isolate which could be entered into the APIWEB online platform, which could, in turn, provide identification from the API Database (Table 2.4).

Figure 2.2: bioMérieux API® 20 E V5.0 identification strips for samples EF1-EF6 after overnight incubation

Table 2.4: API 20 E results for Samples EF1-EF6

Sample	7 Digit Profile	Taxon	% ID	ID Comment
EF1	5307773	*Serratia odorifera*	69.7	Acceptable identification to the genus
EF2	5306561	*Serratia marcescens*	94.2	Very good identification to the genus
EF3	5307561	*Serratia marcescens*	96.3	Excellent identification to the genus
EF4	5306561	*Serratia marcescens*	94.2	Very good identification to the genus
EF5	5306761	*Serratia marcescens*	95.7	Excellent identification to the genus
EF6	5306761	*Serratia marcescens*	95.7	Excellent identification to the genus

Identification to the genus level was determined for all cultures, with likelihood very high of all six isolates being *Serratia* spp.

Sensitivity to Disinfectants

The MIC was determined for each cleaning agent with each of the three strains tested. Results are summarised in Table 2.5.

Table 2.5: Minimum inhibitory concentration (MIC) of 7 cleaning agents against three (3) bacterial samples. Red text indicates a minimum inhibitory concentration above the maximum recommended use concentration, orange text indicates a minimum inhibitory concentration above the minimum recommended concentration, but below the maximum recommended concentration.

Chemical	Active Ingredient	Recommended Use	EF2 MIC	EF3 MIC	***S. marcescens*** **ATCC 13880^T MIC**
Multiklene	NaOH & 2-butoxyethanol	2%	5%	5%	2,5%
Resolvit	QAC	1-3%	1,25%	0,63%	0,04%
Alkafoam Cl	NaOH & KOH	0,5-3%	0,63%	0,63%	0,16%
Topax 66	NaOH & NaOCl	2-5%	0,31%	0,31%	0,08%
Aerofoam PA	H_2O_2 & CH_3CO_3H	0,25-3%	0,63%	0,31%	0,31%
Argonox	H_3PO_4 & 2-butoxyethanol	2-5%	0,31%	0,31%	0,16%
Virukill	Polydimethyl ammonium chloride	1%	0,125%	0,25%	0,016%

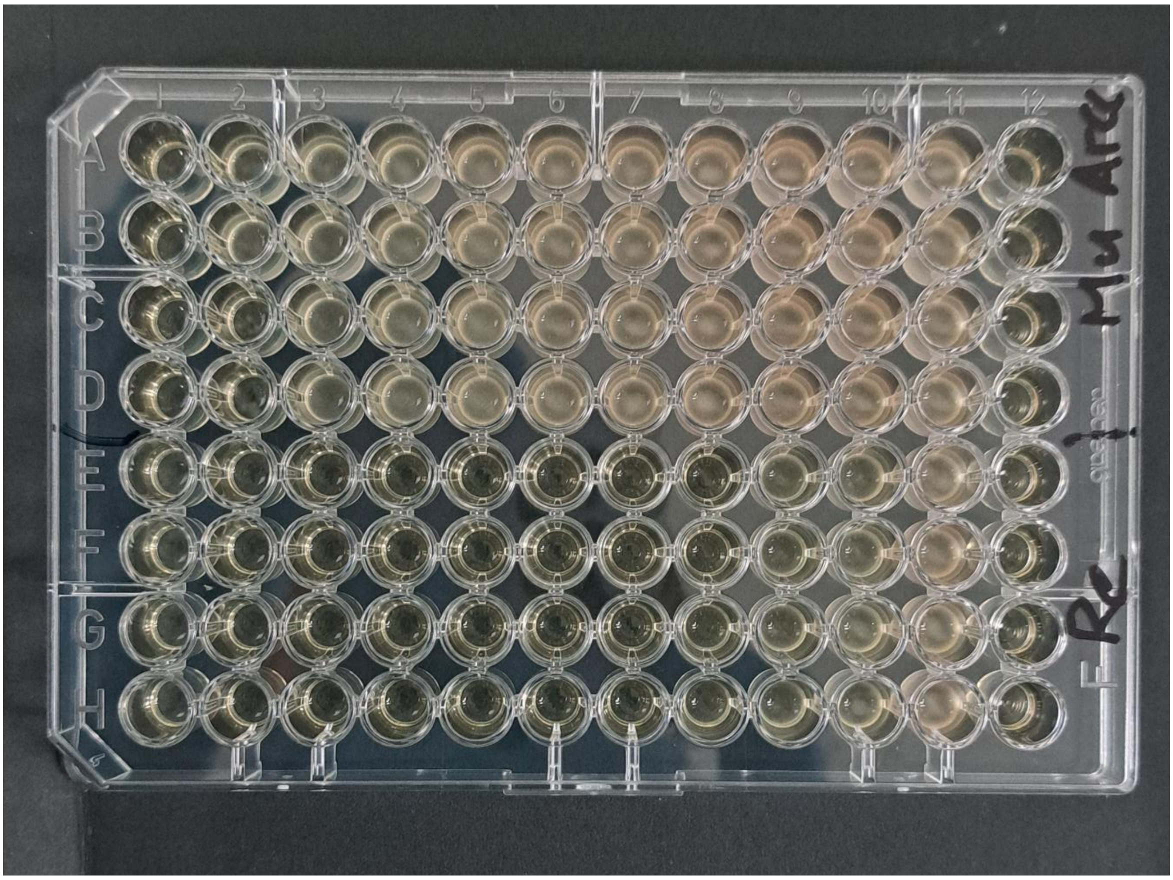


Figure 2.3: Multiwell plate used to determine minimum inhibitory concentration of Multiklene and Resolvit on Serratia marcescens ATCC 13880^T after overnight incubation.

The MIC for Multiklene was above the recommended use concentration of 2% for all three strains tested (Fig 2.3). The MIC for other agents were below the maximum recommended use concentration (Table 2.5). For Resolvit, Alkafoam Cl, and Aerofoam PA, the MIC for EF2 was higher than the minimum recommended use concentration. For Alkafoam Cl and Aerofoam PA the MIC for EF3 was higher than the minimum recommended use concentration.

For all three strains, Virukill had the lowest MIC of the products tested. The type strain showed comparatively higher sensitivity to all products than the selected strains, but more so to agents previously used in the factory (Table 2.5).

2.4 Discussion

All six isolates of the most resistant bacteria were determined to be members of the genus *Serratia*, using the API method. Five of the isolated were determined to be *Serratia marcescens. Serratia marcescens* is considered an opportunistic pathogen and has been isolated from many environments where isolates have harboured antibiotic resistance genes, especially genes associated with efflux pumps (Sandner-Miranda *et al.*, 2018). Antibiotic resistant *S. marcescens* are commonly isolated from hospitals (Bertrand and Dowzicky, 2012). Disinfectant resistant strains of this species have also previously been isolated from food processing environments (Langsrud *et al.*, 2003).

