ANTIMICROBIAL EFFECT OF SELECTED PROBIOTIC STRAINS ON *SALMONELLA TYPHIMURIUM* AND *E. COLI* O157:H7 ISOLATED IN A COMMERCIAL SLAUGHTERHOUSE IN WINDHOEK, NAMIBIA

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ABSTRACT

In recent years, Lactic Acid Bacteria (LAB) have gained popularity due to their preservative and antimicrobial activities. Therefore, there has been a growing interest in researching and developing new antimicrobial agents from various sources to control and eliminate pathogens such as *Salmonella typhimurium* and *E. coli* O157:H7 in food niches. The two pathogens are the major causative agents of foodborne diseases especially in meat products. Therefore, this study aimed to evaluate the antimicrobial activity of selected commercial probiotic strains of *P. acidilactici* and *P. pentosaceus* against *S. typhimurium* and *E. coli* O157:H7 isolated from beef samples using monoculture and co-cultures design. Beef samples were collected from Meatco Abattoir and antimicrobial activity of the probiotics strains was evaluated using pour plate methods over general and selective media such as Salmonella Shigella (SS) agar and MacConkey agar. The optical density (OD) was measured at 600 nm to study the growth kinetics of microbial mass for the probiotic and pathogenic strains in mono and co-cultures models. The co-cultures of *S. typhimurium* and *P. acidilactici* gave the significant reduction of 1.2 log cfu in *S. typhimurium* population, indicating that, it is more sensitive to the tested *Pediococcus* strains than *E. coli* O157:H7. Both LAB strains showed antagonistic activities against *S. typhimurium* and *E. coli* O157:H7, although none of the LAB showed a complete inhibition. However, *P. acidilactici* had a greater antimicrobial activity compared to *P. pentosaceus*. The OD value of *S. typhimurium* co-culture decreased with 2.24 log cfu when it was co-cultured with *P. acidilactici* and 2.28 log cfu when it was co-cultured with *P. pentosaceus*. Meanwhile, OD value of *E. coli* O157:H7 decreased significantly with 2.22 log cfu when it was co-cultured with *P. pentosaceus* and 2.33 log cfu with *P. acidilactici* showing their potential bio-preservation in meat products. However, further confirmatory tests are

needed especially in their permitted concentration on the surface of cow carcasses and meat cuts during the storage.

Keywords: Antimicrobial effect, Lactic Acid Bacteria *S. typhimurium, E. coli* O157:H7, Meat, *P. acidilactici, P. pentosaceus.*

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ABBREVIATIONS

ANOVA - Analysis of variance

ATCC -American Type Control Culture

ATP -Adenosine Triphosphate

BPW - Buffered Peptone Water

BSM - Bifidus Selective Medium

CCP - Critical control point

CDC - Centre for Disease Control and Prevention

CFS - Cell free Supernatant

CFU - Colony Forming Units

DNA - Deoxyribonucleic acid

EHEC - Enterohemorrhagic *Escherichia coli*

EIEC - Entero- invasive *E coli.*

EMB - Eosin Methylene Blue Agar

ETEC - Enterotoxigenic *E coli*

FBD - Foodborne diseases

FERG - Foodborne Disease Burden Epidemiology Reference Group

FSIS - Food Safety and Inspection Service

GMP - Good Manufacturing Practices

GN - Gram-negative

GP - Gram-positive

GRAS - Generally Recognized as Safe

HACCP - Hazard Analysis and Critical Control Point

HACCP - Hazard Analysis Critical Control Point

HAS - Hygiene Assessment System

ISO - International Standards Organization

LA - Lactic Acid

LAB - Lactic Acid Bacteria

MA - MacConkey Agar

MAP - Modified Atmosphere Packaging

Meatco - Meat Corporation of Namibia

MRS - De Man Rogosa Sharpe

NA - Nutrient Agar

NB - Nutrient Broth

OD - Optical density

PCA - Plate Count Agar

PMF -Proton Motive Force

PMF - Proton Motive Force

RPM - Round Per Minute

S. Dev - Standard Deviation

SD - Standard Deviation

SPSS - Statistical Package for the Social Sciences tool

SSA - *Salmonella Shigella* Agar

STEC - Shiga toxin–producing *E. coli.*

TNTC - Too Numerous To Count

TPC - Total Plate Count

TSB - Tryptose Soy Broth

UNAM - University of Namibia

USDA - United States Department of Agriculture

UV -Ultraviolet light

UV - Ultraviolet

WHO - World Health Organization

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I would like to dedicate this research to my son, Gevin Vemuna and my mother Maria Ndatambula Shapwa for motivation, and encouragement.

DECLARATION

I, **Kalihulu Martha Shapwa**, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Kalihulu Martha Shapwa **Date**

CHAPTER ONE: INTRODUCTION

1.1. Background of the study

Free reared Namibian cows are regarded as a meeting the requirement for organic meat production (Meatco Annual Report, 2020), thus, making their product attractive to foreign markets. According to Meat Corporation of Namibia (Meatco), the microbiological requirement for meat exports is stringent and varies from country to country and customer to customer (Meatco Annual Report, 2020). Meatco is a meat processing and marketing entity, which supplies meat to both local and international markets (www.meatco.com.na/aboutus). As foodborne diseases are a major challenge faced by meat consumers, ensuring high levels of safety and quality in meat for local and export markets is very crucial. To ensure the betterment of meat quality, Meatco has introduced the Hazard Analysis Critical Control Point (HACCP) system. The HACCP system combined with international standards of food handling and processing such as the Food Safety System Certification (FSSC) 22000 and the British Retail Consortium (BRC), are put in place to meet the consumers' requirements and expectations. The Meatco Compliance department (previously known as quality assurance) ensures that safe and high-quality meat and meat products meet the quality and requirements of countries to which it is exported. Compliance main goal is to ensure that safe and high-quality meat and meat products are supplied in consistent quality, to the performance requirements of its customers and the legal requirements of those countries to which products are exported (Egziabher & Edwards, 2013).

The basis of the meat quality depends generally on the type of bacterial species, combined with the level of microbiological contamination (Tshabalala, 2011). In the

slaughterhouse, major causes of contamination are inadequate hygienic conditions and poor handling that may result in food poisoning in humans (Elshazly & Fathalla, 2016). Therefore, the removal of bacteria-free meat from two contaminated surfaces mainly the hide and gastrointestinal (GI) tract are the major focus for the slaughter process for cattle and other meat-producing animals (Tshabalala, 2011). However, Tshabalala (2011) reported that the combination of the Hygiene Assessment System (HAS) and the Hazard Analysis and Critical Control Point (HACCP) did not prevent contamination of beef carcasses with *Escherichia coli* O157:H7 and *S. aureus*. Faecal contamination of food by mostly members of Gram-negative bacteria like *Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa* naturally occurs in the warm-blooded animal guts and are the most common cause of illness and deaths worldwide (Mostafa, Al-Askar, Almaary, Dawoud, Sholkamy & Bakri, 2018). This as reported by Mostafa *et al.* (2018) led to the increasing awareness of the microbial quality of meat and the use of natural products and nutraceutical such as probiotics to help eliminate these bacteria. There were 89 confirmed cases of a foodborne disease outbreaks among students from Oshakati Secondary School (on 29 January 2019, Northern Namibia) that was linked to beef consumption (Task Force for Global Health, 2019). In Europe in 2016, a total of 16% of foodborne outbreaks was recorded amongst the foods, meat and meat-based products, the latter is considered as one of the main vehicles of these pathogens (Tesson, Federighi, Cummins, de Oliveira Mota, Guillou & Géraldine Boué, 2020). *S. typhimurium* and *E. coli* O157:H7 are food-borne pathogens that have been frequently linked to outbreaks attributed to the consumption of meat, fresh produce, and water that had been contaminated with cattle manure (Schamberger, Phillips, Jacobs & Diez-Gonzalez, 2004). Therefore,

the presence of *E. coli* and *Salmonella* on food is an indicator of faecal contamination (Murry, Hinton & Morrison, 2004).

E. coli O157:H7 is one of the foodborne pathogens of concern for the dairy industry and thus responsible for most foodborne diseases since this bacterium produces different types of potent toxins which cause a wide range of human death (Rodriguez et al., 2005; Hamad, Botros & Hafez, 2017). Salmonellosis and staphylococcal food poisoning are two of the main food-borne diseases. Salmonellosis is often caused by *Salmonella* contaminated foods such as meat products, eggs and dairy products (Zhang et al., 2016). Studies of possible antimicrobial effects of LAB in co-culture with pathogenic bacteria as targets are rare. A limited amount of research has been completed to determine the effectiveness of interventions under commercially simulated conditions on beef trim to reduce pathogens (Harris Brashears, Garmyn, Brooks & Miller, 2012). Similarly, Mariam, Zegeye, Aseffa & Howe (2017) confirms that studies on possible antagonistic effects of Lactic Acid Bacteria (LAB) in co-culture with pathogenic bacteria as targets appear to be rare. Therefore, the objective of this study was to determine of inhibitory activities of commercial selected LAB species in co-culture with pathogens *S. typhimurium* and *E. coli* O157:H7.

1.2. Statement of the problem

According to Meatco's financial report 2017/18, the group recorded a decrease in revenue of 15.9 % due to *E. coli* and *Salmonella* contaminations as compared to 2016/2017 financial year. Meat Corporation of Namibia annual report 2018/2019 ascribed the loss estimation at N$1.6 million in 2018 alone on meat cuts that tested

positive for *Salmonella* ssp. and *E. coli* O157:H7. As a result, the contaminated meat could not be exported to intended markets. The excision of the rectum and tied off coupled with decontamination of carcasses and meat cuts have been applied to reduces contamination. Yet, they could not eliminate all microbial contamination. As a preventive action, Meatco has been using hot water wash on carcasses right before chilling. Nevertheless, this method alone could not eliminate all microbial contaminants. The same method described by Snijders et al. (1985) has been used. In 2015, Meatco introduced commercial lactic acid solution wash at slaughter floor, the lactic acid, however, reduced the positive number by a mere 20%. There are few reported studies on protocols and methods on how to eliminate these contaminations in meat samples (Shiningeni, 2017).

1.3. Objectives of the Study

The objective of this study is to determine the antimicrobial activity of selected commercial probiotic strains against *E. coli* O157:H7 and *Salmonella* in beef using a co-culture experiment design.

The specific objective was:

The specific objective is to investigate the antimicrobial activity of probiotic *Pediococcus* species against of *S. typhimurium* and *E. coli* O157:H7 present in beef samples using monoculture and co-culture approaches.

1.4. Hypotheses of the study

The hypotheses of this research were:

H_1: The selected commercial probiotic strains can exhibit adequate antimicrobial activity to eliminate the growth of *S. typhimurium* and *E. coli* O157:H7 in single and co-culture design (Tshabalala, de Kock & Buys, 2012).

H_0: The selected commercial probiotic strains cannot exhibit adequate antimicrobial activity to eliminate the growth of *S. typhimurium* and *E. coli* O157:H7 in single and co-culture design (Tshabalala et al., 2012).

1.5. Significance of the study

This study might unveil the immense potential of the antimicrobial activities of commercial LAB to fill the gap as alternative and evidence of beneficial effects of probiotic application in the Namibian meat production industry.

1.6. Limitation of the study

Not all known commercial probiotics was used in the study, as well as not all beneficial properties of probiotics will be evaluated. Equally, not all antimicrobial activity of commercial probiotics was evaluated as much as not all antimicrobial characteristics of LAB will be determined in the study.

1.7. Delimitation of the study

This research will only focus on antimicrobial activity of the two selected commercial probiotics in eliminating and reducing *Salmonella* ssp. and *E. coli* O157:H7 in beef in co-culture models in a Windhoek slaughterhouse.

CHAPTER TWO: LITERATURE REVIEW

2.1. Introduction

Beef (meat) composition is perfect for the growth of a wide type of microorganisms which may cause the spoilage of meat and meat products, making meat and meat products one of the most perishable foods (Hernández-Aquino et al., 2019). Meat and meat products are therefore perishable because they can be easily deteriorated due to unavoidable contamination during the production process (İncili, Karatepe & İlhak, 2020). They have a suitable environment for microbiological growth, they can easily be contaminated by some microorganisms including pathogens, hence, they should be properly stored, processed, and packed and distributed in order to prevent microbial growth (İncili et al., 2020; Poffe, Vaes, Van Den Eynde, & Verachtert, 1981). Their sensitivity led to the usage of fewer chemical additives and natural preservatives to reduce contamination in meat, due to consumers demands for organic or minimally processed foods (İncili et al.,2020). Contamination by foodborne pathogens in meat and meat products can result in a range of human health problems in addition to economic losses to producers due to recalls from market places which can be one of the important challenges faced by producers of meat products (Syne, Ramsubhag & Adesiyun, 2013). The Meat Board of Namibia has the mandate to facilitate the export of livestock, meat, and processed meat products. About 52% of Namibian land is utilised by cattle farmers and an additional 33% by small-stock farmers (Egziabher & Edwards, 2013). Namibia's excellent red meat (beef, mutton, goat, and game) originates from free-ranged animals, without any addition of growth stimulants, antibiotics, or animal by-products. Further, Meatco employs ±600 individuals which makes it one of the biggest employers in the country in agriculture sector, the facilities

worked at 100% capacity employing 326 temporary workers, (Meat Board of Namibia, 2006; Egziabher et al., 2013).

The processing operations of Meatco consist of different sections where animals are slaughtered and processed into meat products. According to the Compliance Department, Meatco only uses two methods to aid in reducing contamination, the first is a physical method where operators inspect for faecal contamination, and the second, is a chemical method which applies lactic acid solution onto carcasses just before they are chilled. Workers are assigned to specific workstations and the carcasses move on a conveyor system from station to station until the process is completed. Raw material, grinding of meat where the contamination could spread throughout the entire muscle, post-processing handling or different equipment, lack of refrigeration facilities, lack of enough suitable methods of transportation and transportation lacking refrigeration which are used from the point of production till marketing coupled with improper storage are the main sources of contamination by different microbes (Zaki, 2016). While ensuring that Meatco meets the demands of current and future markets, its processing operations also maintain continuous compliance with international quality standards and export requirements. Meatco's biggest clients are currently South Africa, the United Kingdom, Norway, Germany and Switzerland to a lesser extent. The quality of human life against food safety is gathering more and more attention and requires continuous improvements. This is in line with food borne outbreaks and these are killing people in both developed and developing countries. However, food outbreaks are becoming more common in Africa with the recent observation in South Africa of listeriosis from Vienna. The World Health Organization (WHO) reported 978 laboratory-confirmed listeriosis

cases of which 674 patients and 183 (27%) deaths. Of these cases, people that are at higher risks are pregnant women, the elderly and immunocompromised persons.

E. coli and *Salmonella* are members of the family *Enterobacteriaceae* and are divided into many sub-groups. They are rod-shaped, Gram-negative, non-spore-forming and considered as pathogens when isolated from a food product or human (Harris et al., 2012). The muscle of a healthy animal is essentially sterile, but even under the most stringent conditions, the muscle can become contaminated during the harvest process from the environment, hide, or from direct contact with the intestinal tract contents (Harris et al., 2012). To evaluate the microbiological quality of meat, the quantification of *E. coli* and the presence of *Salmonella* have frequently been used because these microorganisms are considered good indicators of quality and food safety worldwide (Da Silva, Horvath, Silveira, Pieta & Tondo, 2014). *Salmonella* spp. has been identified as the most important contaminant of food and the leading bacterial agent responsible for foodborne outbreaks in several countries (Da Silva et al., 2014).

Food poisoning is considered one of the most common causes of illness and death in both developed and developing countries (Mostafa, Al-Askar, Almaary, Dawoud, Sholkamy & Bakri, 2018). Food safety, on the other hand, is one of the major health concerns due to outbreaks of food-borne diseases (Mostafa et al., 2018). Bintsis (2017) confirms that foodborne diseases have become a global issue, a unified and joint approach is therefore needed by all countries. It has been acknowledged that most foodborne illnesses are preventable despite being complex in their biology, analysis and epidemiology. Hence, there is increasing demand for natural products

that can serve as alternative food preservatives (Sanlibaba, Güçer & Şanlıbaba, 2015). Due to the outbreak of foodborne diseases worldwide, food safety has become one of the major concerns in public health, since the safety of artificial preservatives used in food is equally becoming a major concern (Sanlibaba et al., 2015). This awareness of healthy foods has led to increasing interest in natural food products and nutraceuticals such as probiotics which are applied to help reduce pathogens that cause foodborne diseases (Bajpai, Han, Rather, Park, Lim, Paek, Lee, Yoon, & Park, 2016). Additionally, there is a rise in consumer demands for natural or minimally processed foods, thus, the need for alternative beef safety interventions (Kirsch, Tolen, Hudson, Castillo, Griffin, & Taylor, 2017). Snijders, Van Logtestijn, Mossel and Smulders (1985) point out that good manufacturing practice (GMP) during slaughter may include all measures that are necessary to produce meat with the lowest possible microbial contamination. They further emphasised that; this is only attainable if the whole process is strictly controlled. Youssef, Yang, Badoni & Gill (2012) add that no matter how carefully a plant handles beef carcass, bacteria still contaminate the carcasses, some of which could potentially be faecal pathogens such as *Salmonella* or *E. coli* O157:H7. Primary interventions in reducing bacterial contamination of beef carcasses include employing effective sanitary dressing procedures during slaughter and packaging processes (Buege & Ingham, 2003). However, Schamberger et al. (2004) went on to explain that one of the most promising methods to reduce pathogenic microorganisms in livestock is one in which antagonistic bacteria are used. The method involves, application of co-cultures which has demonstrated significant advantages that are being explored to reduce the prevalence of *Salmonella* and *E. coli* O157:H7 in cattle (Schamberger et al., 2004).

Lactic acid bacteria (LAB) produce various antimicrobial, which includes lowering of pH, hydrogen peroxide and production of bacteriocins (Şanlıbaba et al., 2015).

2.2. Meat quality and microbial population in a slaughterhouse

Microbial contamination of beef carcasses occurs during the conversion of live animals to the carcass and to meat (Rodriguez, 2006). Beef carcasses are initially sterile and are only contaminated with microorganisms through the transmission of organisms either from the exterior of the live animal, from the environment, or the product surface (Rodriguez, 2006). A wide range of microorganisms can populate red meat especially beef which can be potentially pathogenic. The most important of these are *E. coli,* such as O157:H7, *Salmonella* spp. and *Campylobacter* spp. making the meat unfit for human consumption (Tshabalala, 2011). Hence, these bacterial species are used to evaluate the microbiological quality of meat worldwide (da Silva, Horvath, Silveira, Pieta & Tondo 2014). Abattoirs such as Meatco have implemented systematic controls (e.g., HACCP) from farm-to-fork to reduce risks associated with the consumption of contaminated meat (Meatco annual report, 2018).

To circumvent microbial contamination in the slaughterhouses, different decontamination technologies both physical, chemical and biological have been investigated and reviewed for use during the past decades (Christiansen, Krag & Aabo, 2018). Prevention of pathogen growth on meat products has previously focused on the traditional approaches. However, the challenges to the meat industry to control, reduce, or eliminate such pathogenic bacteria in the production and processing of meat products for human consumption has persisted (Dormedy,

Brashears, Cutter, & Burson, 2000). Granting that many intervention technologies are applied to beef carcasses, very few interventions have been validated to be effective in ground beef products (Smith, Mann, Harris, Miller &. Brashears, 2005). This led to vast increasing interest in natural food products and nutraceuticals such as probiotics, which consequently increased awareness of healthy foods (Park et al., 2016). Bacteria such as *Lactobacillus*, *Bifidobacterium* spp., *Lactococcus*, *Bacillus*, and *Streptococcus* and yeast such as *Candida* are the most commonly used probiotics (Rahimpour, Darsanaki & Salehzadeh, 2017; Park et al., 2016).