The virulence of *Serratia spp.* strains isolated from food products have been shown to be potentially harmful and even lethal (Singh *et al.*, 1997). *Serratia marcescens* is a well-known cause of opportunistic and nosocomial infections in medical settings, made worse by their proficiency in resistance acquisition (Zivkovic Zaric *et al.*, 2023). In their persistent presence, *S. marcescens,* in an environment and biofilms can be a refuge for disinfectant-resistance and antibiotic-resistance genes. The potential also exists for these genes to be transferred to bacteria which are more clinically harmful through horizontal gene transfer (Mc Carlie *et al.*, 2020).

Bacterial loads of fish products are an important factor used when determining their safety for consumption. As with most foods, fish products have legislated allowable limits of bacteria. An increase in resistant bacteria, or a continued decrease in effectiveness of disinfectants can cause products to be rejected for sale and decrease the availability of food. Alternatively, only some of the most resistant bacteria may survive and proliferate, leaving a relatively smaller population that may be much more harmful.

The two strains isolated from the factory showed decreased sensitivity to disinfectants that had been previously used in the environment they had been isolated from, pointing towards environmental selection and acquisition of resistance to these agents (Langsrud *et al.*, 2003). Multiklene, which is the most used agent in this facility, did not show sufficient effectiveness against the isolated strains, with the MIC for EF2 and EF3 being 2.5 times the recommended usage concentration of this cleaning agent. Even the laboratory type strain, which would have a lower intrinsic resistance as well as less chance to acquire resistance, showed an MIC which was greater than the recommended use concentration. The disinfectant, in this case, does not appear to be effective.

Resolvit, a QAC-based sanitiser, showed very good effectiveness against the type strain, but was not effective at its minimum recommended use concentration against one of the isolated strains. In contrast, Virukill, a polydimethylammoniumchloride sanitiser, showed the best effectiveness against all three strains tested.

Argonox, an acidic H_3PO_4 & 2-butoxyethanol disinfectant-detergent, which had not been used in the facility showed better effectiveness in reducing the strains compared to Alkafoam Cl, an alkaline NaOH & KOH disinfectant-detergent, which had been used in the facility (also other agents with the same active ingredients). The disinfectants had identical MICs with regard to the type strain. The isolated strains were, therefore, less sensitive to cleaning agents common in the environment they were isolated from.

The strains had potentially been selected for by environmental pressure by having a greater intrinsic resistance to the active ingredients or had acquired resistance in the environment, both of which would explain the increased sensitivity compared to the type strain (Zhang *et al.*, 2017).

The effectiveness of Virukill at a very low concentration is of value due to the nature of cleaning in the industry. Generally, cleaning work is done by less-educated individuals who are often oblivious to the importance of correct dilution rates and effective concentrations. Even educated staff can make errors in dilution or disregard the importance under certain conditions. The repeated exposure of a bacterial population to low, ineffective concentrations of disinfectant exerts a selective pressure on the population which results in resistant cells becoming more prominent in the population (Zhang *et al.*, 2017). Disinfectants which are able to work effectively at lower concentrations are practically and economically valuable in that they decrease the opportunity for suboptimal dilution.

Importantly, various disinfectants showed the ability to inhibit the growth of more resistant bacteria compared to the chemicals that had been in use at the time. Additionally, multiple different active ingredients had MICs below their recommended dilution, indicating that a more diverse cleaning program would likely be effective and could be implemented.

Chapter 3 : Antibiotic and Disinfectant Resistance Gene Screening

3.1 Introduction

Multidrug resistant (MDR) bacteria and disinfectant resistant bacteria have increased dramatically and could lead to significant harm to the medical industry, but also to the food supply chain. The continued use and misuse of cleaning agents causes an increase of resistant bacteria which could easily be spread through the food chain. With the modern food chain being a global enterprise, bacteria are able to travel continents in food products. Not only is there a concern that foodborne pathogens can spread in this manner, but also that resistance genes are able to spread. Resistant bacteria give rise to resistant populations by both vertical and horizontal gene transfer. Studies have shown co-resistance to disinfectants and antibiotics in bacteria (Kim *et al.*, 2018). Induction of disinfectant resistance can also cause antibacterial resistance. A risk therefore exists that food exposed to disinfectant resistant bacteria may cause a foodborne disease or opportunistic infection which is resistant to antibiotic treatment. A common co-resistance mechanism is the increase of efflux pumps. Increased expression of efflux pump activity can be transferred by horizontal gene transfer and is often triggered by an oxidative stress response.

Gene screening is an effective tool to detect the potential for resistance by the presence of genes that are associated with antibiotic or disinfectant resistance. The plasmid-encoded gene *qacB* encodes the efflux pump QacB, which confers resistance to monovalent lipohilic cations, and is primarily found in *Staphylococcus aureus* and show limited sequence variation (Wassenaar *et al.*, 2015). The gene *adeF* is part of the AdeFGH resistance-nodulation-cell division (RND) efflux pump system and encodes a membrane fusion protein. The gene *kpnF* encodes an efflux pump conferring oxidative stress tolerance and antibiotic resistance in *Klebsiella pneumoniae.* TEM-1 is a β-lactamase and a common determinant for antibiotic resistance. It confers resistance to penicillins and early cephalosporins.

3.2 Materials and Methods

Genomic DNA extraction was performed using a modified Labuschagne-Albertyn method (Annexure B) on all six isolates from Chapter 2 (EF1-EF6) (Labuschagne and Albertyn, 2007). These strains underwent polymerase chain reaction (PCR) screening for multiple genes associated with acquired resistance to disinfectants and antibiotics. The primers used to screen for each gene can be found in Table 3.1. PCR was performed using the GeneTechnologies™ G-Storm thermocycler using reaction volumes in Table 3.2 and conditions in Table 3.3.

Table 3.1: Summary of primers used in resistance gene screening

Primer	Melting Temperature (°C)	Annealing Temperature (°C)	Primer Sequence	Product size (bp)
QacG Forward	60.0	≈ 55	GAT CGC CACC ACC ATG TTG A	≈ 300
QacG Reverse	59.1	≈ 54	CAC CGA GGC TTT GGA CAG TA	
AdeF Forward	59.6	≈ 54	GGC TAA GTT CTT TAT AGA CCG CC	≈ 200
Adef Reverse	62.1	≈ 57	CCA AGA CGG TGG CGG TG	
Kpnf Forward	62.6	≈ 57	TGG GAC TGG CGA TCG TGC	≈ 300
Kpnf Reverse	60.7	≈ 55	GAT CAC CAT CCC CGT CAG C	
TEM-1 Forward	62.6	≈ 57	TAT TCA ACA TTT CCG TGT CGC CC	≈ 800
TEM-1 Reverse	61.6	≈ 56	GGC CCC AGT GCT GCA ATG	
qacE Forward	57.4	≈ 53	GCG AGCA AAA AGG CAG CAA T	≈ 300
qacE Reverse	55.2	≈ 50	GCT GGC TTT TTC TTG TTA TCG C	
qacF forward	57.2	≈ 53	TGT AGT TGT GGC TGG CTA CG	≈ 300
qacF Reverse	57.4	≈ 53	GCT AGA AAC CCT CGG CTA CC	
qacED1 Forward	57.1	≈ 53	TTA CTA AGC TTG CCC CTT CCG	≈ 200
qacED1 Reverse	57.9	≈ 53	AGG CAG CAA TTA TGA GCC CC	
mdfA forward	57.2	≈ 53	CTT GCT GAC AGT GCT GGA GA	≈ 800
mdfA Reverse	57.2	≈ 53	GGT CAC CAC CAT CGA ACA GT	

Table 3.2: Addition order and volumes of reactants used in resistance gene screening polymerase chain reaction

Reactant	**Volume (µL)**	**Final Concentration**
Target gene forward primer	1	0.4µL
Target gene reverse primer	1	0.4µL
dNTPs	1	0.4µM
NEB 10x Thermopol™ reaction Buffer	2.5	-
Template DNA	1	10-40ng
Thermo Fisher Scientific AmpliTaq™ Gold DNA Polymerase	0.3	1.5 units
Nuclease free water	18.2	-
Total Volume	25	

Table 3.3: Thermocycler reaction conditions for polymerase chain reaction amplification of resistance genes

Step	**Temperature (°C)**	**Time (s)**	**Number of cycles**
Initial denaturation	95	120	1
Denaturation	95	30	35
Gradient Anneal	50-60	60	
Extension	72	90	
Final extension	72	120	1
Hold	4	Until stopped	-

To identify the presence of genes and verify PCR amplification, agarose gel electrophoresis was used. A 1% w/v agarose gel was made by dissolving 3 g agarose in 297 ml of 1xTris-acetate-EDTA (TAE) buffer, followed by the addition of 1 µL of ethidium bromide. The electrophoresis was run at 90 volts for 34 minutes. The order of loading can be seen in Table 3.4. Visualisation was done using a BioRad™ Gel Doc EZ Imager.

Table 3.4: Order of PCR products added to agarose gel for electrophoresis

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Target Gene	Ladder	Empty	qacG	AdeF	Kpnf	TEM-1	qacE	qacF	qacED1	mdfA	qacG	AdeF	Kpnf	TEM-1	qacE	qacF	qacED1	mdfA
Strain	N/A	N/A	EF2	EF2	EF2	EF2	EF2	EF2	EF2	EF2	EF3	EF3	EF3	EF3	EF3	EF3	EF3	EF3

3.3 Results

Preliminary screening for various genes associated with antibiotic and/or disinfectant resistance revealed amplification had taken place where primers were used to amplify the genes *qacG*, *Kpnf*, and *mdfA* in the strain EF3 (Figure 3.1)

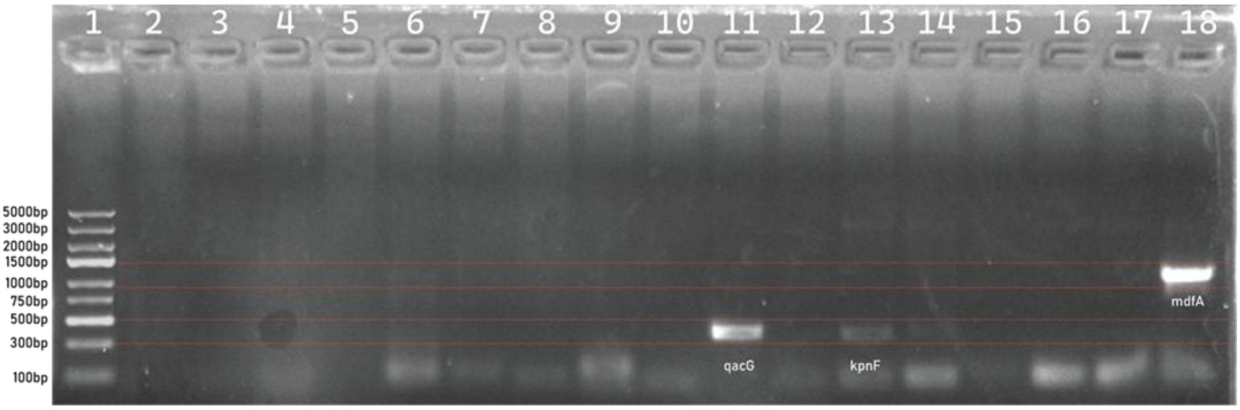


Figure 3.1: Gel electrophoresis of multiple DNA amplification products. Lane 1 contains a DNA Ladder, and lane 2 is left empty. Lanes 3-10 products of strain EF2 and Lanes 11-18 products of strain EF3. Amplification targets were: Lane 11-qacG, Lane 13-kpnF, Lane 18-mdfA.

In the strain EF3, DNA amplification products were shown via electrophoresis in lanes 11 and 13 for the genes *qacG* between 300 and 500 bp. For the same strain, DNA amplification products targeting the *mdfA* gene were seen in lane 18 between 1000 and 1500 bp. A light band was also seen between 300 and 500 bp in lane 13, which contained amplification products for *kpnF*.

3.4 Discussion

The primary aim of the project was to investigate the development of an improved cleaning and disinfection protocol in a factory. The efforts to detect possible resistance genes in the isolated bacteria were an effort to screen for possible resistance genes in the isolated bacteria and is not intended to be a full study of the resistance gene profile of the isolated bacteria.

The EF3 strain was selected for by using a cleaning agent containing glutaraldehyde and QACs (Chapter 2). The *qacG* gene is known to confer resistance to quaternary ammonium compounds, including in staphylococci isolated from the food industry (Heir *et al.*, 1999). The gene is approximately 330 bp in length. The protein encoded by this gene is part of the Small Multidrug Resistance (SMR) family, which are efflux systems that transport QAC compounds, other detergents, and antibiotics (Bay *et al.*, 2008). The proteins QacC, QacG, QacH and QacJ, are members of this SMR family. The genes associated with these proteins have shown variability in their sequences and show similarity to many other *smr* genes (Wassenaar *et al.*, 2015). The *qacG* gene is well known to occur on plasmids in the genus *Staphylococcus* (Wassenaar *et al.*, 2015). The acquisition of genes associated with disinfectant resistance is possible through horizontal gene transfer, which is the primary method of formation of resistant populations (Ochman *et al.*, 2000). The acquisition of resistance genes and their incorporation into the genome of the bacteria is a major part of the formation of multidrug resistant populations (Mc Carlie *et al.*, 2020).

The *kpnF* gene is approximately 330 bp long and encodes an SMR-type efflux pump (Srinivasan and Rajamohan, 2013). It was first characterised in multidrug resistant *Klebsiella pneumoniae,* often associated with nosocomial infections. It has been shown to increase resistance to multiple antibiotics including erythromycin, tetracycline, rifampin, and streptomycin. It is also associated with resistance to QAC disinfectants (Srinivasan and Rajamohan, 2013).

TEM-1 is a β-lactamase which confers resistance to penicillin and cephalosporins. It is a well-known determinant used for antibiotic resistance. They are very common in resistant bacterial strains (Salverda *et al.*, 2010).

The presence of two *smr* genes associated with co-resistance to disinfectants and antibiotics, along with a gene associated with many resistant bacterial strains, may indicate a potentially highly resistant strain, or the accumulation of resistance markers in the strain. The accumulation of resistance genes via horizontal gene transfer is a phenomenon that has been seen in *Serratia marcescens.* The accumulation of drug resistance genes leads to multidrug resistant populations that are harder to control with antibiotics, may be opportunistic pathogens or cause nosocomial infections (Moradigaravand *et al.*, 2016). *Enterobacteriaceae* such as *Serratia marcescens* are intrinsically more sensitive to carbapenems, but the emergence and continued proliferation of carbapenem resistant *Enterobacteriaceae*, especially in hospital settings, have created a looming health crisis (Nordmann *et al.*, 2012; Cristina *et al.*, 2019).