Most research has focused on organic acids and acidified sodium chlorite (ASC) applied in spray, which efficiently reduce pathogen loads on beef carcasses or cuts (Harris et al., 2012). LAB has been used due to its inhibitory characteristic toward various pathogenic bacteria and spoilage organisms during the growth phase and refrigerated storage in associative cultures (Smith et al., 2005). Previous studies used LAB as organic acids and ASC as decontaminants of beef, which produced conflicting results, where different concentrations such as 2%, 4% and 5% were used and yet they did not reduce populations of *E. coli* O157:H7 or *Listeria monocytogenes* (Harris et al., 2012).

2.3. Spoilage and contamination of carcass meat in slaughterhouse

Fresh raw meat must be properly stored, processed, packed, and distributed in monitored conditions to prevent microbial contaminations as meat products are a perfect environment for the growth of a wide variety of microorganisms (Iulietto, Sechi, Borgogn & Cenci-Goga, 2015). The main sources of meat contamination in

slaughterhouses are the intestinal tract and the skin of the animal (Dave & Ghaly, 2011). The process of meat deterioration starts at the time of the slaughtering of cattle and progresses till the consumption of meat. Deterioration occurs as a result of a combination of high temperature and the presence of microorganisms (Zaki, 2016). Therefore, control measures need to be taken at the abattoir level and outside the slaughterhouse.

2.3.1. Control measures at the abattoir level

Interventions for carcass decontamination in the meat industries may include programmes to fight bacteria at all levels from farms to forks such as Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), and HACCP (Rodriguez, 2006). The research further explained that implementing critical control points (CCP) within HACCP may include the use antimicrobial intervention methods designed to reduce microbial loads on carcasses during slaughter operations (Rodriguez, 2006). Animal hides are one of the significant sources of microbial contamination during dressing (Tshabalala, 2011). Meatco SHEQ department explains that spraying the cattle before entering the abattoir aims at removing ticks and washing off faeces from hides. Tshabalala (2011) reports that the hygienic status of dressed carcasses in most abattoirs is largely dependent upon the general slaughterhouse hygiene and the skills of the workers. She adds that workers' hands could be the source of carcass contamination but this can be improved by training workers on sanitation, personal hygiene and hand washing techniques. Facilities must have cleaning procedures in place, which must be continuous. Since some microorganisms have the ability to settle on surfaces and survive in adverse conditions, cleaning with soap and water is not enough to effectively remove all food

residues or other contaminants caused by biofilm formation (Hernández-Cortez, Palma-Martínez, Gonzalez-Avila, Guerrero-Mandujano, Solís, & Castro-Escarpulli, 2017)

2.3.2. Prevention of contamination outside slaughterhouse

One way to prevent *E. coli* O157:H7 infections is by cooking ground beef thoroughly and avoiding ingestion of raw milk coupled with good personal hygiene. Another way is avoiding cross-contamination, keeping foods at a safe temperature, avoiding foods and water from unsafe surfaces and cooking food thoroughly (Makvana & Krilov, 2015; Pradesh, 2019). Hand washing must be practised after handling raw beef and meat products. At the same time, utensils, cutting boards and hands should be washed in soapy hot water (Pradesh, 2019). Europe is governed by Food Safety Laws which is enforced by food inspectors and environmental health officers, who examine the food and collect samples for testing (Hygiene & Level, 1961). Namibia is also governed by a food safety policy gazetted on July 15, 2014, which provides sufficient food safety guarantees on all food products traded nationally, or exported to other countries (Food safety policy, 2014).

2.4. Meat consumption from slaughterhouse

Meat is consumed worldwide and serves as a source of food protein, therefore, a large number of the products are prepared from animals (Poffe et al., 1981). Many people in many parts of the world eat meat as their major source of protein and valuable qualities of vitamins and it is also essential for the growth, repair, and maintenance

of body cells, and necessary for our daily activities (Zerabruk, Retta, Muleta & Tefera 2019).

2.5. Food Poisoning

Sarkania & Bhalla (2013) defined food poisoning as an illness caused by the consumption of food or water contaminated with bacteria and/or their toxins, or with parasites, viruses, or chemicals. Firstly, food poisoning is commonly caused by infectious organisms including but not limited to various bacteria, viruses and parasites or their toxins. The infections by the above happen at any point during food processing or production thereby causing contamination (Umadevi, Pavan & Bhowmik, 2013). Secondly, when a pathogen is ingested with food, it multiplies in the human host, alternatively when a toxigenic pathogens establishes itself in a food product and produces a toxin, then ingested by the humans (Bintsis, 2017). Third and lastly, symptoms varying in degree and combination, include abdominal pain, vomiting, diarrhoea, and headache; more serious cases can result in life-threatening neurologic, hepatic, and renal syndromes leading to permanent disability or death (Sarkania et al., 2013).

2.6. Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are related by their common morphological, metabolic, and physiological characteristics. They comprise Gram-positive, acid-tolerant, non-sporulating, non-respiring rods or cocci (Tshabalala, 2011). LABs are generally recognised as safe (GRAS). They are microorganisms that play a crucial role in preservation either as natural microflora or as starter cultures added under controlled conditions (De Vuyst & Leroy, 2007). LAB exert preservative properties mainly due

to the production of organic acid and hydrogen peroxide (Gao et al., 2019). Ayivi et al. (2020) report that the genus *Lactobacillus* has recently been reclassified into 25 genera of which, three most promising probiotic strains include members of the genera *Lactobacillus, Bifidobacterium,* and *Enterococcus* (Pieniz et al., 2014; Uyeno, Shigemori & Shimosato, 2015).

Lactobacillus, being the largest genus consisting of more than 80 recognised species, (Gupta, Jeevaratnam & Fatima, 2018). Rahimpour, Darsanaki & Salehzadeh (2017) concur that *Lactobacillus* and *Bifidobacterium* spp. are the main genera of LAB and are commonly used as probiotics. Notably, not all LABs are necessarily probiotic (Rzepkowska et al., 2017). LAB play an important role in the environment, food and clinical microbiology (Pieniz et al., 2014). They are reported to be found in numerous raw materials that are used to produce fermented foods like milk, meat, and flour and even in the gut of herbivorous animals and humans as symbiotic organisms (Gupta et al., 2018). For thousands of years, LAB have been recognised for their vital role in the food industry due to their fermentative functions. Lactic acid is the main product associated with pH reduction through the fermentation processes of various foods (Pieniz et al., 2014). The fermentative functions and pH reduction are the primary antimicrobial effect (Ammor et al., 2006). LAB have been used for more than 4000 years to extend the shelf life (Gupta et al., 2018).

LAB have been classified into different genera/species based on their acid production characteristics and their growth at optimum temperatures (Ayivi et al., 2020). Their classification also depends on the morphology, growth at a different temperature, the configuration of lactic acid produced, ability to grow in high salt concentration

and acid or alkaline tolerance (Tshabalala, 2011). This taxonomic classification includes the phylum Firmicutes, class *Bacilli*, and order *Lactobacillales* (Quinto et al., 2014). The homofermentative LAB such as *Lactococcus* and *Streptococcus* yield two molecules of lactates from one glucose molecule (Gupta et al., 2018). Heterofermentative LAB can yield lactate, ethanol, and carbon dioxide from one molecule of glucose such as *Leuconostoc, Weissella* and some *Lactobacilli* (Gupta et al., 2018). In Addition, *Lactobacilli* and *bifidobacteria* play the most important roles in the development of smell, taste, and texture of fermentation products (Rahimpour et al., 2017).

Since, LABs are a type of bio-preservative that were previously reported to be useful for preventing the growth of pathogenic microbes on meat products and are classified as Generally Recognized As Safe (GRAS) for use on carcasses, for human consumption (Kirsch et al., 2017; Bintsis, 2018). To date, they still play an essential role in the bulk of food fermentations (Oliveira, Oliveira & Glória, 2008). Bio-preservation is crucial for the maintenance of microbiological quality and safety of meat and meat products (Da Costa et al., 2019). In addition, LABs are essential in most food applications. The food industry has been seeking strains with superior characteristics and properties to improve sensory and product quality (Ayivi et al., 2020). LAB were first isolated from milk (Sanlibaba, et al., 2015). Further, Snijders et al. (1985) reveal that LA can be produced in meat products through fermentation. LAB has also long been isolated from meat products where they are termed locally isolated LAB, hence they are the best applicants for improving the microbiological safety of these foods. Since they are well adapted to the conditions in meats, they are more competitive than LAB isolated from other sources (Oliveira et al., 2008).

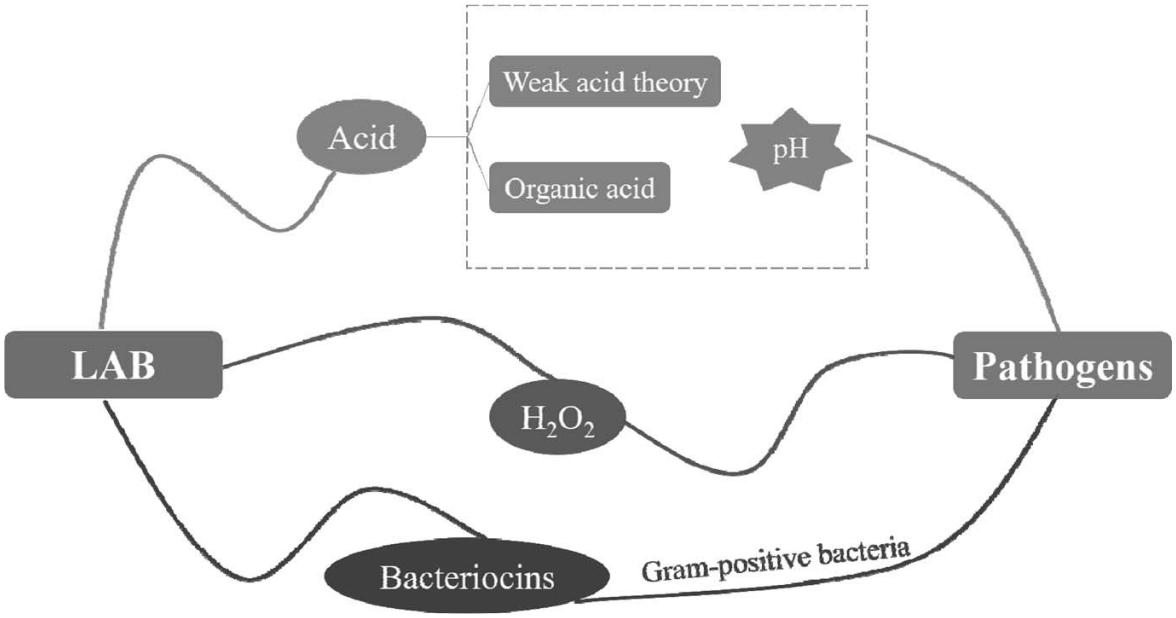


Figure 1 Mechanism of LAB activity against pathogens (Gao et al., 2019)

Lactic acid has three main qualities, namely, it is a natural product; it is physiological, and not toxic. Therefore, it is commonly used in the meat industry, as a decontaminant, (Hossain et al.,2017). Lactic acid sprays are applied to beef cuts and trimmings, to control contamination with pathogens, particularly *E. coli* O157:H7 and *Salmonella* (Youssef et al., 2012). As reported above, because some strains of LAB produce bacteriocins they exert antagonistic properties against many microorganisms, which include both spoilage and pathogenic bacteria. These bacteriocin-producing LAB may play an important role as natural preservatives to enhance meat shelf life and safety by inhibiting spoilage and pathogenic bacteria (Djenane, Martínez, Blanco, Yangüela, Beltrán & Roncalés, 2005). Likewise, Tebyanian, Bakhtiari, Karami1 & Kariminik (2017) also add on the benefits of LABs that *Lactobacillus* plays a critical role in the immune system, such as local control immune responses, allergic and inflammation diseases by increasing the activity of macrophages and immunoglobulin IgA production. According to Gutiérrez-Cortés,

Suárez, Buitrago & Díaz-Moreno (2017), sugar metabolism produces lactic acid which are metabolites released to the environment to reduce its pH and at the same time inhibiting the development of some populations of undesirable microorganisms. Then, H_2O_2 reacts with Oxygen (O_2) thereby forming carbon dioxide (CO_2) reducing free O_2 and creating an anaerobic environment that can reduce the development of anaerobic populations.

2.6.1. LAB antimicrobial activities

Inhibition or reduction of microbial growth using LAB is gaining momentum (Castellano et al., 2017). Gupta et al., (2018) state that probiotic LAB produces numerous anti-microbial compounds. LAB are well known to produce various compounds, such as hydrogen peroxide, carbon dioxide, diacetyl, bacteriocins and bacteriocins like substances known for their antimicrobial activities (Sanlibaba et al.,2015). With lactic acid and acetic acid bacteriocin and bacteriocin-like being the best (De Vuyst & Leroy, 2007). Significantly, sugar metabolism produces organic acids, especially lactic acid (Gutiérrez-Cortés et al., 2017). These are released into the environment reducing its pH, preventing the development of some populations of undesirable microorganisms (Gutiérrez-Cortés et al., 2017). *Lactobacillus* sp. acts by rapidly colonising in intestinal epithelial which they disorder growth and proliferation of entero-pathogens together with the production of bacteriocins, Lactic acid and reducing pH (Sanlibaba et al., 2015). Due to its low pH, lactic acid is toxic to many bacteria, fungi, and yeasts. Additionally, different microorganisms are also sensitive to lactic acid (Sanlibaba et al.,2015). Furthermore, hydrogen peroxide (H_2O_2) has an oxidizing effect over sulfhydryl groups of membrane proteins and lipids, consequently, damaging the cell wall of some microorganisms (Gutiérrez-

Cortés et al., 2017). A report by Schmid, Saengerlaub & Mueller (2016) revealed that these antimicrobial agents must be stable for processing, and hence should not change the sensorial properties of food, as well as the deliciousness. Consequently, antimicrobial effect of LAB has been utilised by humans for many years and has enabled the manufacturer to extend the shelf life of many foods via fermentation processes (Saranraj et al., 2013). Moreover, most research has focused on the production and application of bacteriocins from *Bifidobacteria*, *Lactobacillus* and *Pediococcus* (Cheikhyoussef et al., 2009). However, very little research on LAB produced from natural vegetables has been done (Gaamouche et al., 2014).

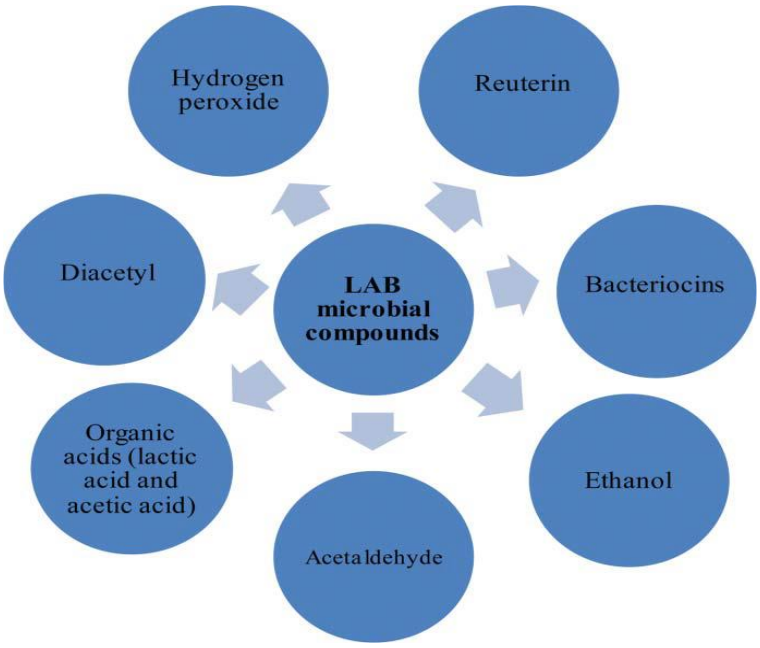


Figure 2 Some of the antimicrobial substances excreted from LAB (Özogul & Hamed, 2018)

Although bacteriocins have the ability to inhibit bacterial growth, they are less likely to induce resistance (Castellano et al., 2017). Some studies have evaluated the effect

of LAB Cell-Free Supernatants (CFS) on the reduction or elimination of food borne pathogens. It was demonstrated that bacteriocins produced by the probiotics may exhibit antimicrobial activity which would result in suppression of the growth of the indicator organisms (Kadhim Isa & Razavi, 2018). Previous study by Oliveira et al, (2008) report that LAB produce bacteriocins that are responsible for the antagonists' effects that reduce bacterial growth as observed in the research. When administered in food individually or in combination, some LAB appear to be effective in controlling pathogen growth in various food products (Smith et al., 2005).

2.6.2. Lactic Acid (LA)

Acetic and Lactic acids are two of the most studied and more widely used among different organic acids for carcass decontamination (Dickson & Acuff, 2017). Lactic acid is produced by the L- or D-isomer form of LAB (Sanlibaba et al., 2015). Pharmaceutical applications prefer L-lactic acid and as starting material in the production of biopolymers, unlike D-lactic acid, which is toxic for humans, therefore not preferred. Furthermore, the two isomers differ in antimicrobial activity, L-lactic acid being more inhibitory than the D-isomer (Sanlibaba et al., 2015). The most important mechanism by which LAB inhibit pathogens through acid production where bacteria are slowly inactivated at the same time as the pH gets lower (Gao et al., 2019). In the same way, they act by providing an acidic environment that is unfavourable for the growth of most ram-negative pathogens that are responsible for food poisoning and food borne diseases (Özogul & Hamed, 2018).

The type of species or strain, culture composition and growth conditions influence the production of lactic acid and reduction of pH (Sanlibaba et al., 2015). Thus,

decontamination of meat can be attained by sprays or washes with water or antimicrobial solutions such as acetic acid or lactic acid (Dickson et al., 2017). Consequently, the reduction in microbial numbers on the meat surface is due to physical removal of the microorganisms, a killing effect of the decontaminating solution or a combination of both factors depending on the treatment (Dickson et al., 2017).

Lactic acid plays a vital role in the agricultural, food, and clinical sectors (Ayivi et al., 2020). The use of organic acids such as lactic acid and carcass washes using hot water are a few of the most common intervention techniques within the cattle industry (Laury et al., 2009). The molecular basis for bacterial attachment has been reviewed by (Castillo et al., 1998). Beneduce, Spano, & Massa (2003) reported that once bacteria are attached to meat, rinse solutions such as acetic acid or trisodium phosphate (TSP) were not effective in removing a large part of the contaminating bacteria. Lactic acid is more effective than acetic acid in reducing *E. coli* O157:H7, but effective as acetic acid in reducing *S. typhimurium* on beef carcass surfaces (Dickson et al., 2017).

2.6.3. Bacteriocins of LAB

Bacteriocins are ribosomal-synthesised antimicrobial peptides with narrow to broad antimicrobial activity. They are produced by some species of Gram-positive bacteria including LAB (Da Costa et al., 2019). Bacteriocins inhibit the growth of pathogens by creating pores in the membrane of the pathogenic bacteria resulting in disrupting the permeability of the membrane (Rossi, 2016). Bacteriocins are extremely specific against closely related bacteria making them widespread amongst LABs

(Castellano et al., 2017). Since the majority of LAB have GRAS status they have great potential application in food bio-preservation and for this reason, they are by far most studied due to the food origin (Castellano et al., 2017), with a high emphasis on milk and dairy products derivatives, while meat and meat products isolates were less studied (Da Costa et al., 2019). Above all, Rossi (2016) narrates that historically bacteriocins application in meat and poultry has been used as a barrier technology in the same way its application in fresh produce has recently been explored.

Studies by Castellano et al., (2017) explain how several approaches were used to classify bacteriocins, which sub-divided them into three classes based on their mode of action and structure. Class I bacteriocins including lanthionine as such in nisin, class II bacteriocins of small heat-stable, non-lanthionine containing peptides, Class III bacteriocins of relatively large molecular weight and heat stability was suggested (Saranraj, Naidu & Sivasakthivelan 2013). Class IV complex bacteriocins contain chemical moieties such as lipid and carbohydrate (Sanlibaba et al., 2015). The most thoroughly studied bacteriocin to date is nisin and it is produced by *Lactococcus lactis*, its biggest application as food additives has been used in certain foods including cheese, canned food and cured meat (Sanlibaba et al., 2015). Most LAB bacteriocin-producing microorganisms appear to have a relatively narrow inhibitory spectrum or broad-spectrum, nisin and pediocin are active against a wide range of bacterial spectra (Saranraj et al., 2013, Sanlibaba et al., 2015). Moreover, nisin is the only bacteriocin licensed as a food preservative in over 50 countries and the only bacteriocin that is approved with FDA and GRAS status for use in food products (Castellano et al., 2017). Rossi (2016) further emphasised that the effectiveness of bacteriocins weakens through storage, and proteolytic degradation.

2.6.4. Mode of action of Bacteriocins

Most bacteriocins interact with the cell membrane anionic lipids of the target bacteria, causing their permeabilization through the formation of pores (Da Costa et al., 2019). This interaction ultimately causes the death of the target cell, promoting the degeneracy of the proton motive force (PMF) and the inhibition of amino acids transport (Da Costa et al., 2019). They further explain that several processes such as the accumulation of ions and metabolites, and adenosine triphosphate (ATP) synthesis involve the proton motive force (Da Costa et al., 2019). Ex-situ and in-situ production are the two common approaches used by food producing company in the application of bacteriocins for food bio-preservation (Solomakos et al., 2008). In ex-situ production, involves the addition of either purified or semi-purified bacteriocins as food preservatives, which is dependent on legal approval/regulations or the use of a previously fermented product with a bacteriocin-producing LAB strain as an ingredient in the food (Da Costa et al., 2019).

In situ production, involves the inoculation of food with bacteriocinogenic LAB strains, the ability of these strains to grow and produce bacteriocins in the products being crucial for its successful application (Da Costa et al., 2019). The mechanism of action of nisin involves binding to the peptidoglycan layer resulting in the formation of transient pores, which causes the weakening of the cell membrane this allowing leakage of intracellular metabolites and dissipation of membrane potential (Solomakos et al., 2008).

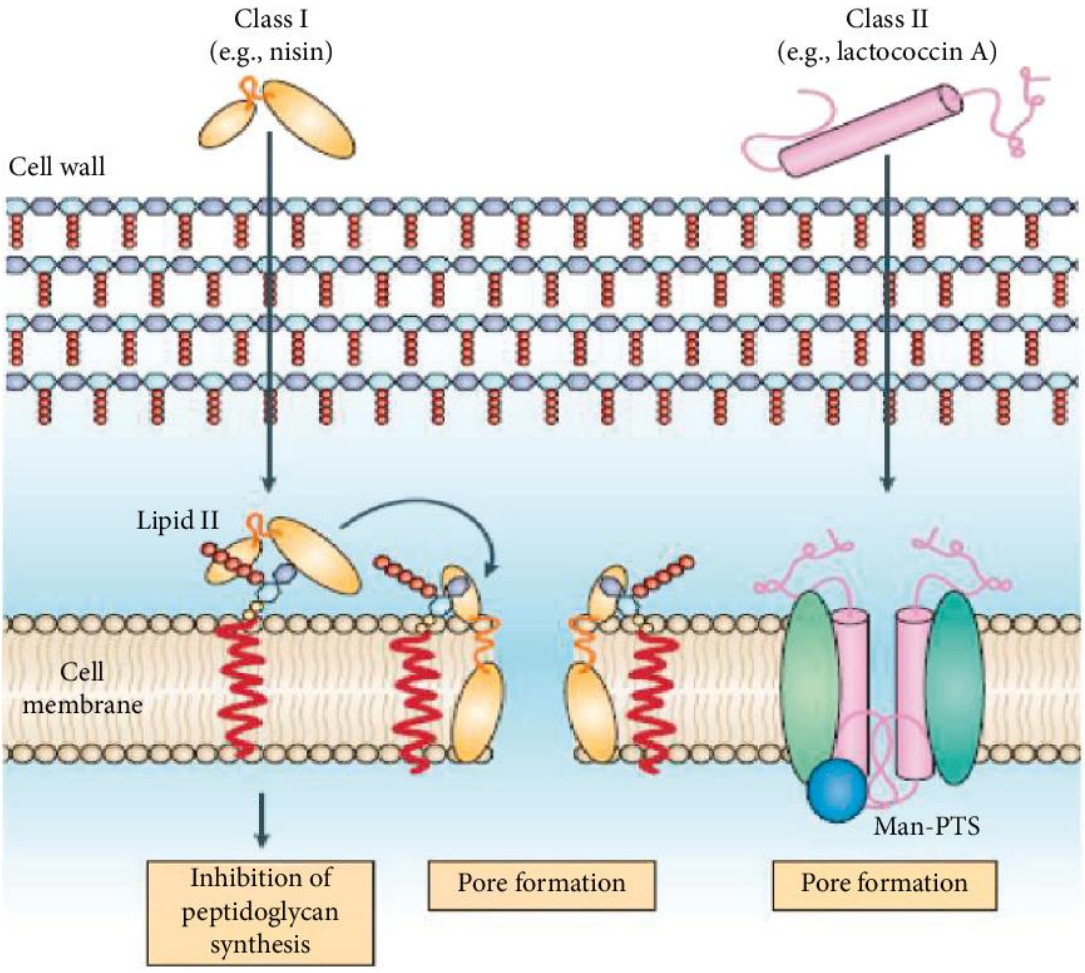


Figure 3 Mechanism of action of bacteriocins on Gram-positive bacteria (Da Costa et al., 2019).

2.7. *Salmonella*

Salmonella is Gram-negative, non-spore-forming, rod-shaped and their cells move by means of a peritrichous flagella (Gut et al., 2018). They are facultative anaerobes and are members of the Enterobacteriaceae group (Forsythe et al., 2000, Food Safety Authority of Ireland, 2011). There are about 2000 different serotypes of genus *Salmonella*, since there are some new serologically distinct strains frequently isolated this number is increasing (‘Food poisoning and other food-borne hazards’, 2000).

Based on their 16S rRNA sequence analysis, the genus *Salmonella* is further classified into two main species, *Salmonella enterica* and *Salmonella bongori* (Characteristics, 2011; Eng et al., 2015). *Salmonella enterica* has led to further classification into subspecies, including *enterica, salamae, arizonae, diarizo- nae, houtenae* and *indica* (Gut et al., 2018). *Salmonella enterica* is the most significant for human health (Characteristics, 2011). Zhang, Jeza and Pan (2008) claim that *Salmonella* has more than 2,300 closely related serovars known, of which *S. typhi* and *paratyphi* are pathogenic exclusively to humans. They cause systemic infections and typhoid fever. On the other hand, *S. typhimurium* also causes gastroenteritis. *Salmonellas* optimum growth temperature is about ±37 °C, they are fairly heated sensitive being killed at 60 °C in 15-20 minutes, and they do not grow at a temperature below 8 °C (Zhang, Jeza & Pan, 2008). Salmonellas are characterised by their ability to ferment glucose with the production of acid and gas, and their inability to attack lactose and sucrose ('Food poisoning and other food-borne hazards', 2000).

2.7.1. *Salmonella* Pathogenesis

Once human ingest food or water contaminated (Gut et al., 2018)with *Salmonella,* infections may occurs, the organisms multiply over the period of 1-3 weeks in the small intestine, they may further breech the intestinal wall, and spread to other organ systems and tissues (Zhang et al., 2008). Once the bacteria enter the lamina propria it multiples and produces inflammatory mediators recruits neutrophils and which further triggers inflammation, inflammation then causes release of prostaglandins from epithelial cells and Prostaglandins cause electrolytes to flow into lumen of the intestine, diarrhea is then caused in response of the water flows into lumen (Jong

2000). *Salmonellas* are motile and short 1-2 pm ('Food poisoning and other food-borne hazards', 2000), thus they uses their flagella as a mode of movement as well as chemotaxis to target cells, the enterocytes (Gut et al., 2018). Investigation by Eng et al. (2015) stated that during *Salmonella* invasion of non-phagocytic human host cells, it in fact induces its own phagocytosis in order to gain access to the host cell, which remains an outstanding characterises. Pathogenesis of *Salmonella* into the host cell depends on its ability to persists, if the strain however lacking this ability are non-virulent (Eng et al., 2015). It has been reported that the serotype involved determines the severity of *Salmonella* infections in human combined with health status of the human host e.g. children below the age of 5 years, elderly people and patients with immunosuppression are more susceptible to Salmonella infection than healthy individual (Eng et al., 2015). *Salmonella* then attaches to the receptive epithelial cells and internalization into lamina propria which promotes the release of pro-inflammatory cytokines, the latter causes acute inflammatory responses which lead to ulceration, diarrhoea and the destruction of the mucosa (Gut et al., 2018).

2.7.2. Virulence factors of *Salmonella*

Salmonella is able to colonize its host through attaching, invading, surviving, and bypassing the host's defence mechanisms with the help of virulence factors either individually or in combination with others (Jajere, 2019). The presence of virulence capsular polysaccharide, Vi, in *S. Typhi* is the major distinction between *S. Typhi* and *S. Typhimurium* (Parween, Yadav & Qadri, 2019). The inhibitions phagocytosis and conferring serum resistance are mode of action for Vi capsule this is due to *shielding the O-antigen from antibodies* (Johnson, Mylona & Frankel, 2018). Colonization, adhesion, invasion, production of toxins (LPS) (endotoxin, enterotoxin, cytotoxin),

and survival inside the host cells are various stages of infection involved in virulence factors in *Salmonella typhi* (Achparaki et al., 2012). *Salmonella*'s gene that encode for the virulence factors may be divided into two major categories, genes, which are located on chromosomes, (like stn) primarily *Salmonella* pathogenicity islands (SPIs) and lastly genes which are located on the virulence plasmid (Jajere, 2019). *Salmonella* enterica subspecies is classified into serovars, based on O (lipopolysaccharide) and H (flagellar) antigens. *Salmonella* serovars are further classified into two groups: typhoidal and nontyphoidal *Salmonella* (NTS). Infection in human can results in two outcomes which mainly depends on the infecting serovar (Johnson et al 2018). Intracellular survival and replication of *Salmonella enterica* serovar *typhimurium* are important virulence determinants and the bacteria can be found in a variety of phagocytic and non-phagocytic cells in vivo (Ibarra & Steele-Mortimer, 2009).

The pathogenicity of *Salmonella* is interceded by various genes *encompassing invA, fimA, stn, spvR, spvC, spiC* and *pipD* (Nikiema et al., 2021). Factors such as flagella, capsule, plasmids, adhesion systems, and type 3 secretion systems (T3SS) encoded on the *Salmonella* pathogenicity island (SPI)-1 and SPI-2 and other SPIs have been demonstrated to play variety of roles in the pathogenesis of *Salmonella* infections (Jajere, 2019). In *Salmonella* seventeen SPIs (SPI-1 to SPI-17) have been identified which contribute to the virulence of *Salmonella* along with several genes like *Spv* operon which are located on the plasmid (Jajere, 2019). The *invA* gene of *Salmonella* is also intricated in invasion of host epithelial cells, located on the pathogenicity island 1 (SPI1) and has been widely studied for its ability to promote virulence and as a biomarker for the detection of *Salmonella* spp. (Nikiema et al., 2021)

These factors bring about actin cytoskeletal rearrangements resulting in membrane ruffling and bacterial uptake into the epithelial cells which is due to activation of membrane GTPase (Parween et al., 2019). *HylE* gene produces *HylE* protein, which is another important virulence factor for *Salmonella*, some studied confirmed that production of *HylE* by *Salmonella* and other enteric bacteria plays a crucial role in the pathogenesis of *S. Typhi* (Jajere, 2019)

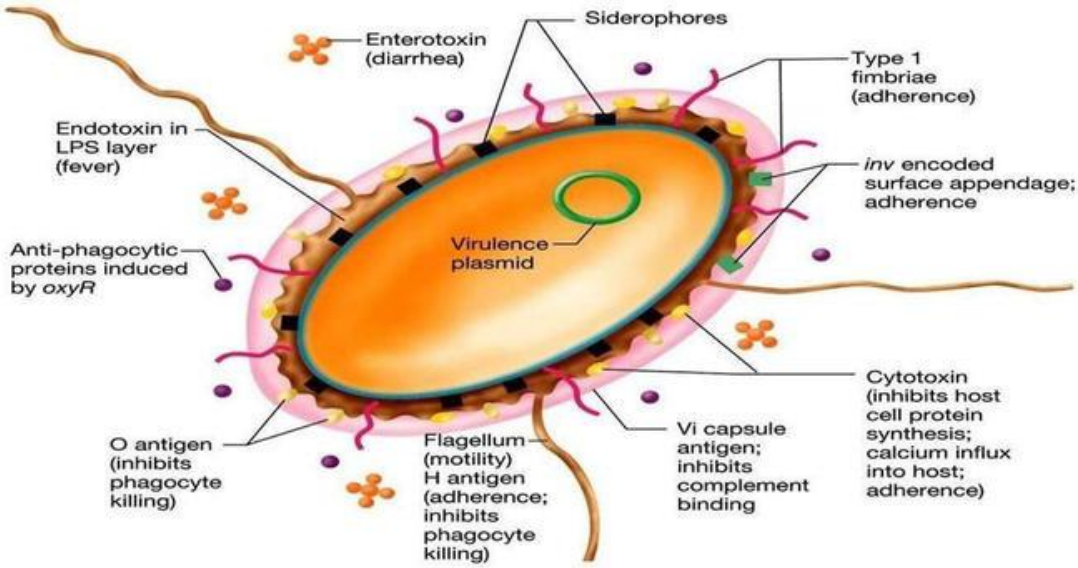


Figure 4 Virulence factors of *Salmonella typhi* (Achparaki et al., 2012).

2.7.3. *Salmonella* Laboratory Diagnosis

The six serotypes mentioned above by Gut et al. (2018) are detected on the basis of their antigenicity. The presence of *Salmonella* O and H antigens is tested by Widal method, that is quite useful in areas where resources are limited. They further explained that the Widal method can cross-agglutinate with other non-*Salmonella* Enterobacteriaceae bacteria but does not distinguish *Salmonella* species or their serotypes.

2.8. *Escherichia coli* O157:H7

E. coli are a large and diverse group of bacteria of the family Enterobacteriaceae. Like *Salmonella*, they are also found in the lower intestine of warm-blooded organisms (Daly, 1986). The genus *Escherichia* was isolated by Dr Theodor Escherich, the species *E. coli* was named after Dr Theodor Escherich (Woodward, 2015). He further discovered that organisms are rod-shaped, Gram-negative bacilli and explains that they comprised literally hundreds of serotypes (Daly, 1986). They are non-fastidious organisms, bile-tolerant that are easily cultured on routine laboratory media (Adamu, Shamsul, Desa and Khairani-Bejo, 2015). There are several serotypes found in E*nterohemorrhagic Escherichia coli* (EHEC), which are linked with human diseases such as O26:H11, O91:H21, O111:H8, O157: NM, and O157:H7. Of these serotypes, *E. coli* O157:H7 is being the most frequently isolated from patient worldwide (Dias, Vera Junn, Eunsung & Mouradian, 2008).

The first confirmed case of *E. coli* O157:H7 was in the United States of America in 1975 from a woman who had bloody diarrhoea and the first isolated case from cattle was reported in 1977 (Tshabalala, 2011). The first outbreak of *E. coli* 0157:H7 was in 1982, the same year it was identified as a human pathogen (Tshabalala, 2011; Stein & Katz, 2017). The first outbreak of *E. coli* O157:H7 was caused by undercooked meat reported from humans which caused gastrointestinal illness. Thereafter, the bacterium became globally distributed. Most identified outbreaks were community-acquired and transmitted by the food and water routes (Stein et al., 2017).

Generally, not all *E. coli* are harmful. They aid in food digestion; production of vitamin K and B-complex vitamins and they prevent the colonisation of harmful

bacteria within the intestine of their hosts. They become part of the normal flora (Daly, 1986). Apart from strains that exist as harmless symbionts, a variety of diseases in animals and humans are caused by some pathogenic *E. coli* strains that can temporarily be found in the gastrointestinal tract. Additionally, these pathogenic *E. coli* strains are more likely to express virulence factors. This characteristic differentiates them from those that predominate in the enteric flora of healthy individuals and animals (Donnenberg & Whittam, 2001). One of these *E. coli* strains is *E. coli* O157:H7 which is known as an important human pathogen (Padhye & Doyle, 1992).

E. coli O157:H7 is linked to illnesses like diarrhoea, urinary tract infections, pneumonia, and other clinical diseases (Daly, 1986). Entero-pathogenic diarrhoea causing *E. coli* reveals that they are grouped into four categories. This categorisation is based on their virulence properties, clinical syndromes, differences in epidemiology, and distinct O:H serogroups (Padhye et al., 1992). The four main categories are entero-pathogenic *E. coli* (EPEC), entero-invasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), and Enterohemorrhagic *E. coli* (EHEC). The serotype *E. coli* O157:H7 is Enterohemorrhagic and has become a major concern for cattle producers and their customers (Daly, 1986). This strain produces Shiga toxins that can damage the lining of the human intestine and other tissues. It is important to note that even though *E. coli* O157:H7 causes illness in humans, the bacteria do not cause disease in cattle (Daly, 1986).

2.8.1. *E. coli* 0157 Morphology and Pathogenesis

Nunnelly (2012) defined *E. coli* O157:H7 as a Gram-negative, flagellated, rod-shaped organism. The *E. coli* O157:H7 strain can be distinguished based on antigens associated with that strain, this can be done using an "O" and "H" naming system. The "O" designation describes the particular antigen associated with the cell wall of the microbe, while the "H" designation refers to the flagella antigen of the cell. This O:H combination is called the serotype. The pathogenicity of STEC is determined by several virulence factors that are encoded by chromosomal pathogenicity islands, phage chromosomes integrated in the bacterial genome as well as plasmids (Nunnelly 2012). Pathogenic forms of *E. coli* is associated with human and animal diseases (Donnenberg & Whittam 2001), which primarily inhibit the intestinal tract of warm-blooded animals including humans (Adamu et al., 2015), with the prevalence of *E. coli* O157:H7 in the feedlot cattle faeces of dairy calves and is low (Dean-Nystrom et al., 1997).

E. coli O157:H7 infect human and can display different clinical manifestations, where some infected individuals present intestinal and extraintestinal manifestations and some remain asymptomatic (Stein et al., 2017). The strains of serotype O157:H7 belong to a pathogenic Enterohemorrhagic *E. coli* (EHEC) which cause hemorrhagic colitis, bloody or non- bloody diarrhea, and hemolytic uremic syndrome in humans, where cattle are significant reservoirs of EHEC O157:H7 strains (Dean-Nystrom et al., 1997). There are different strains *E coli* which are associated with number of typical diarrheal illnesses which includes the Enterotoxigenic *E coli* (ETEC), Enteroinvasive *E coli* (EIEC), and Shiga toxin–producing *E coli* (STEC). Of the STEC The *E. coli* strains has incubation period which ranges from 10 hours to 6 days

however incubation period for *E. coli* O157:H7 is usually 3 to 4 days (Dean-Nystrom et al., 1997). USDA's Food Safety and Inspection Service (FSIS) reported that if as little as 1 cfu of EHEC O157:H7 is detected in 25 g of ground beef is considered adulterated.

2.8.2. *E. coli* O157:H7 Laboratory Diagnosis and Treatment

Culture isolation techniques used to differentiated *E. coli* O157:H7 from other *E. coli* due to inability to ferment sorbitol, lack of β–glucuronidase enzyme and slow or no growth at temperatures above 44 °C, even though in some cases atypical O157 strains may ferment sorbitol (Beneduce, et al., 2003). Padhye & Doyle (1992) in the same way explained that biochemical reactions of *E. coli* 0157:H7 isolates show similar characteristic of *E. coli*, apart from sorbitol fermentation and β-glucuronidase activity. Moreover, they further explained that about 93% of *E. coli* isolates of human origin ferment sorbitol within 24 h where *E. coli* Ol57:H7 does not similarly, 93% of *E. coli* strains possess the enzyme that is crucial for the development of a rapid fluorogenic assay for *E. coli*.