The amplification products from the strain EF3 were not purified from the gel and confirmed so these may be non-target genes. To confirm the presence of resistance genes, the amplification would have to be done along with a positive control, and the resulting amplification products could be excised from the agarose gel. After amplification and sequencing, the amplification gene may be compared for similarity. Many resistance genes such as the *qac* genes are well-conserved in different bacterial species and sequence similarity have been seen for different resistance genes, especially efflux protein encoding genes (Wassenaar *et al.*, 2015).

These results serve as a preliminary screening and highlight the need for more in-depth analysis of resistance genes and also adds another reason why cleaning protocols need to be adapted urgently.

Although emerging technologies such as phage treatment or competitive bacteria show some potential, the only truly viable option, which is also immediately actionable, is improved biosecurity and sanitation practices (Bragg *et al.*, 2018).

Chapter 4 : Cleaning Efficacy After Results-Based Development of a Cleaning Schedule for a Seafood Processing Facility

4.1 Introduction

When cleaning a food processing area, one should not only consider the risks of foodborne diseases but also the survival of disinfectant-resistant bacteria. Disinfectant resistance is a growing problem which cannot be prevented by theoretical means alone (Bragg *et al.*, 2018). It is important to ensure that practices that can prevent bacterial growth and prevent the formation of resistant bacteria are feasible at an industrial scale. Bacteria present in a fish processing facility may easily gain resistance from horizontal gene transfer from any resistant bacteria present in the environment. This can be amplified when suboptimal applications of cleaning agents are used for extended periods, thus exposing the bacteria to sub-MIC levels of the chemicals which can result in the rapid development of resistance.

During the development of an effective cleaning program many different factors need to be considered. The environment may be one that is prone to the formation of biofilms due to unreachable areas or constant moist conditions, such as when large volumes of water are used in the process. The surfaces may be either smooth, or more jointed and angular, leaving more crevasses in which bacteria can grow. Large areas may be difficult to clean, or some higher areas may be hard to reach. The specific bacteria found in the environment may show resistance or extreme abundance due to the nature of the raw materials which are being processed.

A marine fish processing facility would have an abundance of bacteria entering with the raw material, which is in the form of wild-caught fish. Fish processing involves large volumes of water, which leads to a constantly wet environment. Due to many factors influencing traditional seafood processing in South Africa, the workforce is generally minimally schooled and although hygiene is deemed to be important, the reality of disinfectant resistance is not a widely known issue. Cleaning agents themselves are generally used for years at a time and a very small variety of chemicals are used.

With access to a laboratory and minimum inhibitory screening on some of the most resilient bacteria from the facility (Chapter 2), it is possible to create a new cleaning program, that incorporates the data from MIC testing to provide optimum site-specific cleaning.

The aim of this chapter was, therefore, to develop a novel cleaning procedure based on the laboratory data obtained from samples collected from the facility. Once such a novel process has been designed, the next major objective was to implement this in the factory and evaluate the efficacy of the new procedures.

4.2 Materials and Methodology

Data from Chapter 2 was evaluated, and a cleaning schedule was devised with the goal of decreasing bacterial load, increasing the variety of active ingredients, and being easily understandable.

The cleaning procedure was updated to the procedure below (Fig 4.1).

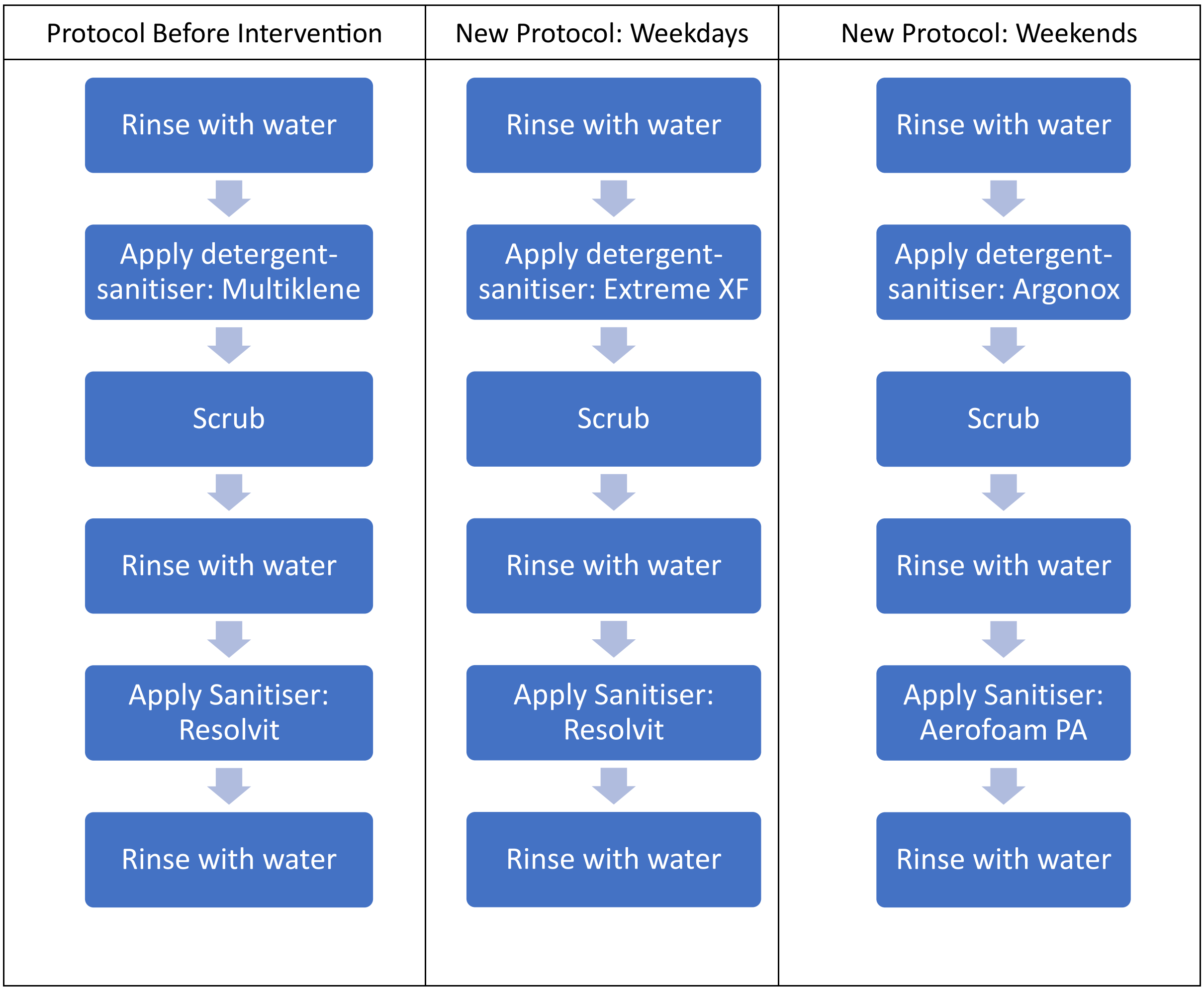


Figure 4.1: Cleaning procedures at fish processing facility before intervention (left) and after intervention, where one procedure is used on weekdays (middle) and another on weekends (right).