E. coli 0157 cab be diagnosed using selective media e.g. MacConkey agar base with sorbitol, alternatively serologic diagnosis using enzyme immunoassays may (ELISA) be used to detect serum antibodies to the O157:H7 lipopolysaccharide (Makvana & Krilov 2015), there after strains can then be identified by serotyping using specific antisera(Jong 2000). A sensitive isolation method, using enrichment and selective procedures may be selected for low infectious dose of *E. coli* O157:H7 in foods, this led to studies of further development of selective enrichment culture media specific for *E. coli* O157:H7, these culture media contains bile salts, novobiocin, cefclidine

and cefixime as selective agents (Beneduce, et al., 2003). Pending isolation of the organism from cultures, patient who are suspected of having a systemic infection with *E. coli*, especially infants may receive intravenous antibiotic treatment(Makvana & Krilov 2015)

2.9. *Salmonella* spp. and *Escherichia coli* O157:H7 contamination in slaughterhouses

Meat is an important source of protein and in the same way also a potential source of disease if not properly prepared or handled (Schmid et al., 2016). Fresh meat is a sensitive product, it needs to be packed in appropriate packaging materials with sufficient high gas barrier properties to avoid food borne diseases (Schmid et al., 2016). *Salmonella* is a leading cause of foodborne disease and is often associated with the consumption of foods of animal origin (Zhou, Li, Hou, Wang, Paoli, & Shi, 2019). Similarly, Schamberger et al. (2004) report that *E. coli* O157:H7 is a food-borne pathogen that has been commonly associated with outbreaks attributed to the consumption of meat, produce, and water that had been contaminated with cattle manure.

According to research done by Smith et al. (2005), Centres for Disease Control and Prevention (CDC) report that *Escherichia coli* O157:H7 causes an estimated 73,000 cases of illness and 61 deaths each year while *Salmonella* is associated with 40,000 reported cases of illness per year in the USA alone. Therefore, *Salmonella* and *E. coli* O157:H7 are common sources of foodborne diseases, resulting in most death worldwide (Zhou et al., 2019). The goal is to minimise bacterial contamination of

the carcass and eliminate contamination effectively from the slaughter processes (Buege & Ingham, 2003).

The intestinal tract of most mammalian species, including humans and birds, are colonised by *Escherichia coli*, *E. coli* is one of its main inhabitants, most of which is harmless, however, a small proportion of them are a cause of disease worldwide (Rigobelo, Ávila & Karapetkov, 2013). Animal slaughterhouses, the process workers, and the processing environment are the major sources of the initial microbiota found on meat carcasses. Due to this colonisation of bacteria in the gut of animals, in recent years more research are done aiming at strategies to prevent the colonisation of pathogens or reduce their population in the gut (Rigobelo et al., 2013). It is imperative that we recognise how environmental and processing conditions can affect the presence of food-borne *Salmonella* and *E. coli* O157:H7 in food (Berry & Cutter, 2000). Human health is, therefore, threatened by the direct and indirect spread of *Salmonella* and *E. coli* O157:H7 between animals and humans (Zhou et al., 2019). Buege and Ingham (2003) explain that no matter how carefully processes are carried out, contamination of bacteria to the carcass occurs, some of these bacteria can be pathogens such as *Salmonella* and *E. coli* O157:H7.

Hides are the primary source of carcass contamination most of the interventions have been focused on post-harvest. Therefore, the rate of diffusion of fermentable substrates from within the meat to the surface depends on the growth of bacteria on the surface (Tshabalala et al., 2012). In recent years, cattle packing plants have implemented several intervention strategies to decrease bacterial contamination on carcasses e.g., organic acid rinses, steam treatment (Callaway et al., 2008).

Antimicrobial interventions selected and used in food production depends upon factors such as desired effect, legal limits of use, cost, and effect on the food (Wheeler & Bosilevac, 2014). Beef packing plants introduced lactic acid solutions as a decontaminant, the solution may also be applied to beef cuts and trimmings, to control contamination with pathogens, specifically *E. coli* O157:H7 and *Salmonella* (Youssef et al., 2012). Most commercial plants for both beef and lamb use organic acids solution at 1.5 to 2.5 % solution for pre-chilled carcasses, treatments are most effective when applied warm (50 - 55 ° C) on carcasses (Wheeler et al., 2014). Meatco currently uses hot water, knife trimming and lactic acid solution at 4 – 5 %. Hot water and lactic acid solution are prepared and applied to the carcass on the slaughter floor just before chilling whilst knife trimming forms part of the critical control point (CCP). Meatco started using the lactic acid solution in 2015 and according to the Meatco monthly report compiled by the SHEQ department, the *S. typhimurium* and *E. coli* O157:H7 detection drastically reduced in meat samples by 35%. However, Meatco still wants to achieve zero detection on both *E. coli* O157:H7 and *S. typhimurium. E. coli* O157:H7 on lean and adipose tissues of beef carcasses can also be reduced when treated with 1%, 3%, or 5% acetic acid, lactic acid, or citric acid, where spraying with commercial antimicrobial compound Beefxide (containing blended lactic and citric acids) was recorded to reduce the populations of *E. coli* O157:H7 and *Salmonella* by 1.4 and 1.1 log cfu/100cm^2 on inoculated fresh beef application for 20s (Wheeler et al., 2014).

Company bankruptcy, loss of work and financial loss which sometimes result in business closure are some of the aftermaths of positive pathogen detections from meat samples from exporting abattoirs (Klaus, 2007). A recent example is the

listeriosis outbreak in 2017–2018 from *Listeria monocytogenes* food poisoning that resulted from contaminated processed meats produced by Tiger Brand in South Africa. Therefore, to the best of my knowledge, it can never be over emphasised that prevention is the key, food handlers are advised to practice good hand washing, storing food at the right and monitored temperature, and keeping food preparation areas clean.

2.10. Survival and growth of foodborne pathogens in meat in the slaughterhouse

Farm animals and wild animals are frequently found to carry *E. coli* STEC and *E. coli* O157 in a wide range. But for the most part, *E. coli* STEC and *E. coli* O157 typically do not cause disease in animals. The primary habitat of *E. coli* is the intestinal tract in both both in human and warm-blooded animals. All transmission to humans is by means of ingestion of contaminated foods (Rigobelo et al., 2013). Furthermore, infections of *E. coli* O157:H7 to humans can be attained in number of way which includes person-to-person transmission, through foodborne & waterborne routes, through farm visits & animal contact and very rarely from improperly chlorinated swimming pools (Stein & Katz, 2017). *Salmonella* infection on meat can be due to contamination during the slaughtering, dressing and deboning processes, alternatively during processing, transport, storage and from cross-contamination (Djordjević et al., 2018). Mincing pork and beef also allows the migration of surface bacteria throughout the product (Djordjević et al., 2018).

During the slaughtering processes, raw meat and minced beef can be directly contaminated by cattle faeces and human cross-contamination. Ground beef hamburgers, ready-to-eat cold meats including poultry, pork and beef products,

cheese, milk, butter, yoghurt, ice cream, etc. have acted as vectors (Adamu et al., 2015). Numerous preservation techniques e.g. vacuuming of chilled meat, have been developed in the food industry to prevent and control *Salmonella, E. coli* O157:H7 and spoilage microorganisms in fresh meat products, improving and extending its shelf life at the same time making the meat safer for consumption (Djordjević et al., 2018)**.** During production, slaughterhouses implement preventive hygienic measures aimed to achieve a low initial adulteration and control the safety of meat. These may include chemical preservatives which have a reputed inhibitory effect on other foodborne pathogens such as *Salmonella, L. monocytogenes*, and *S. aureus* (Patarata et al., 2020). The growth of *E. coli* O157:H7 can be controlled by the lactic acid spray which may be applied to beef cuts and trimmings, to control and reduce contamination (Youssef et al., 2012). Inactivation of EHEC in food can be achieved by thermal processing, application of high-pressure processing, use of high-energy gamma rays as food irradiation, ozone coupled with chlorine, one of the most commonly used disinfecting agents, and Electrochemically Activated Water (EAW) (Adamu et al., 2015).

Research by Saeedi et al. (2017) on strategies to decrease *E. coli* O157:H7 risk in animals conclude that there are only two licensed vaccines actively and currently applied in cattle and two unapproved ones for humans and cattle immunisation; this is one of the most promising interventions strategies that can be applied to minimise the exposure of livestock to the pathogen before they reach the slaughter houses. Cattles that are delivered to Meatco for slaughter are not vaccinated, farmers are also not aware of this vaccine (Meatco livestock procurement Department). However, with continuous improvement, food safety is getting more attention, with emphasis

on both inactivation of foodborne pathogens and or keeping microbial count in food below regulatory standard (Gao et al., 2019). Meatco does conform to microbial specifications set by both its own customers and from the government. Whenever *S. typhimurium* and *E. coli* O157:H7 counts are detected on meat, the batch is not shipped to any customers, thus, it is the company loss. The latter conforms to the policy passed in 1994 by USDA-FSIS which instituted a zero-tolerance policy for *E. coli* O157:H7 in ground beef (Laury et al., 2009).

2.11. Vacuumed packed beef in slaughterhouse

Seideman et al. (1983) defined vacuum-packaging as the distribution of beef primal cuts in oxygen impermeable bags under vacuum which results in a product that is maintained in an oxygen-deficient environment to achieve its preservative effect. Vacuum-packaging of beef is the standard method used in the food industry: removing oxygen from the packaging prevents the growth of aerobic organisms responsible for spoilage and allows storage of meats for many weeks (Renerre & Labadie, 1987). In the past years, beef distribution methods have changed from the shipment of carcasses as quarters to the shipment of primal or sub-primal cuts from areas of production to areas of consumption (Seideman & Durland, 1983). Vacuum packaging, modified atmosphere packaging (MAP) and refrigeration have become widespread to improve and extend the shelf-life of fresh meat and conserve the sensory characteristics inherent to the product for a period longer than its Shelf-life (Kalchayanand et al., 1989). Nassu et al. (2010) explain that gas-packaging's is aimed to slow respiration rates of food products that are packaged in high barrier material in which the gaseous environment has been changed. Vacuum-packaging and gas-packaging reduce the microorganisms which maximise the sensory quality

of the product, including the colour, odour and deliciousness (Nassu, 2010). Therefore, there are two factors of foremost importance in red meat packaging, the meat colour and microbiological content (Matche, 2001). The retail industry uses the top three methods which are overwrapping with permeable film, vacuum-packaging and gas-packaging as a result meat is displayed in an attractive, hygienic, and convenient form in plastic materials which allow easy consumer evaluation (Renerre et al.,1987). Most of all, consumers relate the bright red colour of the meat to freshness and discolouration to bacterial growth (Renerre et al.,1987). Moreover, the application of vacuum packaging results in increased technology a development which has closely paralleled the use of the "boxed beef" notion, consequently, it prolongs the shelf-life and deliciousness of the beef throughout extended periods of shipment and storage (Siedeman et al., 1983). To extend the shelf-life of fresh meat, vacuum-packaging and refrigeration have become popularly used (Siedeman et al., 1983). Therefore, storage temperature and the type of packaging material greatly influence beef spoilage microflora (Tshabalala, 2011). *Lactobacilli, Leuconostoc, Enterobacteriaceae* and *Brocothrix thermosphacta* are the major bacteria associated with spoilage of vacuum-packaged refrigerated meat at pH of 5.5 to 5.8 and temperatures nearing 0 °C (Kalchayanand et al., 1989).

The spoilage is linked with sour, acid, and cheesy odours from the production of short-chain fatty acids and other organic acids (Kalchayanand et al., 1989). To minimise deterioration and extend the shelf-life of meat and meat products, special care should be applied during handling. Kalchayanand et al. (1989) further explain that the number and type of bacteria initially present and their further growth in the ecological conditions applied during storage, particularly temperature, pH and

gaseous atmosphere determine the shelf-life of meat and meat products (Kalschne et al., 2015). In addition to the latter, the two major important factors during the packaging of red meat, are colour and microbiological content. Therefore, the packaging material selected has to be selected very carefully to protect these qualities of meat and its products (Matche, 2001). Equally important, a change in colour, undesirable odour and slime formation are the main criteria for rejection in the meat industry (Hernández-Macedo et al., 2011).

Blown packs are defined as the vacuum-packs of chilled meat that leads to pack distension which produces gas from microbial deterioration (Hernández-Macedo et al., 2011). The "blowing" of packages of vacuum-packaged chilled meat by microorganism is currently one of the challenges facing the meat industry, which hinders the slaughter and marketing of bovine meat (Hernández-Macedo et al., 2011). The loss of vacuum and subsequent entry of oxygen into the package could result in an environment even more deleterious to meat quality than the total absence of packaging. The advantages of vacuum packaging are that meats are generally quite stable at low temperatures which prolong the shelf life of meat (Hernández-Macedo et al., 2011) while MAP has a shorter shelf-life (Renerre et al.,1987), preservation of muscle colour in its freshest state due to lack of oxygen, preservation of improved hygiene due to the exclusion of external contamination; provision of a means of prolonged edibility and not limited to providing of an ideal environment for the ageing of beef (Siedeman et al.,1983).

The drawbacks of vacuum packaging are but not limited to, the process is costly, the impermeable films used compared to direct shipment of carcasses, and vacuum

packaged meat can endure more abuse due to its dominance in gas control. Nassu et al. (2010) conclude in their research that more studies about interactions of the packaging with the meat and the use of emerging technologies and nanotechnology are required. It is important to note that this was not the focus of this research. Most export abattoirs such as Meatco that are involved in the exportation of fresh meat make use of vacuum-packaging to protect their meat during transportation. Therefore, the shelf-life of meat depends on the quality of the packaging material, the number and type of bacteria initially present, and the ecological conditions applied during storage, predominantly temperature, pH and gaseous atmosphere (Kalschne et al., 2015).

2.12. Current Intervention Strategy in slaughterhouse

Over the past 20 years, probiotics have been the topic of many studies and researches (Rigobelo et al., 2013). In 2015, the probiotic market has reached $33.19 billion and was expected to reach $46.55 billion by 2020, demonstrating a 7.0% annual growth rate. The Asia pacific dominated the probiotic market followed by Europe in 2014, this was credited to the dietary supplements increasing demand (Park et al., 2016). LAB play an important role in food, agricultural, and clinical applications (Britis, 2018). The use of LAB has been successful in reductions of various pathogens growth in ground beef and live cattle, this is because of its ability to produce antagonistic and antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins (Dow, Alvarado & Brashears, 2011). Several studies have been conducted to understand how LAB can successfully inhibit other bacteria as well as their various areas of application in the food supply (Dow et al., 2011). Even though there are reports demonstrating how probiotic bacteria could reduce the levels of *E.*

coli O157:H7 in cattle, there is little information available about the ecological variables affecting their performance (Schamberger et al., 2004). LAB bacteriocins are safe, therefore, they are by far the most studied due to the food origin and GRAS status of producer strains (Castellano et al., 2017).

Bio-protection antagonistic microorganisms or their metabolic products were being used, which control undesirable (pathogen and contaminant) organisms that extend shelf-life and enhance food safety without altering the sensory characteristics of the food product (Castellano et al., 2017). Different treatments have been designed and studied to decontaminate beef carcasses, these include the use of sanitising agents, such as hot water or organic acid sprays, the two have been intended with the purpose of reducing pathogens on beef carcasses (Castillo et al., 1998). During dressing, carcasses can get contaminated with bacteria and pathogens, however, if cleaning and disinfection are inadequate, considerable quantities of dirt which include hair or ingesta may still be present (Snijders et al., 1985). The control of pathogens in cattle depends on interventions intended to decrease bovine faecal shedding, this is because pathogens are widely distributed in cattle's (Schamberger et al., 2004) and some interventions may have a direct bacterial effect (Rigobelo et al., 2013).

While past studies mostly focused on microbial safety and shelf life of ground beef, very few studies were conducted using a culture-independent approach, additionally only few studies investigated the inhibitory effect of LAB on *E. coli* O157:H7 in ground beef products (Goodarzi, Hovhannisyan & Barseghyan, 2016; Weinroth et al., 2019). Reduction or elimination of *E. coli* O157:H7 ground beef products is imperative concern in the beef industry, since ground beef products are common

sources of *E. coli* O157:H7 (Goodarzi et al., 2016). To date there is little data published detailing the antimicrobial efficacy of LAB food safety interventions for inhibiting members of *S. typhimurium* and *E. coli* O157:H7 on fresh beef during handling and storage prior to retail (Kirsch et al., 2017).

CHAPTER THREE: RESEARCH METHODS

3.1. Research Design

In this study, quantitative data was used to determine the mean of colonies which was reported in cfu/g and the testing of the antimicrobial activity of selected commercial probiotic strains against *E. coli* O157:H7 and *Salmonella typhimurium* in beef using monoculture and co-culture design and the determination of optical density thereof. Figure 4 below shows a schematic flow chart for the main steps and methods used in the study.

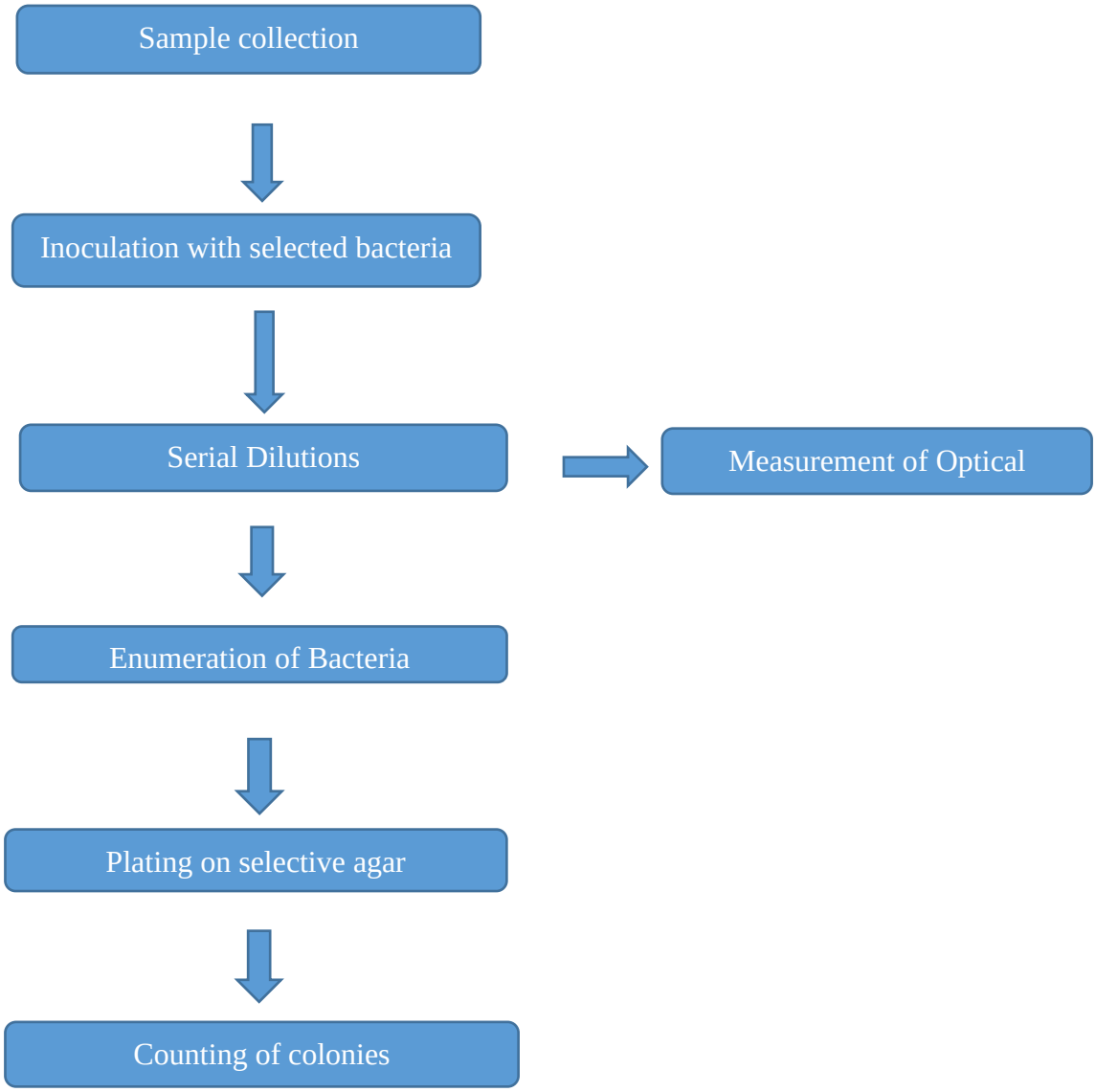


Figure 5 Schematic flow chart of research methods that were used in the study

3.2. Microorganisms, sample collection and site description

This study was laboratory-based research focused on three components; the enumeration of lactic acid bacteria in de man Rogosa Sharpe (MRS), the enumeration of two pathogens *Salmonella typhimurium* and *E. coli* O157:H7 in Salmonella Shigella agar and MacConkey agar selective media and total aerobic counts (TAC) in general plate count agar (PCA). Lastly, the inhibitory test was done in the co-culture method. The methods used above were adopted from Meatco laboratory in-house test method that were modified from ISO 4833:2003.