To evaluate the effectiveness of cleaning, analysis was done on a Saturday after a week (Monday to Friday) of normal single-shift production, with nightly cleaning as well as after the first application of the new cleaning procedure. TSA contact plates were taken on Saturday morning after the old cleaning protocol and again after the new cleaning protocol had been implemented.

Figure 4.2 : Seafood processing facility after old cleaning protocol (left), during new cleaning protocol (middle), and after (right) cleaning using new cleaning protocols.

4.3 Results

Contact plates were exposed to a variety of surfaces before and after the new cleaning program. Visual inspection of the factory before taking contact plates did not display obvious lack of hygiene. Inspection after cleaning looked marginally better visually, but the differences were unremarkable (Fig 4.2). Plates were incubated at room temperature for 48 hours before evaluation. All plates were photographed, and plates were scored according to colony counts. Figures 4.3-4.6 are provided to illustrate the stark contrast in hygiene before and after application of new cleaning protocol. Results are summarised in Table 4.1. below.

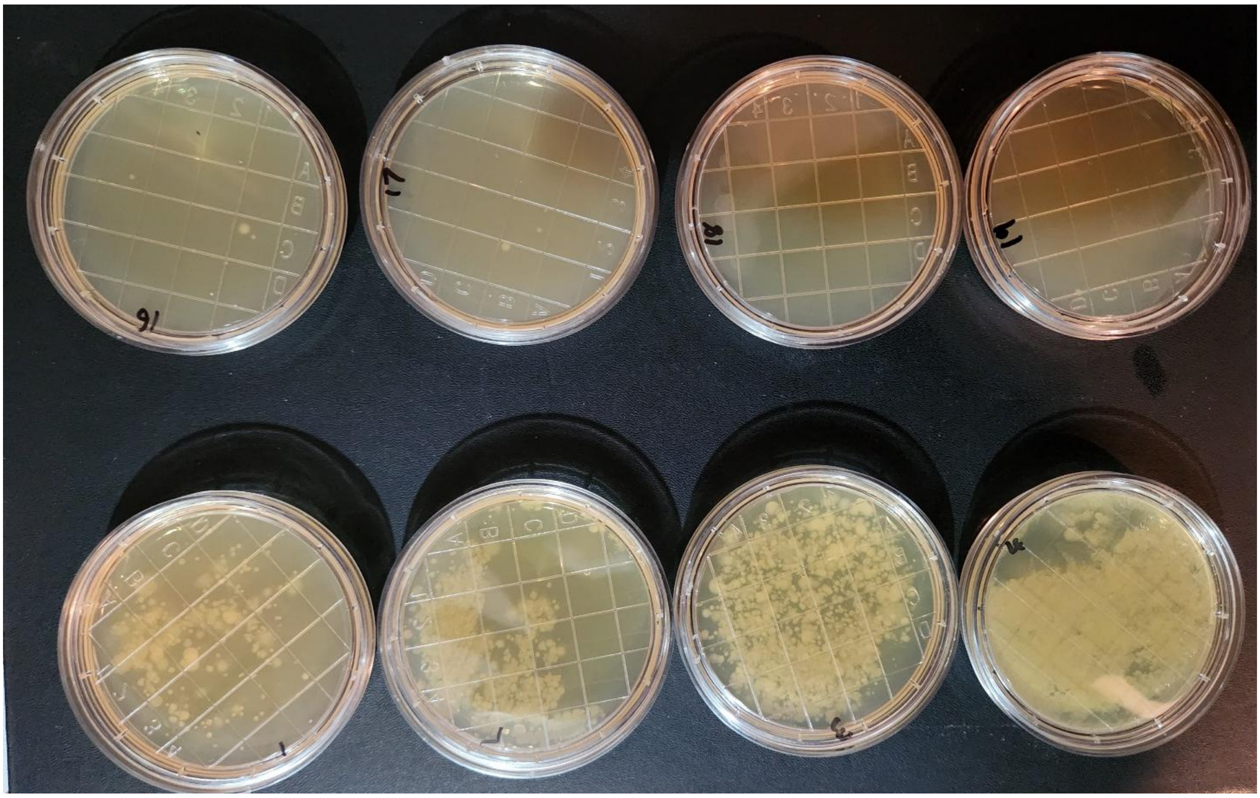


Figure 4.3: Contact plate samples before (bottom) and after cleaning using the new cleaning protocol (top). From Left to right: L1 Bones chute, L1 Water chute, L1 Under packing table, L2 Line electronic interface.

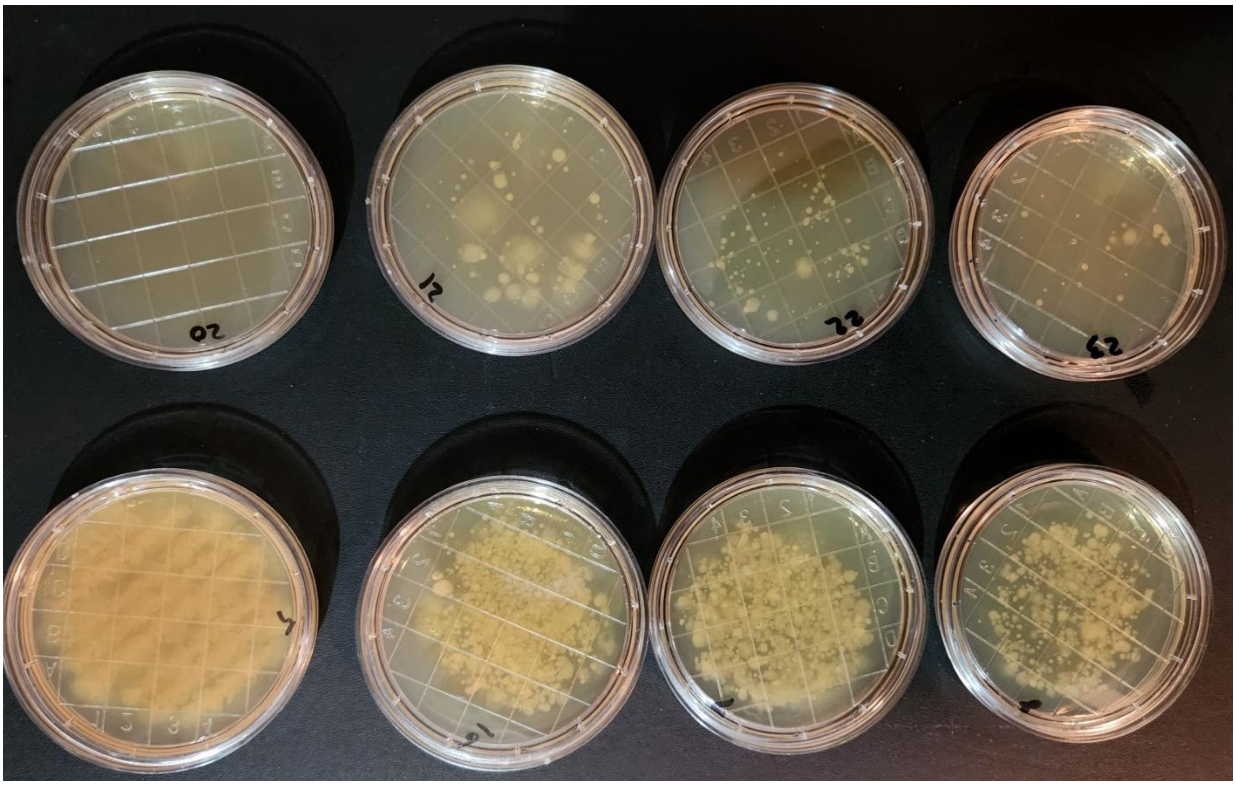


Figure 4.4: Contact plate samples before (bottom) and after cleaning using the new cleaning protocol (top). From Left to right: L2 Skinning machine conveyor, L2 Top of Packing Table, L3 Wall between filleting and packing, L3 Motor cover.

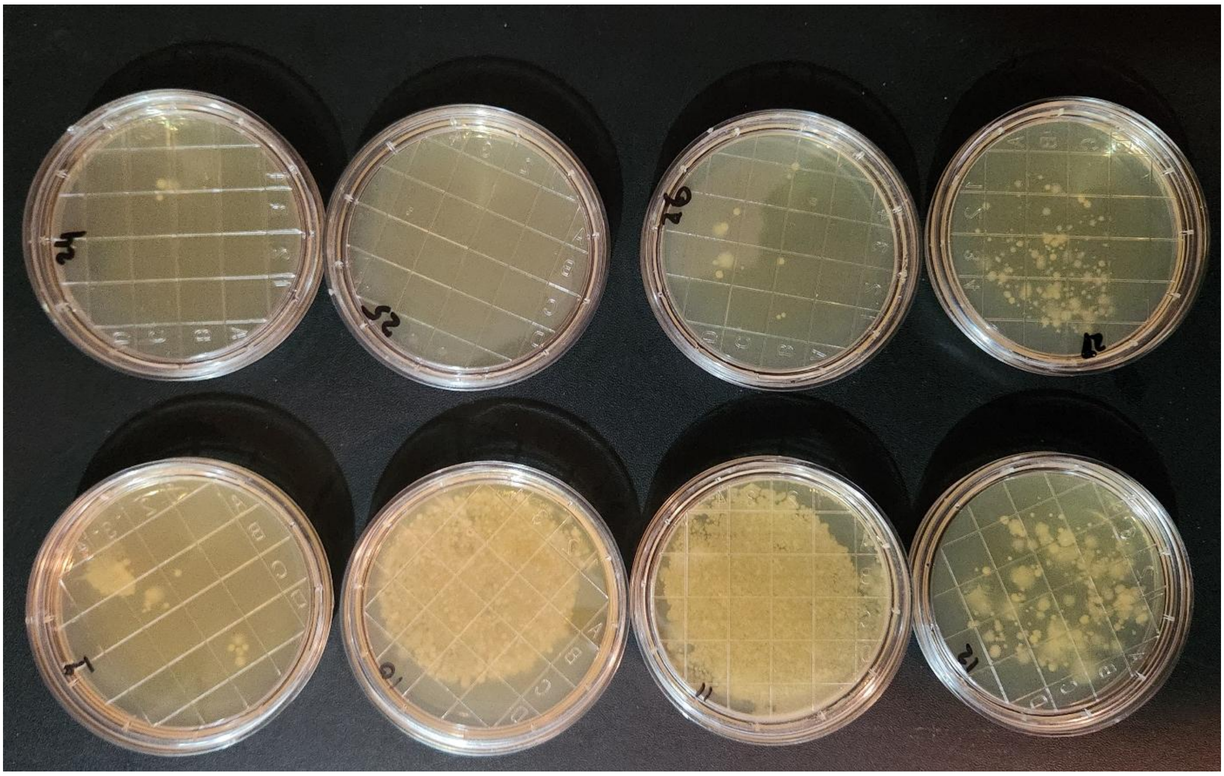


Figure 4.5: Contact plate samples before (bottom) and after cleaning using the new cleaning protocol (top). From Left to right: L3 End-of-line scale, Plate Freezer 1, Mincer Drum, H&G line Table.

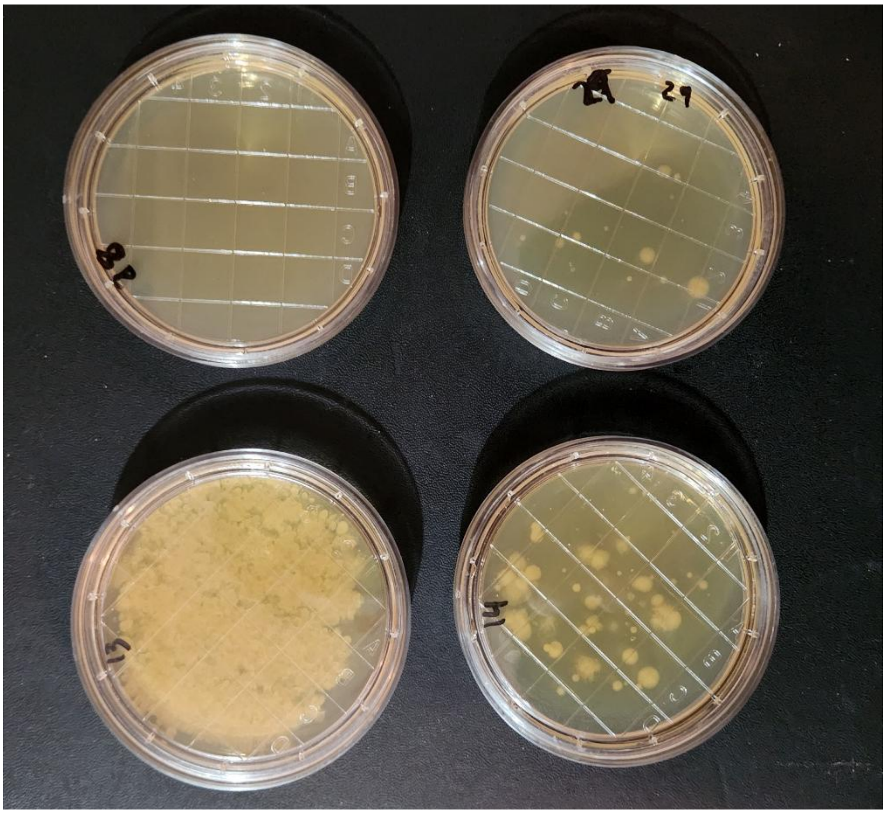


Figure 4.6: Contact plate samples before (bottom) and after cleaning using the new cleaning protocol (top). From Left to right: Sorting area conveyor, Sorting Area floor.

Bacterial growth was decreased by the new cleaning protocol, indicating effective cleaning. Growth was observed on seven of the fourteen plates taken after cleaning, but all were below 100 cfu/plate.

Table 4.1: Summary of colony abundance on contact plates before and after cleaning using new cleaning protocol. "-" = no growth, "+" = slight growth, <100 cfu/plate; "++"= high growth, >100 cfu/plate; "+++"= abundant growth, >300 cfu/plate.