The probiotic bacteria used to experiment were *Pediococcus acidilactici* ATCC 8042 and *Pediococcus pentosaceus* ATCC 33316. Pathogens used in this study were *S. typhimurium* ATCC 14028 and *E. coli* O157:H7 ATCC 43888. All strains were purchased form a local agent (Anatech, Windhoek, Namibia) and were kept at 4 °C until they were ready for use (as per ATCC culture instructions). All the strains were revived as per manufacturer instructions (Microbiologics, Microorganism Maintenance Plan ISO 11133 and ATCC culture instructions). Initially, *Pediococcus* swab strains were transferred to10 mL of MRS incubated for 24 hours, at 37 °C. To obtain the *E. coli* O157:H7 and *Salmonella* cocktails, *S. typhimurium* ATCC 14028 and *E. coli* O157:H7 ATCC 43888 were cultured in 10 ml of Nutrient broth and incubated for 24 hours at 37 °C. To determine the initial concentration of the cocktails and the concentration to be used in spiking the beef samples, the aliquots were serially diluted in Nutrient broth (BioLab, UK) and MRS broth (Oxoid, Canada). Nutrient agar and MRS agar were used to obtain pure colonies and incubated aerobically for 24 hours at 37 ± 2 °C (ISO 4833:2003).

Sampling was performed on separate visits during production at Meatco over five months between July-November 2020. A sampling of meat was done by cutting strips (meat trimmings) from the outside of the selected cuts. The trimmings were selected as compared to primal cuts, as they are easy to spike and distribution of the co-cultures and to attain uniform distribution of the added compounds. On each visit, samples were taken from different sampling tables as described by Rivera-Pérez, Barquero-Calvo & Zamora-Sanabria (2014). All samples were collected 48 hours post-slaughter (Djordjević et al., 2018). Meat samples were randomly selected and pooled as one sample of ± 50 g each. Samples were then aseptically transferred to sterile stomacher bags. The collected samples were placed in a cooler box and immediately transported to Meatco Laboratory where they were stored under refrigeration (Kirsch et al., 2017). The study was conducted utilising the method for horizontal enumeration of microorganisms using the pour plate technique (ISO 4833: 2003; ML/TM/05). For total plate count, microorganisms were enumerated according to International Organization for Standardization (ISO) 4833: 2003. The same procedure was followed for *S. typhimurium* using Salmonella Shigella Agar (SSA) and *E. coli* O157:H7 using MacConkey agar (MA). The cocktails of both pathogens and LAB (inoculated in nutrient broth and MRS broth) used for optical density measurement using photo UV spectroscopy (Gene sys 20, Thermo, USA).

3.2.1. Preparation of strains

Strains were prepared using ISO 6887-1:1999 (which covers Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension, and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions). Room temperature of the

laboratory was kept at < 25 °C with the aid of an air conditioner. From the examined plates, a single colony was selected and inoculated to the prepared broths as follows separately, a single colony each of *S. typhimurium* ATCC 14028 and *E. coli* O157:H7 ATCC 43888 was inoculated into two vial containing nutrient broth while the third vial was kept as a control. They were all incubated aerobically for 24 hours at 37±2 ^{o}C. For LAB, a single colony was inoculated in 10 ml MRS broth likewise for the 2 pathogens 10 ml of NB was used for inoculation using a single colony. Both MRS broth and NB were incubated for 24 hours at 37±2 ^{o}C. Nutrient broth and MRS broth were both prepared as per manufacturer instruction and were distributed in 6 vials of 10ml each, being 3× 10 ml Nutrient broth (NB) and 3× 10 ml MRS broth (Microbiologics, microorganism maintenance Plan ISO 11133 and ATCC culture instructions). The nutrient broth was inoculated with pathogens while MRS was inoculated with LABs. There was growth and turbidity observed after incubation for 24 hours at 37 ^{o} C.

Preparation of the co-cultures (Figure 5) from the prepared cultures of NB and MRS broth above, 8ml vials of NB were prepared and incubated. The following abbreviations were used in reporting of co-cultures: *Sal – Salmonella, E.c - E. coli* O157:H7, *P.a - Pediococcus acidilactici* and *P.p- Pediococcus pentosaceus* (ISO 6887-1:1999). The two selective agars used in the research above do not differentiate between the LAB and pathogen grown together.

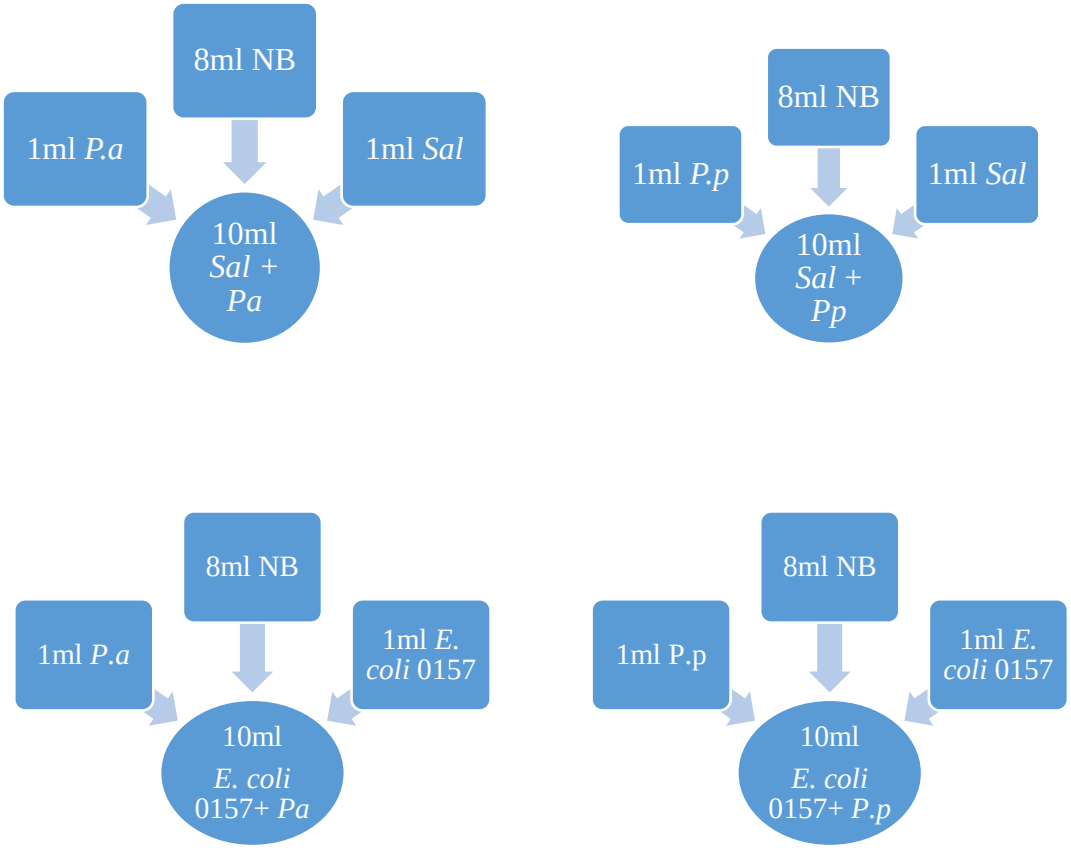


Figure 6 Schematic flow chart showing preparation of co-cultures.

3.3. Microbial Enumeration and Measurement of Optical Density

The number of viable cells was estimated from the inoculated aliquots with serial tenfold dilutions of nutrient broth. Serial dilutions were prepared out of both monoculture and co-cultures. Two plates were prepared from each dilution by pouring 1ml (of the monoculture/co-cultures) into the selective agar appropriate for each microbial species using pour plate method (ISO 6887-1:1999). ISO 4833 was followed with changing the growth media, incubation time and incubation period, where LABs were grown on MRS agar, *S. tymphimurium* on Salmonella Shigella agar and *E. coli* O157:H7 on MacConkey agar**.** Salmonella Shigella and MacConkey agar were both incubated for 24 hours at 37 ^{o}C (Brashears et al., 2003) and MRS agar

incubated for 48 hours at 35 ºC (Meatco test methods ML/TM/11). After incubation, colonies grown in both media were counted. The optical density of both monoculture and co-culture was measured using the spectrophotometer (Single Beam UV-VIS Spectrophotometer, India) at 600nm as described by (Kadhim Isa & Hadi Razavi, 2018). Optical density was measured at 10^{-1} and 10^{-6} for pathogens and 10^{-1} and 10^{-7} for LAB. Each sample was read five times with blank resetting the reading.

3.4. Inoculation of Meat samples

3.4.1. Inoculation of the Meat sample

Serial dilution was done for both pathogens and LAB and the dilution which gave counts of 15-150 of isolated colonies was, thus, selected for co-culture. Co-cultures were selected of 10^{-7} for *S. typhimurium and E. coli* O157:H7 and 10^{-6} for both *P. acidilactici* and *P. pentosaceus* (ISO 6887-1:1999). Cocktail were prepared as shown in Figure 6.

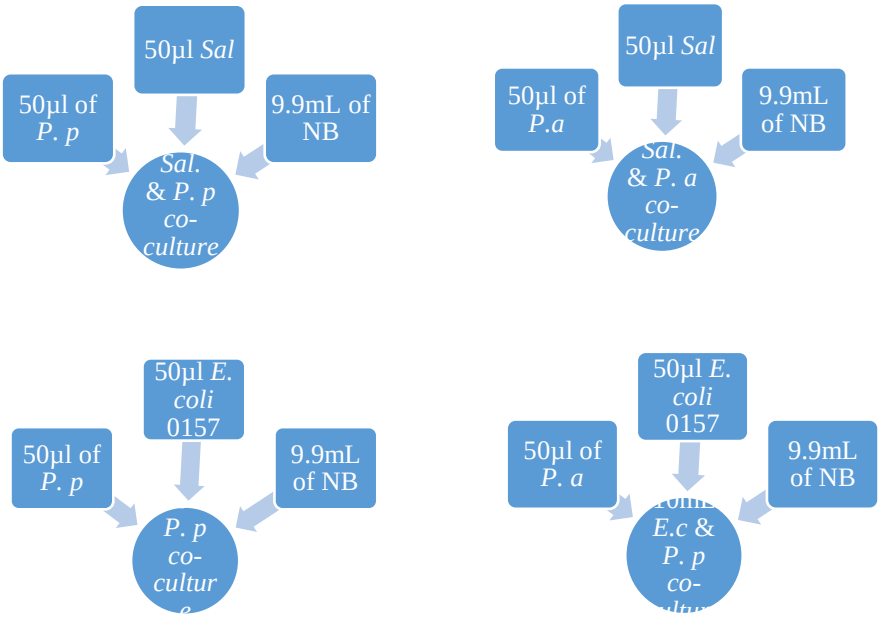


Figure 7 Schematic flow chart of inoculation of meat samples

All the inoculated samples and the control were then kept in the refrigerator for 48 hours at 4 ^{o}C. On the day of analysis, 25 g of the sample was measured in stomacher bags (Seward 400, Seward, London, United Kingdom), and the cocktail of co-culture was added and blended in 225 ml sterile BPW (CM0509, Oxoid, Hemisphere, England) using a stomacher machine (Seward 400, Seward, London, United Kingdom) to achieve an initial 1:9 dilution (Kirsch et al., 2017).

Further dilutions were prepared in Nutrient Broth and 1 ml aliquots plated onto selective media (ISO 6887-2:2003) (Salmonella Shigella agar selective agar for *S. typhimurium* and MacConkey agar selective agar for *E. coli* O157:H7) both plates were incubated for 18-24 hours at 37 ^{o}C (Meatco method, ML/TM/07). All inoculated co-cultures of *S. typhimurium* and *E. coli* O157:H7 samples were also plated on PCA (ISO 4833:2003). *Salmonella Shigella* agar and MacConkey agar had growth. For the enumeration of total aerobic counts (TAC), Standard plate count agar (PCA) (CM0463, Oxoid, New Hampshire, UK) was used and the plates were incubated for 72 hours at 30 ± 1°C. After incubation, SSA and MacConkey agar plate with counts ranging from 15-150 colonies counted. Equally, PCA plates containing 15-300 colonies were counted and recorded in the worksheets (ISO 4833:2003).

3.5. Preparation of LAB cell free supernatant (CFS)

About 50 ml MRS broth was prepared and 9 ml were aliquoted in seven bottles, to the first three bottles of 9 ml were inoculated with 1 ml of *P. acidilactici*, to the next three bottles were inoculated with 1ml of *P. pentosaceus* (ISO 6887-2:2003). The last bottle was left uninoculated to serve as a control. All the bottles were incubated for 24 hours at 37 ^{o}C. *P. acidilactici* and *P. pentosaceus* were aseptically aliquoted

into eppendorf tubes for centrifugation at 8000 rpm for 4 minutes. The supernatant was pipetted out from eppendorf tube into a sterile dilution bottle leaving the pellet behind, the optical density measurement was then taken and recovered. Fifty millilitres (50 ml) of NB were prepared and dispensed into four dilution bottles each with 8 ml. To the first bottle of 8 ml of NB, 1 ml of *S. typhimurium* and 1ml of CFS *P. pentosaceus* were inoculated, the second bottle with 8ml of NB, 1 ml of *S. typhimurium* and 1 ml of CFS *P. acidilactici* were inoculated, to the third bottle with 8 ml of NB 1 ml of *E. coli* O157:H7 and 1 ml of CFS *P. pentosaceus* and the fourth bottle was inoculated with 8 ml of NB, 1ml of *E. coli* O157:H7 and 1ml of CFS *P. acidilactici.* After inoculation of the pathogens and CFS LAB to NB, all bottles were mixed with vortex for 1 minute (ISO 6887-2:2003).

3.6. Inoculation and enumeration of meat samples with co-culture CFS LAB and pathogens

A total of 20 samples weighed 25 g each and were labelled accordingly. Co-cultures were vortexed and inoculated on the meat samples, whereby, each meat sample corresponding to one co-culture was added (Meatco method, ML/TM/05). All the stomacher bags with spiked meat samples from all treatments were wrapped and stored in the fridge at 4 °C for 15 days. Every third day, samples were analysed according to the number of days indicated in Table 1 (Meatco method, ML/TM/07).

Table 1. Co-culture of *S. typhimurium* and *E. coli* O157:H7 with LAB strains analysis up to 15 days storage.

Days of storage	Samples Number tested	Co-culture
3	4	*S. typhimurium* + *P. acidilactici* *S. typhimurium* + *P. pentosaceus* *E. coli* O157:H7 + *P. acidilactici* *E. coli* O157:H7 + *P. pentosaceus*
6	4	*S. typhimurium* + *P. acidilactici* *S. typhimurium* + *P. pentosaceus* *E. coli O157:H7* + *P. acidilactici* *E. coli O157:H7* + *P. pentosaceus*
9	4	*S. typhimurium* + *P. acidilactici* *S. typhimurium* + *P. pentosaceus* *E. coli* O157:H7 + *P. acidilactici* *E. coli* O157:H7 + *P. pentosaceus*
12	4	*S. typhimurium* + *P. acidilactici* *S. typhimurium* + *P. pentosaceus* *E. coli* O157:H7 + *P. acidilactici* *E. coli* O157:H7 + *P. pentosaceus*
		S. typhimurium + *P. acidilactici* *S. typhimurium* + *P. pentosaceus* *E. coli* O157:H7 + *P. acidilactici* *E. coli* O157:H7 + *P. pentosaceus*

Salmonella Shigella agar and MacConkey agar media were used and kept at ± 45 ^{o}C in the water bath for about an hour. The nutrient broth was cooled and dispersed into

dilution bottles of 9 ml each. Enumeration was done for the samples withdrawn at specific intervals (e.g., on day 3, day 6, day 9, day 12 and day 15). On the third day, each sample was removed from the fridge and about 225 ± 1 ml of the BPW was added aseptically and immediately homogenised steward Stomacher at 160 rpm for 2 minutes (Meatco method, ML/TM/01). Serial dilution was done, SSA was poured over samples of co-culture of *S. typhimurium* with LAB and MacConkey agar was pour plated over samples of co-culture of *E. coli* O157:H7 with LAB. Plates were incubated at 37 ^{o}C for 24 hours (Meatco method, ML/TM/01).

3.7. Optical density Measuring of co-culture

Approximately, 50 ml MRS broth was prepared and distributed in 9 ml vials. To the first three bottles of 9 ml, 1 ml of *P. acidilactici* was pipetted, to the next three bottles, 1 ml of *P. pentosaceus* was added and the last bottle was left uninoculated to serve as a media control (ISO 6887-2:2003). All the three bottles were incubated for 24 hours at 37 ^{o}C (Meatco method, ML/TM/01). After incubation, the 1 bottle of each inoculated LAB was prepared and transported in a cooler box to UNAM for centrifuging. All the samples (LAB) were loaded on the centrifuge, which was set at 8000 rpm for 4 minutes. When serial dilution was done optical density was measured at 10^{-1} CFS LAB. MRS was used as a blank control in the spectrophotometer. The absorbance wavelength was then set at 600 nm. Each sample reading was recorded five times with blank resetting the reading. The supernatant was pipetted out from Eppendorf tube into cuvettes bottle using a sterile tip until the mark leaving the pellet with precipitate is left behind, the blank was transferred first. The absorbance was recorded, thereafter, the average and the standard deviation was calculated.

It is important to note that with OD measurements, the spread of light is recorded which depends on the quantity of light received that determines the spread of light by the biomass of the cells, giving a direct concentration of microbial cells present in the aliquot. The less light received at the detector, the more spread of light (Sutton, 2011). Furthermore, OD 600 was used in this research because we are measuring bacteria in the late growth of logarithmic phase, stationary or decline phase, at which it measures both live and dead cells, and we suspect the accumulation of debris and metabolites (Krumm, 2019).

3.8. Data Analysis

Data in all phases of the study were compiled in Microsoft word excel and organised for statistical analysis. The mean number of colonies in cfu/g and standard deviation were calculated using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). If the p-value is less than or equal to the significance level, the null hypothesis must be rejected and conclude that not all population means are equal. Consequently, if the p-value is greater than the significance level, it means the research data do not have enough evidence to reject the null hypothesis that the population means are all equal.

3.9. Research Ethics

An official permit from Meatco (Appendix 1) was sought to have access to the production area and use of Meatco Laboratory for experimental work. Research ethics were strictly observed and followed in this study by obtaining an ethical clearance from the UNAM Research Ethics Committee (Appendix 2).

CHAPTER FOUR: RESULTS

4.1. Microbial population counts

Pathogens and LABs were individually grown on their selective media, their results are presented in Table 2.

Table 2: Monoculture enumeration of both pathogens and LAB strains

Organism	Dilution	Counts cfu/g	St deviation
S. typhimurium	10^{-6}	7.69×10^{7}	±7.92
E. coli O157:H7	10^{-5}	1.79×10^{7}	±13.73
P. acidilactici	10^{-6}	1.85×10^{8}	±16.96
P. pentosaceus	10^{-6}	2.61×10^{8}	±12.70

S. typhimurium was grown on *Salmonella Shigella* agar (SS) and gave black typical colonies (Figure 7a) and *E. coli* O157:H7 was grown on Eosin methylene blue (EMB) agar and MacConkey agar (MA) and gave typical red colonies (Figure 7b). *P. pentosaceus* and *P. acidilactici* were both grown on MRS agar and both gave typical creamy colonies as shown in Figure 8 a & b.