Location	**Sample numbers (before & after)**	**Before Cleaning**	**After Cleaning**
Line 1 Bones Chute	1 & 16	+	-
Line 1 Water Chute	2 & 17	++	-
Line 1 Under Packing Table	3 & 18	++	-
Line 2 Electronic Interface	4 & 19	+++	-
Line 2 Skinning Machine Conveyor	5 & 20	+++	-
Line 2 On Packing Table	6 & 21	+++	+
Line 3 Wall between Filleting and Packing	7 & 22	+++	+
Line 3 Conveyor Motor Cover	8 & 23	++	+
Line 3 End-of-Line Scale	9 & 24	+	+
Plate Freezer 1	10 & 25	+++	-
Mincer Drum	11 & 26	+++	+
H&G Line Table	12 & 27	++	+
Sorting Area Conveyor	13 & 28	+++	-
Sorting Area Floor	14 & 29	+	+

Bacterial loads decreased after use of new cleaning protocol at both food contact and non-food contact areas. For analysis and display purposes, plates with more than 300 CFUs were assigned a value of 300 CFU.

Average CFU per plate for non-food contact areas was 240 after the old cleaning protocol and 18 after the new cleaning protocol. For food contact areas after the old cleaning procedure the average CFU per plate was 241 and after the new cleaning protocol the average CFU per plate was 24 (Figure 4.7).

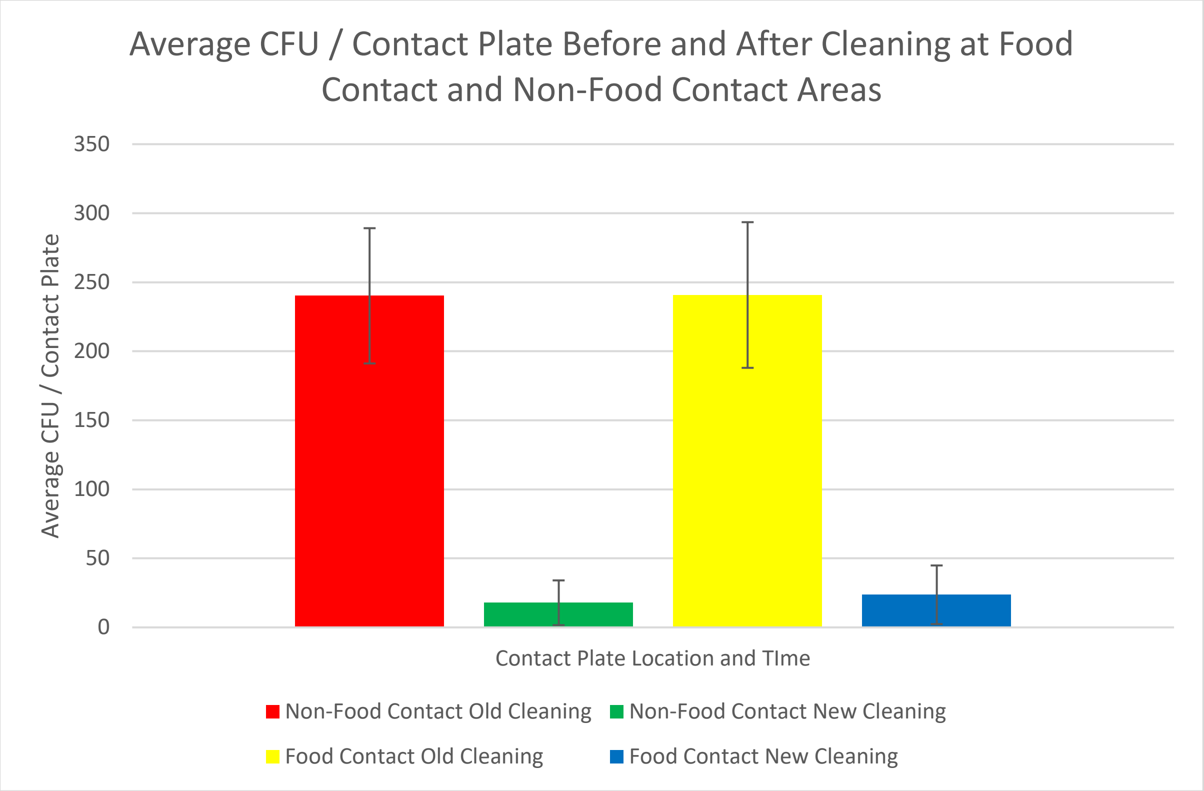


Figure 4.7: Average CFU per contact plate after old cleaning and new cleaning protocols at food contact and non-food contact areas

4.4 Discussion

The current best defence against the looming threat of a post-antibiotic world is good biosecurity (Bragg *et al.*, 2014). Effective use of disinfectants is a crucial part of controlling the spread of harmful organisms. This also holds true for the spread of organisms in food products. Fish and fish products were the most common cause of foodborne disease outbreaks between 2011 and 2014 in the US (Barrett *et al.*, 2017). The largest of these outbreaks was caused by raw fish contaminated with *Salmonella* species, likely during processing, storage or preparation (Barrett *et al.*, 2017). In 2017/2018 South Africa experienced the world's largest listeriosis outbreak, wherein 216 deaths were reported (Tchatchouang *et al.*, 2020). This was eventually traced back to ready-to-eat meat products from a single processing facility (Tchatchouang *et al.*, 2020). This outbreak serves as a reminder of how rapidly foodborne disease can spread from a single origin point due to the nature of the global food chain and the importance of application of scientific principles in a real-world commercial setting.

Good disinfection programmes have been shown to decrease the spread and impact of infectious coryza in poultry farming (Bragg, 2004). The same principles of improved biosecurity can be used to decrease the spread of harmful bacteria in food processing environments which in turn should decrease foodborne disease outbreaks.

After adjusting the cleaning programme at a fully operational, industrial fish processing plant, the decrease in bacterial load was immediately evident. A decrease in bacterial load was seen on every point tested during the trial with the only change in cleaning being the cleaning agents used. The decrease was seen on food-contact surfaces as well as on non-food contact surfaces, which is important for the prevention of cross-contamination during processing. Notably, the chemicals used during the industrial trial were also previously shown in laboratory experiments to eliminate more resistant bacteria (Chapter 2). Using multiple different active ingredients should assist in slowing down resistance development and be effective on a wider range of organisms (Ray and Bhunia, 2014).

Implementation of a cleaning program that would benefit the facility in the short term with improved cleaning and in the long term with decreased resistance, was done in a relatively smooth and easy manner once the threat of disinfectant-resistant bacteria and the effectiveness of the proposed disinfectants against these organisms was established. The bacterial loads are expected to decrease even more should there be other simultaneous measures to improve hygiene such as better training on physical cleaning methods, improved water quality for use in cleaning and improvements in staff hygiene. The proliferation of information regarding site-specific bacterial risks should be strongly considered if improved biosecurity is to be achieved. The industrial sector must accept the use of scientific principles as it will improve food safety and food quality. After a scientific theoretical base was formed, the facility could be prescribed a site-specific cleaning regiment that is easy to follow. Communication between researchers and industry needs to be open to ensure that theory is put into practice.

Chapter 5 : Summary and Conclusions

5.1 Summary

Bacterial populations in South African marine fish processing facilities have not been studied well. Food processing facilities are known to harbour potentially harmful organisms (Ray and Bhunia, 2014). Hazard Analysis and Critical Control Point (HACCP) based food safety management systems in the US are estimated to have decreased foodborne disease by 20% in the first seven years following their adoption (Weinroth *et al.*, 2018). These systems focus on identifying risks of contamination in food processing and controlling these hazards using pre-requisite programs, which include cleaning programs (Weinroth *et al.*, 2018). The usage of scientific input is critical to this system, which has also become compulsory for food handlers in South Africa.

Bacteria are abundant in an environment where raw, wild caught fish is processed since they are constantly introduced with the raw materials. It is, therefore, important for cleaning to be consistent and effective to prevent the build-up of microbial contaminates (Ray and Bhunia, 2014). Biofilms harbouring potentially harmful bacteria, including species of the genus *Vibrio,* also readily develop in seafood processing environments (Shikongo-Nambabi *et al.*, 2010). These have been shown to be controllable using oxidising disinfectants such as H_2O_2 and ozone (Shikongo-Nambabi *et al.*, 2010).

The application of good disinfection protocols that consider different target organisms and the environment to be cleaned is of critical importance. Also important is the consideration of selection pressure imposed by these cleaning agents and their potential to speed up the development of disinfectant resistant populations (Zhang *et al.*, 2017).

Antibiotic resistance has been shown to increase in populations exposed to chlorine disinfectants (Hou et al. 2019). Chlorine disinfection also promotes the exchange of antibiotic resistance across genera (Jin *et al.*, 2020). Chlorine disinfectants are very common in food processing environments due to their ease of use, their status as a trusted disinfectant, and their low cost (Ray and Bhunia, 2014). Another group of increasingly common disinfectants are the QAC-based disinfectants. They have been in use for a long time but are only increasing in popularity, especially since the COVID-19 pandemic (Zheng *et al.*, 2020). This widespread use of QACs has been linked to the spread of QAC resistance, as well as to antibacterial resistance (Hegstad *et al.*, 2010; Buffet-Bataillon *et al.*, 2012; Kim *et al.*, 2018).

The continued careless, uncontrolled use of these disinfectants would develop even more resistance development in these environments. The only way to prevent this is responsible biosecurity measures and well-developed disinfection plans that take all factors of bacterial physiology and resistance into account.

During the project, bacteria from a food-processing facility were isolated. Due to their abundance, the most resistant were selected for using MIC screening. Commercial cleaning agents were then tested to determine which of these agents would be the most effective. From these agents a new cleaning plan was created. This cleaning plan had to consider the safety of operators, cost of cleaning agents, storage of cleaning agents, application methods, and any cross-interactions the chemicals may have with each other. A new cleaning protocol was developed and implemented at the facility. The cleaning protocol was shown to be more effective than the old cleaning protocol. The new cleaning protocol takes into account the development of resistance in bacterial populations and attempts to address this issue using a rotation of active ingredients.

5.2 Future Work

The method for developing a site-specific cleaning program can be applied to other similar factories in the industry. There is also potential to apply the same method in other food processing establishments. Future work may include profiling the microbial community in these processing environments to better understand the formation of biofilms and methods of resistance. Work can also extend to evaluating resistance after a prolonged period of applying the new cleaning plan. Additional work can also be done to investigate the specific mechanisms of resistance in these environments that have gone understudied. Further research may also expand into the larger of South Africa’s seafood processing industries: that of ocean-based freezer vessels where catching, processing, and freezing take place on a seafaring vessel. These factories would potentially harbour significantly more pathogens due to the difficulty in cleaning such a facility and the even greater lack of oversight compared to a land-based factory.

Supplementary Documents

Annexure A: Reactions included in the bioMérieux API® 20 E V5.0 identification strip

Test Code	Active ingredient	Reaction/Enzymes	Results	
			Negative	Positive
ONPG	2-nitrophenyl-ßD-galactopyranoside	ß-galactosidase (Ortho NitroPhenyl-ßd-Galactopyranosidase)	colourless	yellow
ADH	L-arginine	Arginine Dihydrolase	yellow	red / orange
LDC	L-lysine	Lysine Decarboxylase	yellow	red / orange
ODC	L-ornithine	Ornithine Decarboxylase	yellow	red / orange
CIT	trisodium citrate	Citrate utilization	pale green / yellow	blue-green / blue
H_2s	sodium thiosulfate	H_2S production	colourless / greyish	black deposit / thin line
URE	urea	Urease	yellow	red / orange
TDA	L-tryptophane	Tryptophane Deaminase	yellow	reddish brown
IND	L-tryptophane	Indole production	colourless pale green / yellow	pink
VP	sodium pyruvate	acetoin production (Voges Proskauer)	colourless	pink / red
GEL	Gelatin (bovine origin)	Gelatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	fermentation / oxidation (Glucose)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	fermentation / oxidation (Mannitol)	blue / blue-green	yellow
INO	inositol	fermentation / oxidation (Inositol)	blue / blue-green	yellow
SOR	D-sorbitol	fermentation / oxidation (Sorbitol)	blue / blue-green	yellow
RHA	L-rhamnose	fermentation / oxidation (Rhamnose)	blue / blue-green	yellow
SAC	D-sucrose	fermentation / oxidation (Saccharose)	blue / blue-green	yellow
MEL	D-melibiose	fermentation / oxidation (Melibiose)	blue / blue-green	yellow
AMY	amygdalin	fermentation / oxidation (Amygdalin)	blue / blue-green	yellow
ARA	L-arabinose	fermentation / oxidation (Arabinose)	blue / blue-green	yellow

Annexure B: Modified Labuschagne-Albertyn Method for DNA Extraction

- A 1.5 ml microcentrifuge tube is filled with culture and centrifuged at 19800 x g for 10 min at 4°C to harvest the cells.
- The supernatant is discarded, and the pellet is resuspended in 500 µL of lysis buffer consisting of 100 mM Tris-HCL pH 8.0, 50 mM EDTA pH 8.0, and 1% SDS.
- Approximately 200 µL (or 1 full PCR tube) of glass beads is added. The tubes are vortexed for 1 minute before being placed on ice for 1 minute. This is repeated until 5 minutes have passed in total.
- 275 µL of ammonium acetate (7 M, pH 7) is added and the mixture is mixed by vortex. The tubes are then incubated at 65°C for 5 minutes followed by being placed on ice for a further 5 minutes.
- 500 µL of chloroform is added, the sample is vortexed and centrifuged at 19800 x g at 4°C for 2 minutes. From the resulting lysate, the top layer which contains the nucleic acids is removed and transferred to a clean 1.5 ml microcentrifuge tube before 750 µL of isopropanol is added.
- The tube is inverted gently to mix and left to precipitate at room temperature for 5 minutes. The sample is centrifuged at 19800 x g for 2 min at 4°C after which the supernatant is removed by aspiration to be discarded.
- Ice cold 70% ethanol is used to wash the pellet before a final centrifugation at 19800 x g for 2 min at 4°C. The supernatant is discarded by aspiration and the pellet airdried in a vacuum centrifuge.
- The dried pellet is dissolved in 100 µL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and 5 µL RNase (0.5 mg/ml) and incubated at 37°C for 60 minutes. The samples are then stored at -20°C.

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