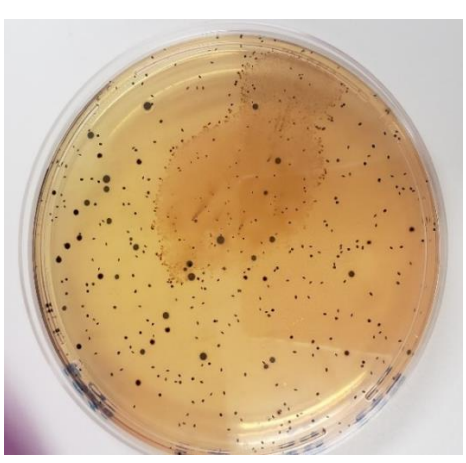
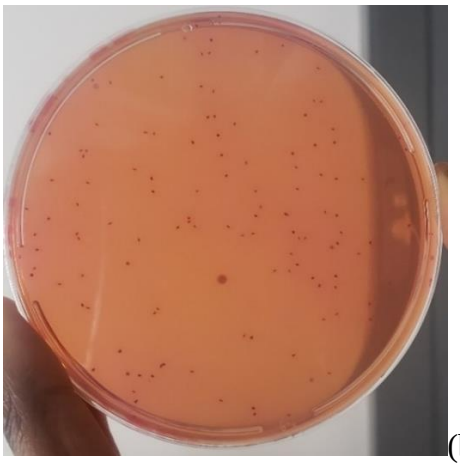
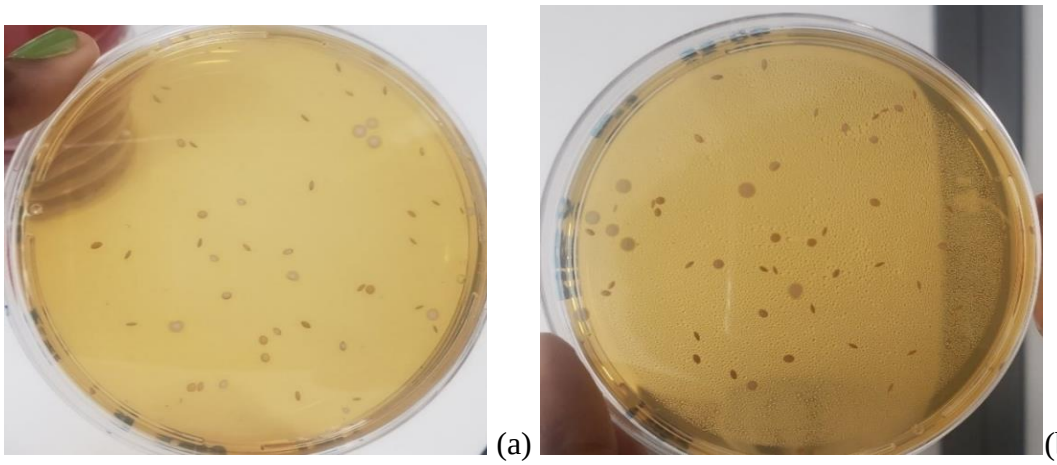
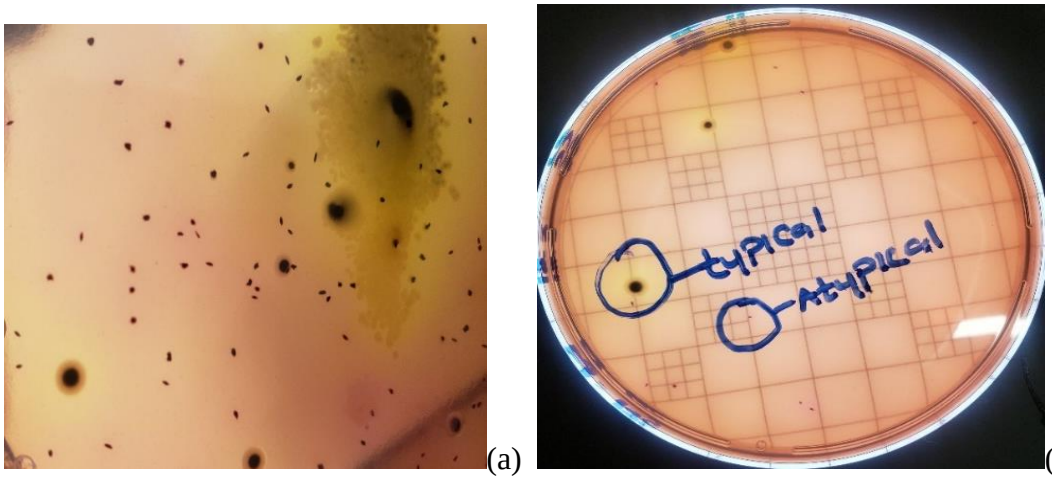

(a)

(b)

Figure 8 *S. typhimurium* colonies (a) and *E. coli* O157:H7 colonies grown on selective agar (b).



(a) (b)

Figure 9 *P. pentosaceus* (a) and *P. acidilactici* (b) both grown on MRS agar after serial dilution.



(a) (b)

Figure 10 Typical and atypical colonies of *S. typhimurium* on SSA.

The figure above shows two types of colonies that grow on Salmonella Shigella agar, Figure 9 (a) shows typical colonies of *S. typhimurium* straw-coloured colonies with black centres while Figure 9 (b) shows both typical and atypical colonies, atypical colonies appears red and smaller in dimention as compared with black colonies conforming to *S. typhimurium*.

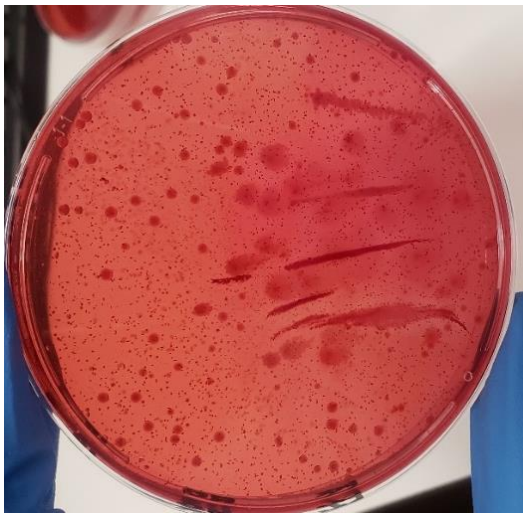


Figure 11 MA agar plates with overgrown colonies of *E. coli* O157:H7.

Typical red colonies conforming to *E. coli* O157:H7, the plate above (MA agar) as shown in Figure 10 is overgrown with >300 colonies.

4.1.1. Isolation and preparation of culture (monoculture and co-culture)

After initial incubation of both pathogens and LAB at 37 ^{o}C for 24 hours, both vials showed turbidity, which is an indication that cultures were still viable and alive. To confirm the latter growth, a loopful was inoculated on NA for pathogens and MRS agar. The growth was confirmed as it can be seen in Figure 7 and Figure 8. Figure 7 (a) show *S. typhimurium* ATCC 14028 grown on Salmonella Shigella Agar and (b) *E. coli* O157:H7 ATCC 43888 grown on MacConkey agar. Figure 8 (a) *P. pentosaceus* ATCC 33316 and (b) *P. acidilactici* ATCC 8042 both grown on MRS agar. Both pathogens and LAB were grown on their respective selective agar for isolation of pure cultures and there was a good recovery observed from all the agar plates. This is a good indication of the quality of the media prepared. For a trial, when one of quality controls fail, none of the results was recorded and the whole test was repeated, this, however only happened once during the research. The quality controls used in the research were

media sterility of agar, diluent control, positive control, negative control, and enrichment broth control. No colonies were observed for all the control plates this is an indication that there was no contamination during the time of analysis. Colonies were then inoculated in both nutrient broth and MRS broth for the preparations of co-culture.

Both media showed a good recovery because dilution of 10^{-1} to 10^{-5} colonies was too numerous to count (TNTC) therefore, they were also not considered for calculation or co-culture.

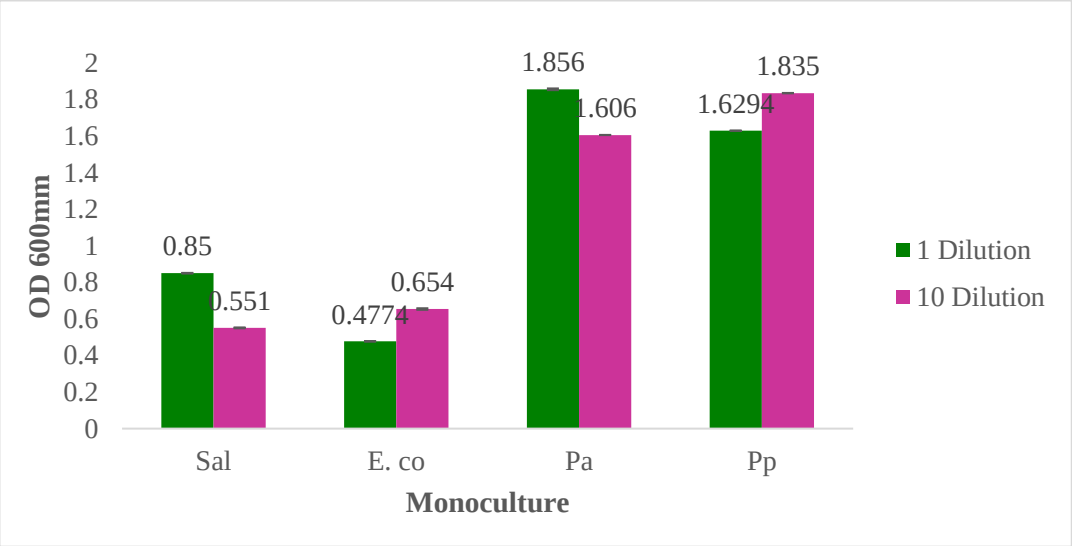


Figure 12 Optical density of mono strains measured at original culture (undiluted) and at 10^{-1} dilution.

The optical density for *S. typhimurium* and *E. coli* O157:H7 (Figure 11) gave a value less than one (0.85 and 0.47, respectively). Whenever optical density value is closer to one (1), it means that the aliquot allowed 10% of light to be transmitted through the sample. This explains the results of both *S. typhimurium* and *E. coli* O157:H7. Meanwhile, *P. acidilactici* and *P. pentosaceus* (Figure 11) gave optical density values higher than one (1.62 and 1.85, respectively). The two values were closer to

optical density value of two (2) which may be owed to only 1% of light was allowed to be transmitted through the sample. This might be because the reovery of *P. acidilactici* and *P. pentosaceus* was very high as compared to *S. typhimurium* and *E. coli* O157:H7. This can be observed in Table 2 and Figures 7 and 8.

When the microbial cultures were diluted, the optical density showed different trends whereby *S. typhimurium* reduced from 0.85 to 0.55 and *E. coli* O157:H7 raised from 0.47 to 0.65 (Figure 11). However, a slight reduction in the optical density for LAB was observed whereby *P. acidilactici* reduced from 1.62 to 1.60 and *P. pentosaceus* from 1.85 to 1.83 (Figure 12).

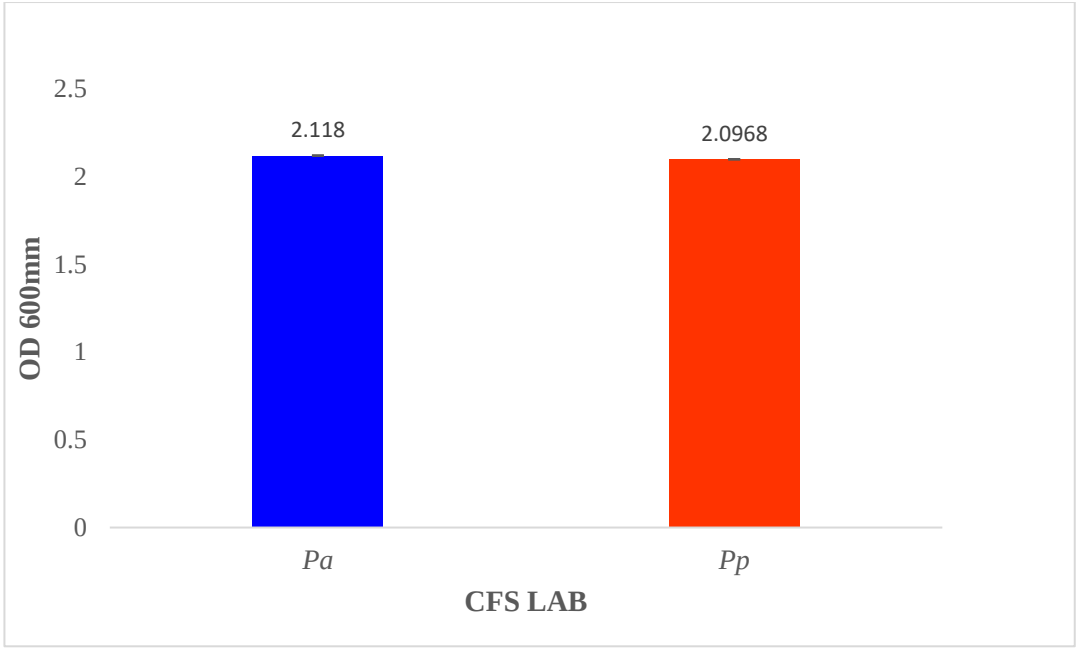


Figure 13 Optical density of undiluted CFS LAB.

4.2. Inhibition of *S. typhimurium* in co-culture system

The results for the inhibition of *S. typhimurium* in co-culture using the two LAB strains are shown in Figure 13 which gave typical black colonies of *S. typhimurium*.

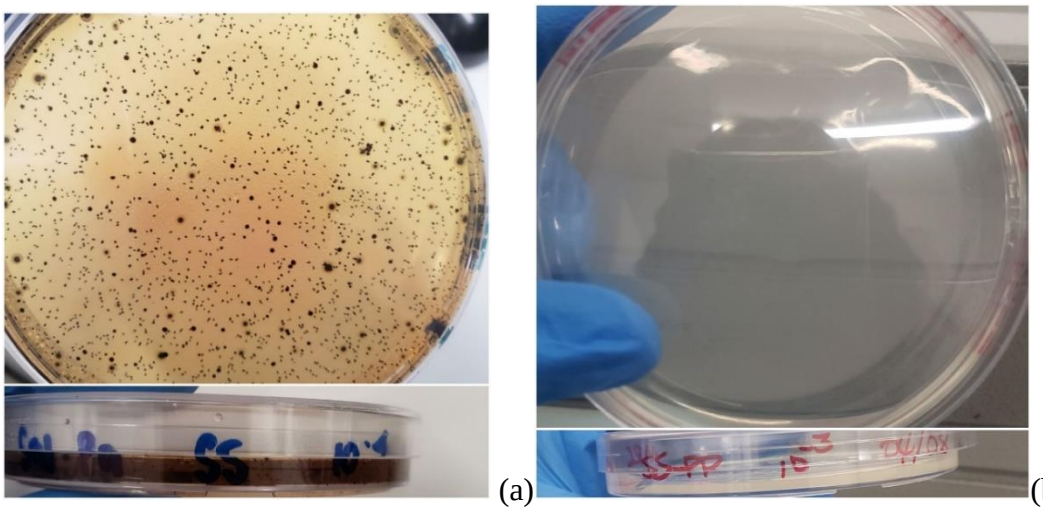


Figure 14 *S. typhimurium* colonies on SSA(a) and PCA (b)

Co-culture of *S. typhimurium* and *P. acidilactici* were grown on Salmonella Shigella agar (Figure 13a), when the same sample was grown on plate count agar (PCA), there were no colonies at 10^{-3} dilution (Figure 13b).

Table 3: Effect of LAB on *S. typhimurium* as co-culture on SSA

Organism	Pathogen dilution	cfu/ml	St deviation
S. typhimurium	10^{-7}	4.1×10^{9}	±17.78
S. typhimurium + *P. acidilactici*	10^{-1}	6.5×10^{2}	±1.41
S. typhimurium + *P. pentosaceus*	10^{-1}	5.3×10^{2}	±2.30

Data in Table 3 shows the counts of viable pathogen cells (cfu/ml) recorded at different dilutions along with treatment, presented as mean of triplicates and standard deviation. After the application of LAB, the population of *S. typhimurium* drastically

reduced from 4.1×10^9 cfu/ml to 6.5×10^2 cfu/ml with co-culture of *P. acidilactici* and finally to 5.3×10^2 cfu/ml with co-culture of *P. pentosaceus.*

Table 4: Effect of LAB on *S. typhimurium* as co-culture on grown SSA and PCA

Strain & co-culture	SSA cfu/ml ± SD	PCA cfu/ml ± SD
S. typhimurium	2.79 ± 0.4^{aX}	1.76 ± 0.8^{bX}
S. typhimurium + *P. acidilactici*	6.46 ± 0.08^{aY}	2.16 ± 0.6^{bY}
S. typhimurium + *P. pentosaceus*	5.29 ± 0.2^{aY}	1.92 ± 0.2^{bY}

*Results were recorded at 10^{-3} for *S. typhimurium* and at 10^{-5} for the co-cultures grown on SSA, ab: The mean values with different letters in the same line are significantly different $P < 0.05$), XY- The mean values with different letters in the same column are significantly different ($P < 0.05$).

When the same samples of *S. typhimurium* co-cultures were grown on PCA there was no reduction of any of the pathogens on the contrary colonies increased. This might be because the PCA support the growth of all microorganisms.

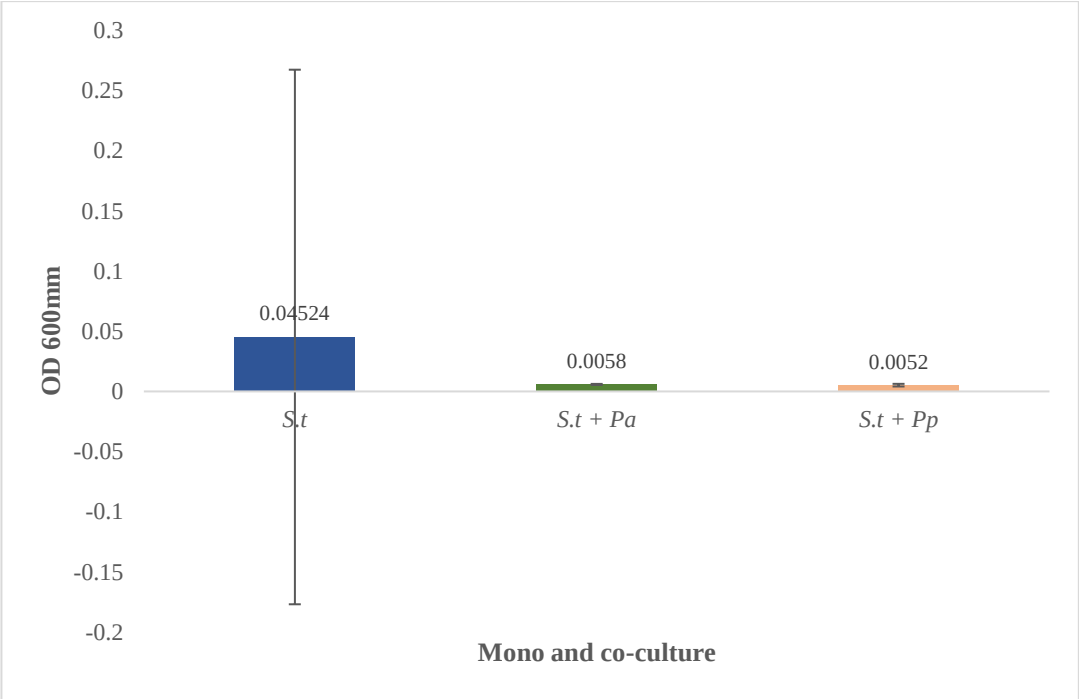


Figure 15 Optical density of *S. typhimurium* with *Pediococcus* species.

The optical density value of both co-cultures is reported in Figure 14. *S. typhimurium* + *P. pentosaceus* gave a higher value of 0.0058 than *S. typhimurium* + *P. acidilactici* of 0.0052 (Figure 14). The very low optical density value from both can be since the bacteria were diluted (*S. typhimurium* and *P. acidilactici* 10^{-7} + *P. pentosaceus* 10^{-6}) before combination to obtain a co-culture system. However, the optical density value for *S. typhimurium* monoculture was high with 0.4524 this can be due to a high number of bacteria in an aliquot ultimately the passing of light is affected.

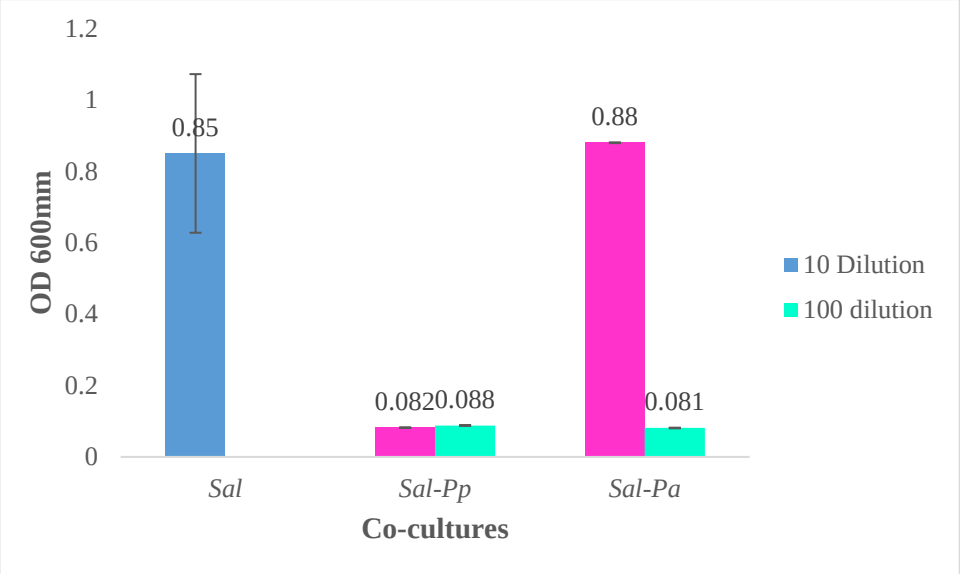


Figure 16 Optical density of diluted *S. typhimurium* with *Pediococcus* species.

In Figure 15, 10 means dilution of 10^{-1} and 100 means dilution of 10^{-2}, the co-culture of *S. typhimurium* with *Pediococcus* species at 10^{0} OD was very low of 0.0058 and 0.0052. It was observed that the OD value for *S. typhimurium* increased after dilution, co-culture of *S. typhimurium* with *P. acidilactici* increased from 0.0052 to 0.088 at 10^{-1} and from 0.0052 to 0.081 at 10^{-2} meanwhile, co-culture of *S. typhimurium* with *P. pentosaceus* increased from 0.0058 to 0.082 at 10^{-1} and from 0.0058 to 0.088 at 10^{-2} (Figure 15). This can be explained as high OD value after dilution can be due to more light passing through the medium as compared to the original medium which allows less light to pass through thus resulting in a low OD value.

4.3. Inhibition of *E. coli* O157:H7 in co-culture system

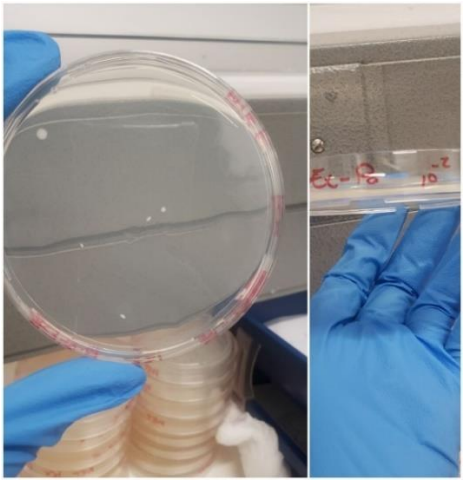
The results for the microbial counts and the inhibition of *E. coli* O157:H7 in co-culture using the two LAB strains are shown in Table 5 and figures 16 and 17.

Table 5: Effect of LAB on *E. coli* O157:H7 as co-culture grown on MA and PCA

Strain & co-culture	MA	PCA
	cfu/ml ± SD	cfu/ml ± SD
E. coli O157:H7	3.14 ± 0.8^{aX}	1.8 ± 0.5^{bX}
E. coli O157:H7 + *P. acidilactici*	2.16 ± 0.7^{aY}	1.63 ± 0.06^{bY}
E. coli O157:H7 + *P. pentosaceus*	2.30 ± 0.7^{aY}	1.75 ± 0.2^{bY}

*Results were recorded at 10^{-4} for *E. coli* O157:H7 and at 10^{-1} for the co-cultures grown on MA and recorded at 10^{-5} for PCA; ab: The mean values with different letters in the same line are significantly different $P < 0.05$) XY: The mean values with different letters in the same column are significantly different ($P < 0.05$).

Microbial count for *E. coli* O157:H7 reaches 3.14 ± 0.8 cfu/ml at 10^{-4} dilution after 24 hours, meanwhile, when it was co-cultured with *P. acidilactici*, reduction of almost six folds reduction of 2.16 ± 0.7 cfu/ml and four folds with *P. pentosaceus* with TPC of 2.30 ± 0.7 cfu/ml after 24 hours at 37 ^{o}C. When the LAB co-cultured with *E. coli* O157:H7 on PCA, the overall trend of the obtained results was different than the observed ones on MacConkey agar (Table 5). As per Table 5, microbial count of *E. coli* O157:H7 reached 1.8 ± 0.5 cfu/ml after 24 hours at 37 ^{o}C, however, the co-culture of *E. coli* O157:H7 with *P. acidilactici* (Figure 16) decreased the *E. coli* O157:H7 count. Meanwhile, the co-culture of *E. coli* O157 with *P. pentosaceus* (Figure 17), a slight reduction in the count was observed from 1.80 ± 0.5 cfu/ml to 1.75 ± 0.2 cfu/ml after 24 hours at 37 ^{o}C.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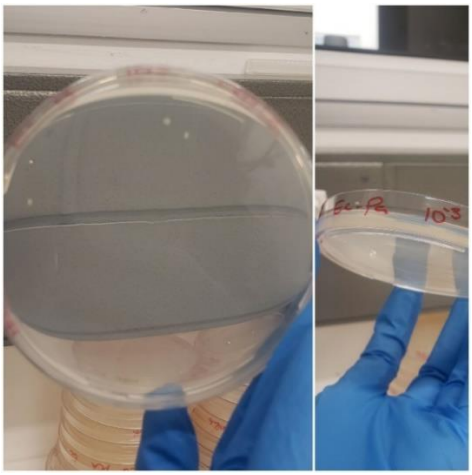
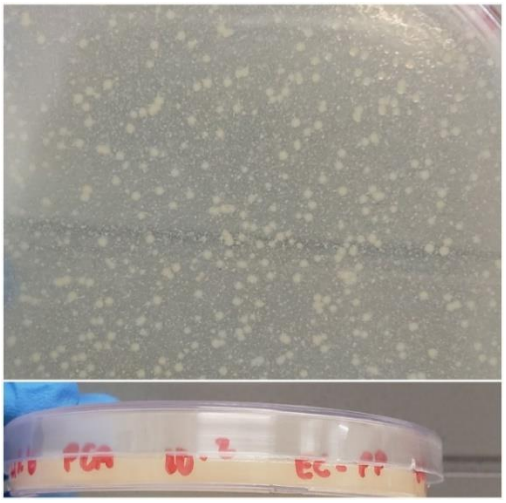

(b)

Figure 17 Co-culture of *E. coli* O157:H7 and *P. acidilactici* grown on PCA with few colonies.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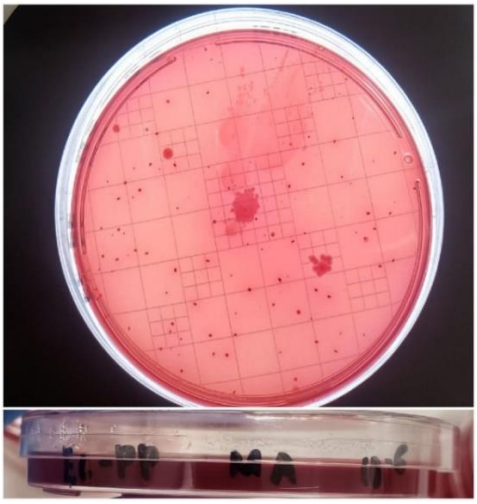
(b)

Figure 18 Co-culture of *E. coli* O157:H7 and *P. pentosaceus* on PCA (a) and MA(b).

Figure 17 shows the samples inoculated with *E. coli* O157:H7 and *P. pentosaceus.* It was grown on PCA (Figure 17a), the plate above was done at 10^{-3} dilution with overgrown colonies this overgrowth was expected as PCA is not a selective media and (Figure 17b), the same sample was grown on MacConkey agar which is a selective media 10^{-6} dilution thus countable colonies of 1.41×10^{6} cfu/ml.

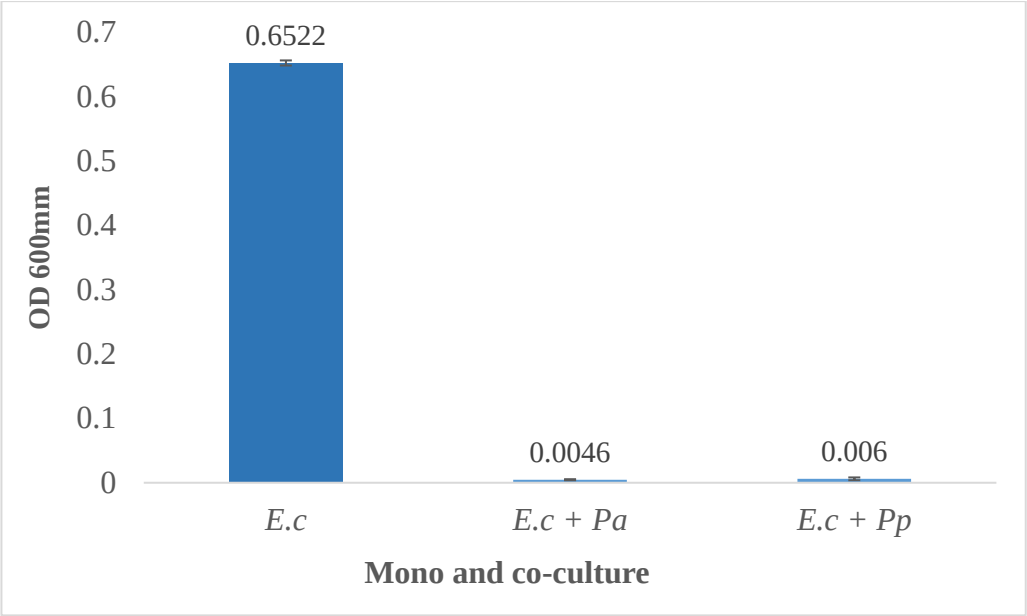

Figure 19 Optical density of *E. coli* O157:H7 with *Pediococcus* species.

The optical density value of both co-cultures is much lower than 0.01 with *E. coli* O157:H7 with *P. acidilactici* giving the lowest optical density value of 0.0046 (Figure 18) and the value is low as compared to the co-culture of *E. coli* O157:H7 with *P. pentosaceus* which gave a high count on MA (1.75 ± 0.2cfu/ml) and optical density value is relatively low (0.006).

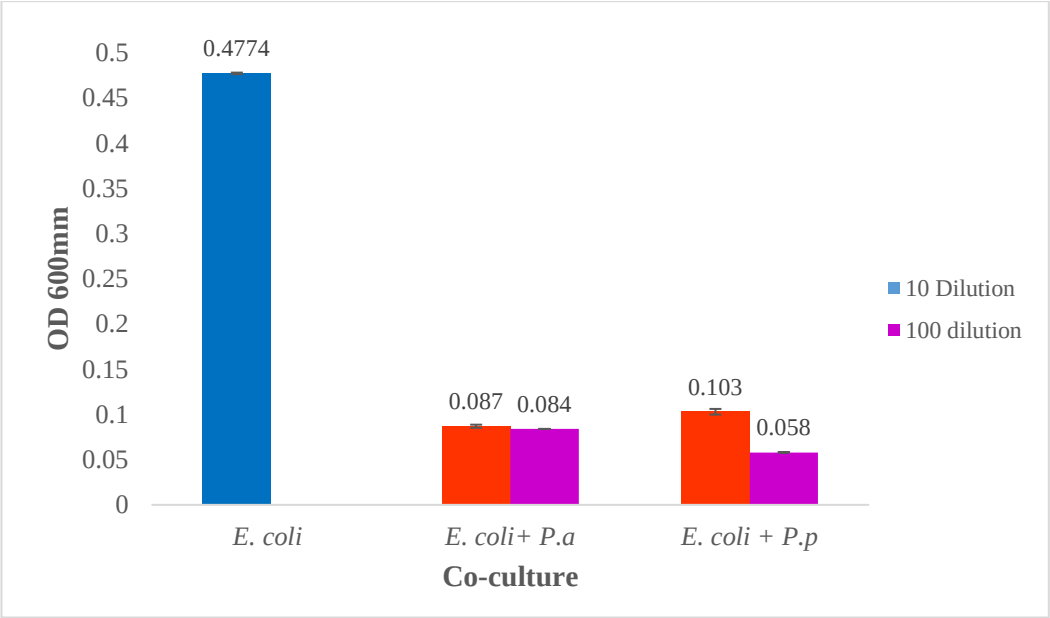

Figure 20 Optical density of *E. coli* O157:H7 co-culture with *Pediococcus* species after serial dilution.

In Figure 19, ten (10) means dilution of 10^{-1} and hundred (100) means dilution of 10^{-2} are presented. At 10^{-1}, the optical density value for *E. coli* O157:H7 with *P. acidilactici* of 0.087 and *E. coli* O157:H7 with *P. pentosaceus* was 0.103. Meanwhile, at 10^{-2}, the optical density value for *E. coli* O157:H7 with *P. acidilactici* was 0.084 and *E. coli* O157:H7 with *P. pentosaceus* was 0.058. With an increase in dilution, the optical density value was reduced as the microbial count in the aliquot was reduced.

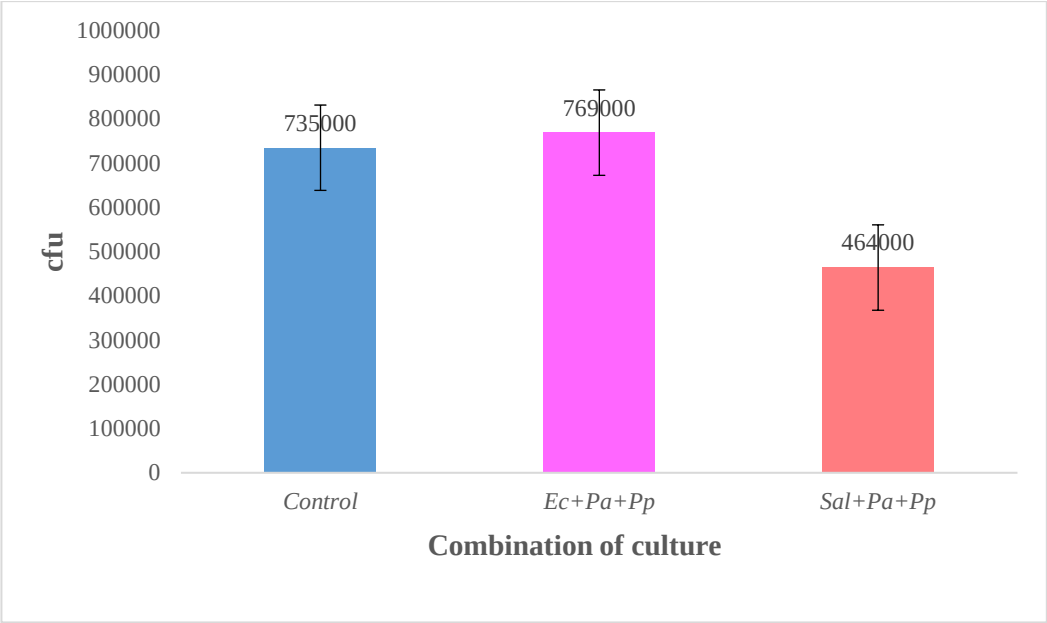

Figure 21 Effect of combination of *P. acidilactici* and *P. pentosaceus* strains with *S. typhimurium* and *E. coli* O157:H7 grown on their selective agar.

Figure 20 shows the effect of the combination of both *P. acidilactici* and *P. pentosaceus* strains with *S. typhimurium* and *E. coli* O157:H7, whereby, each pathogen was grown on its selective agar over 24 hours at 37 ºC. Combination culture of *S. typhimurium* gave a high reduction in colony count of 4.64×10^5 cfu/ml as compared to combination culture of *E. coli* O157:H7 which increased in colony count with 7.69×10^5 cfu/ml as compared to the control with 7.35×10^5cfu/ml.

4.4 Inhibition of *S. typhimurium* in meat samples

The co-culture of *S. typhimurium* with *P. acidilactici* resulted in a significant reduction in *Salmonella* count (Table 6) from 3.96 ± 0.9 cfu/ml to 2.01 ± 1.1 cfu/ml as compared to *S. typhimurium* with *P. pentosaceus* from 3.96 ± 0.9 cfu/ml 2.40 ± 1.5 cfu/ml.

Table 6: Inhibition of *S. typhimurium* in meat samples using *Pediococcus* species

Strain & co-culture	SSA cfu/ml ± SD
S. typhimurium	3.96 ± 0.9^{X}
S. typhimurium + *P. acidilactici*	2.01 ± 1.1^{Y}
S. typhimurium + *P. pentosaceus*	2.40 ± 1.5^{Y}

*Results were recorded at 10^{-4} for both *S. typhimurium* and its co-cultures grown on SSA; XY: The mean values with different letters in the same column are significantly different ($P < 0.05$).

The effect of cell-free supernatant (CFS) of LAB strains on the *S. typhimurium* growth in beef samples is shown in Table 7. The inoculated meat samples were stored at 4 ^{o}C for 15 days to determine the impact of CFS of LAB on the growth inactivation of *S. typhimurium*. During the storage at 4 ^{o}C for 15 days, it was observed that the colony numbers were reduced during the first nine days and then, they took a sharp increase especially *S. typhimurium* + *P. pentosaceus* in Day 12 and Day 15.

Table 7: Inhibition of *S. typhimurium* in meat samples using CFS *Pediococcus* strains.

Number of days stored	*S. typhimurium* cfu/ml ± SD	*S. typhimurium* + *P. acidilactici* cfu/ml ± SD	*S. typhimurium* + *P. pentosaceus* cfu/ml ± SD
3	1.60 ± 0.8	2.05 ± 0.8	1.93 ± 0.5
6	0 ± 0	5.05 ± 5.5	1.3 ± 0.3
9	1.35 ± 0.07	4.55 ± 0.9	7.8 ± 0.1
12	2.74 ± 0.3	5.02 ± 0.7	6.45 ± 2.8
15	3.78 ± 0.3	1.8 ± 0.07	5.3 ± 0.1

*Results were recorded at 10^{-6} for both *S. typhimurium* and its co-cultures grown on SSA; ab: The mean values with different letters in the same line are significantly different $P < 0.05$); XY: The mean values with different letters in the same column are significantly different ($P < 0.05$).

4.5. Inhibition of *E. coli* O157:H7 in meat samples

The co-culture of *E. coli* O157:H7 with *P. acidilactici* gave a slight reduction in *E. coli* O157:H7 count from 2.72 ± 1.2 cfu/ml to 1.70 ± 0.7 cfu/ml ($P > 0.05$) as compared to co-culture of *E. coli* O157:H7 with *P. pentosaceus* from 2.72 ± 1.2to 1.31 ± 0.2 cfu/ml ($P > 0.05$) (Table 8).

Table 8: Inhibition of *E. coli* O157:H7 in meat samples using *Pediococcus* species

Strain & co-culture	MA cfu/ml ± SD
E. coli O157:H7	2.72 ± 1.2^{a}
E. coli O157:H7 + *P. acidilactici*	1.70 ± 0.7^{b}
E. coli O157:H7 + *P. pentosaceus*	1.31 ± 0.2^{b}

Results were recorded at 10^{-6} for both *E. coli* O157:H7 and its co-cultures grown on MA; XY: The mean values with different letters in the same column are significantly different ($P < 0.05$).

The inoculated meat samples were stored at 4 ^{o}C for 15 days to determine the impact of CFS of LAB on growth inactivation of *E. coli* O157:H7. During the storage at 4 ^{o}C for 15 days (Table 9), it was observed that the colony numbers were increased steadily with days.

Table 9: Inhibition of *E. coli* O157:H7 in meat samples using CFS *Pediococcus* species

Number of days stored	*E. coli* O157:H7 cfu/ml ± SD	*E. coli* O157:H7+ *P. acidilactici* cfu/ml ± SD	*E. coli* O157:H7 + *P. pentosaceus* cfu/ml ± SD
3	7.95 ± 0.30^{aU}	5.56 ± 0.3^{bU}	7.32 ± 2.2^{cU}
6	0 ± 0^{aW}	5.90 ± 0.6^{bW}	2.7 ± 0.1^{cW}
9	1.59 ± 0.5^{aX}	3.10 ± 0.3^{bX}	5.8 ± 0.6^{cW}
12	4.0 ± 0.2^{aY}	$1.21 \pm 0.1a^{bX}$	4.93 ± 5.3^{bW}
15	4.9 ± 0.5^{aZ}	7.6 ± 0.4^{bY}	1.04 ± 0.02^{cX}

Results were recorded at 10^{-6} for both *E. coli* O157:H7 and its co-cultures grown on MA; ab: The mean values with different letters in the same line are significantly different $P < 0.05$).U-Z: The mean values with different letters in the same column are significantly different ($P < 0.05$).

Figure 21 shows results for the pathogens co-cultured with the two LAB strains. The co-culture of *S. typhimurium* + *P. acidilactici* + *P. pentosaceus* gave a higher reduction in colony count as compared to the other combination (Figure 21).

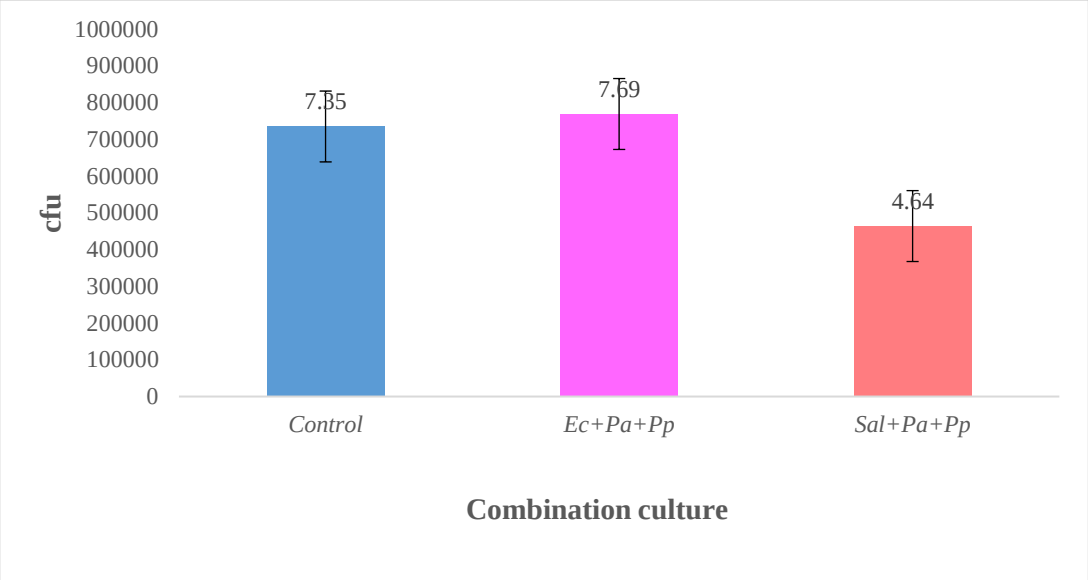

Figure 22 Effect of CFS from combined *Pediococcus* strains against *S. typhimurium* and *E. coli* O157:H7.

CHAPTER FIVE: DISCUSSION

Meatco faces the same challenges as other meat industries of meat recall from the positive result of both *S. typhimurium* and *E. coli* O157:H7. LAB isolated from meat and meat products are the best to be used for improving the microbiological safety of meat and meat products because they are well adapted to the conditions in meat and are more competitive than LAB isolated from other sources (Zaki, 2016). This is especially true because when these isolated LAB are incubated together in the meat and meat products, they are able to compete with the target culture since they are originally adapted to the condition of the meat (Zaki, 2016).

5.1. Microbial counts and growth characteristics

For isolation of pure culture of both pathogens and LABs were grown on nutrient broth and MRS broth, respectively. The latter broths promoted bacterial growth by showing turbidity in the vials. These were serially diluted and subsequently grown on nutrient agar and MRS agar which yielded good, isolated colonies shown in Figures 7 to 9. The first five dilutions for both pathogens and LABs showed a good recovery which gave more colonies that were too numerous to count (TNTC). The microscopic evaluation confirmed small creamy to yellow colonies of similar sizes that appeared on Bromocresol Purple (BCP) agar using the pour plate method as the presence of the *P. pentosaceus* isolate 4I1 which was coccus-shaped (Bajpai et al., 2016).

The LAB colonies observed in our research were also similar to Kalschne et al. (2015), where they observed typical colonies grown on MRS agar plates were white,

circular, and slightly convex, with a small diameter (0.5-2.0 mm), this can be observed in Figure 8. According to research conducted by Zaki (2016), inoculated *Lactobacilli* on MRS agar using pour plate method, colonies with *Lactobacilli*-like morphology were counted, this is to confirm that MRS is perfect for the growth of LAB. During monoculture enumeration high counts ≥ 150 was observed which is interpreted as TNTC, thus, these dilutions were not used in the research. This also shows the good recovery during resuscitation of the strain as mentioned above in the result section. Figure 7a and Figure 7b displayed typical colonies as expected from Material Safety Data Sheet (MSDS). Figure 8 shows typical colonies of both *P. pentosaceus* (Figure 8a) and *P. acidilactici* (Figure 8b) grown on MRS agar.

The results of the microbial populations performed on monocultures were reported in Table 2. From, Table 2, *P. acidilactici* and *P. pentosaceus* gave high counts (1.85×10^8 and 2.61×10^8) with a relatively high standard deviation (SD) of ±16.96 and ±12.70 respectively. This concurs with research where most of the bacterial population was formed by *Lactobacilli,* this is because LAB are naturally dwelling in vacuum packaging environmental conditions, especially when the packs are stored for a long time (Stella et al., 2013). The counts of pathogens (*S. typhimurium* and *E. coli* O157:H7) were very low resulting in a low SD as compared to LAB. To the best of my knowledge, there may be factors that influenced the effectiveness of LAB, such as LAB diversity, dose-response, the quantity of dose and the use of other experimental designs that were used in the present research other than the simple control treatment protocol that may have not been explored.

From Figure 11, the OD values for LAB were greater than one (1) and closer to two (2), (*P. acidilactici* OD = 1.62 and *P. pentosaceus* OD = 1.85), whilst *S. typhimurium* OD = 0.85 and *E. coli* 0157:H7 OD = 0.47). The higher absorbance (>1.5) of LAB could mean that most of the light is absorbed by the samples and an only a small amount of the light is picked up by the detector. Additionally, the absorbance of one (1) means the 90% light has been absorbed, therefore, the ratio is 100/10, giving a log of 10 to 1, at times absorbance could be more than 90% in those cases the absorbance would be more than one (Krumm, 2019).

5.2. Inhibition of *S. typhimurium* in co-culture system

Gao et al. (2019) indicate that LAB especially *Lactobacillus* has been used as antimicrobial agents in foods, therefore, they serve as an excellent preservative for fresh meat, inhibiting the growth of *Listeria monocytogenes, S. typhimurium,* and *E. coli* O157:H7 in beef. The two *Pediococcus* strains used in the research did not completely inhibit both *S. typhimurium,* and *E. coli* O157:H7, instead, they reduced the bacterial count (as reported in Table 3 and Table 5). Therefore, they had a bacteriostatic effect on both pathogens. Furthermore, *P. acidilactici* was more effective in the reduction of the pathogen population.

According to Gao et al. (2019), bacteriostatic substances produced by LAB are thermally stable, thus, they have an inhibitory effect against *Salmonella*. This can be demonstrated in Table 3, samples inoculated with *Salmonella* only gave a count of 4.1 × 10^9 cfu/ml, the co-cultures of both *S. typhimurium* with *P. pentosaceus* and *S. typhimurium* with *P. acidilactici* reduced drastically by 97% and this was recorded at

10^{-1} dilution for both co-cultures. A study by Laury et al. (2009) demonstrated that when lactic acid was applied on carcasses, *S. typhimurium* reduced with 1.0 log cfu/100 cm^2 on beef trimming. From the result, it can be observed that co-culture of *S. typhimurium* with *P. pentosaceus* reduced significantly in colony numbers. When total bacterial count (TBC) and lactic acid bacteria count (LAB) were measured on individual fermented sour meat from inoculating fermentation showed, the changes in TBC and LAB count also showed the same trend in fermented sour meat from natural fermentation (Zhang et al., 2020). TPC were recorded at 10^{-5}, for both co-cultures of *S. typhimurium* monoculture increased 18% with P. *acidilactici* co-culture and 8% with *P. pentosaceus* co-culture. In my opinion, this can be because PCA supports growth of all bacteria so some of the counts observed may not be that of *S. typhimurium* but other bacteria that were present in the meat sample. *S. typhimurium* on both SSA and PCA count gradually increased with co-culture.

At 10^0 dilution the optical density of *S. typhimurium* in co-culture was recorded at 0.4524 as shown in Figure 14. Research conducted by Krumm (2019) explains why microbial growth are measured at $OD_{600,}$ the reason being that different instruments use different light beams, and consequently their detectors are positioned at different distances from the sample whenever a light beam being scattered by a microorganism: a nearby detector still captures the light, while a detector positioned further away does not capture the light.

The pH value in this research was not adjusted nor measured. Similarly, there was no treatment to eliminate the effect of acid or hydrogen peroxide. Thus, it cannot be

concluded that the inhibitory activity is due to the production of antimicrobial compounds by the studied LAB. Tebyanian et al. (2017) studied *Lactobacilli* and different pathogenic bacteria using agar well diffusion method whereby the entero-pathogens bacteria (*E. coli, Staphylococcus aureus, Shigella dysenteriae* and *Salmonella paratyphi*) growth were inhibited in the presence of all *Lactobacillus* strains by measuring the zone of inhibition which was between 12 and 32 mm. The present research focus on measuring reduction in microbial viable count number by colony counts, where there was microbial reduction in microbial number as reported in Tables 4 and 5. The research did not determine whether it was the metabolites or bacteriocin produced by LAB which was able to inhibit the growth of *S. typhimurium* and *E. coli* O157:H7 as compared to research, where the investigated bacteria produced metabolites that inhibited the growth of *E. coli* O157:H7 (Salehzadeh et al., 2017).

5.3. Inhibition of *E. coli* O157:H7 in co-culture system

The co-culture of *E. coli* O157:H7 with LAB was also analysed similarly as *S. typhimurium* but with *E. coli* O157:H7, MacConkey was used as a selective agar. The results were recorded at 10^{-4} dilution. Test tubes inoculated with the monoculture of *E. coli* O157:H7 on MA resulted in a bacterial count of 3.14 ± 0.8 cfu/ml. However, when *E. coli* O157:H7 was co-cultured with *P. pentosaceus*, the microbial count reduced 27% and when co-cultured with *P. acidilactici* resulted in 32% reduction as reported in Table 5. The total plate count (TPC) is generally the most consistent method for detection of sanitary levels of proper treatment of meat and food stuff, additionally, it gives awareness on hygienic measures applied through receiving, handling and preparation of meat (Shaltout, Zakaria, Lamiaa & Ibrahim, 2016). Meanwhile, when *E. coli* O157:H7 was grown on PCA, the results as shown

in Table 5 were recorded at 10^{-5} dilution, the co-culture of *E. coli* O157:H7 with *P. pentosaceus* reduced slightly with 2% on the other hand, the bacterial count of E. *coli* O157:H7 with *P. acidilactici* co-culture reduced with a mere 9%. In a similar report where *E. coli* O157:H7 was co-cultured with *Lactobacillus plantarum* on fresh beef, TPC remained relatively constant with a significant increase ($p<0.05$) between the counts enumerated at day 0 compared to day 5 and day 7 (Tshabalala et al., 2012).

OD measurement of *E. coli* O157:H7 with *P. pentosaceus* (0.006) was almost the same value as that of *S. typhimurium* with *P. pentosaceus* of 0.0058, however, the optical density measurement of *E. coli* O157:H7 with *P. acidilactici* reduced to 95% less than that of *S. typhimurium* with *P. acidilactici* of 94%. When the optical density of serial dilution of at 10^{-1} and 10^{-2} was measured, it slightly reduced for *E. coli* O157:H7 with *P. acidilactici* co-culture of 3% while there was a significant reduction of 43% from *E. coli* O157:H7 with *P. pentosaceus* co-culture.

The Jameson effect model initially proposed that when two intestinal organisms e.g., pathogens were grown in a co-culture liquid medium, each microbe would thrive rapidly at the beginning, as if they were grown as monoculture and then both would stop growing when one microbe reached the maximum level (Gao et al., 2019). In the current study, since there was no complete inhibition (bactericidal effect), it could be deduced that, that each microbes grew to reach the maximum level in counteracting with each other, but LAB was not able to reduce the pathogen growth to a minimum number. Additionally the latter could be because the agar used (PCA) could not differentiate between pathogen and LAB colonies. Bajpai et al. (2016) evaluated the effect of CFS of *P. pentosaceus* on pathogen viabilities using foodborne pathogenic

bacteria, *S. aureus* and *E. coli* O157:H7 and reported that the inhibitory effect observed for the tested pathogens in growth-phase dependent inhibition assay began from the early stationary phase, which could be mediated by secondary metabolites, organic acids, or other compounds produced in the CFS of *P. pentosaceus* 4I1. Similarly, İncili et al. (2020) also found significant growth recorded in the aerobic plate count after 1 week inoculation of the storage period. The same study reported that *L. plantarum* was not capable to grow at 4 °C and a 1 week period is required for detection of growth and the reason of these fluctuations may be due to the possible effects of background microflora.

In the results of the combination of co-culture, both *P. acidilactici* with *P. pentosaceus* and *E. coli* O157:H7 (*Ec+Pa+Pp)* and *P. acidilactici* and *P. pentosaceus* with *S. typhimurium* is shown in Figure 21. There was a significant reduction with the combination of cultures with *S. typhimurium* as compared to a combination of cultures with *E. coli* O157:H7. This also confirms the finding where *S. typhimurium* counts were reduced with 37% as compared to combination of co-culture *E. coli* O157:H7 which reacted differently with an increase of 4%. According to literature, the main bacteriostatic factor probably is the low pH caused by LAB fermentation and this could be the case here. These discoveries are crucial for addressing the problem of foodborne salmonellosis (Gao et al., 2019).

OD was measured at 600nm, this is because at 600nm wavelength is orange light and most bacterial cultures tend to grow more dark orange as their culture grows denser. Since not all cultures are the same colour, their visible density may not even correspond to their actual culture density (Engel, 2011). Furthermore, OD600 is

preferable over to Ultraviolet (UV) spectroscopy when measuring the microbial growth over time of a cell population because, at this wavelength, the cells will not be killed as they are under too much UV light (Krumm, 2019). UV can cause mutation in the DNA, thus, OD of 600nm is safe for the bacterial genome, so mutations are avoided. Equally important, OD 600nm minimise interferences from yellowish broths such as LB, TSB, etc., thus, there is low interference of the broths (Eppendorf, 2015).

Some researchers believe that when the absorbance = 1.00, usually, this is the top end of the linearity curve where the coefficient correlation becomes nonlinear. Although this is not always the case, it depends on many factors, mainly the concentration of the analyte-containing the chromophore. Some researchers accepted that for reliable measurements of optimal optical density (OD) must be lower than 2, Depending on the manufacturer of the UV vis spectrophotometers, a more acceptable OD value may be between 0.1 and 1.5 (Engel, 2011). When their OD values were measured LABs (*P. pentosaceus* and *P. acidilactici*) gave higher OD values as compared to pathogens (*S. typhimurium* and *E. coli* O157:H7), and this can be because LABs were grown on selective broth while pathogens were grown on general broth (Nutrient broth) that promote the growth of all bacteria.

5.4. Inhibition of pathogens in Meat samples

The inoculated meat samples with CFS LAB were refrigerated up to15 days at 4 ^{o}C to determine the impact of LAB on growth inhibition of *E. coli* O157:H7 and *S. typhimurium* over the indicated period. The samples were analysed after every third

day and results did not show any uniformity in the reduction of pathogens or an increase in bacterial number (Table 9).

After three days of storage, the populations of *E. coli* O157:H7 reduced with 30% when it was co-cultured with *P. acidilactici* as compared to 8% reduction when it was co-cultured with *P. pentosaceus*. After 12th of storage, the co-culture of *E. coli* O157:H7 with *P. acidilactici* reduced with 85%. The co-culture with *P. pentosaceus* reduced with 8% after 3 days storage and after 12th day storage there was a drastic reduction of 87%. İncili, et al. (2020) study shed different results to the current research in that *L. plantarum* count did not was constant from first day of storage until day 7 but in the second week, there was significant increase and lastly 2 weeks later the aerobic plate count in LAB-inoculated beef samples remained constant.

The results in Figure 21 show a combination culture which resulted in a 5% increase in *E. coli* O157:H7 combined cultures, and then those observed with *E. coli* O157:H7 monoculture. A similar trend in the antimicrobial activities of LAB was observed with Murry et al. (2004) with the difference in the used method whereby the plate count method was used to determine the reduction in the microbial population. The investigator, however, followed the method by Kirsch et al. (2017) but did not supplement MRS agar with antibiotics. Applying *Pediococcus* to meat samples resulted in 63% reduction of *E. coli* O157:H7 count after application of co-culture of *P. acidilactici*, and 51% reduction with co-culture of *P. pentosaceus*. While the study by Kirsch et al. (2017) , LAB were applied on beef striploin there was a reduction in STEC of 0.4 ± 0.1 log10 cfu/cm^2 ($p < 0.05$). Similar results were obtained by Smith et al. (2005) where combined cultures of *E. coli* O157:H7 resulted in significant

declines that were greater than those observed with individual cultures, but his samples were stored at 5 ^{o}C and a study was carried out in 5-days. At the end of five days, the *Salmonella* count was undetectable by direct plating or by pre-enrichment and subsequent culturing, indicating that the *Salmonella* was eliminated from the ground beef by the combined LAB strains (Smith et al., 2005).

During cell reduction, bacteria undergo a period of adjustment in their new environment when they are inoculated in meat samples, this may include repair of cell damage and bacteria adaptation to utilize nutrients available before they can start to grow and compete for nutrients in the food matrix (Tshabalala et al., 2011). *S. typhimurium* was prone to Oregano extract to presence of MIC of 160 µg/ml, membrane degradation which allows for leakage of ions and increased cell membrane permeability, this was mechanism of action of the Oregano essential oil (Nazareth, 2017).

5.5. Inhibition of *S. typhimurium* in meat samples

With reference to Table 6, there was a significant (P > 0.05) reduction in populations of *S. typhimurium* in meat samples after applying *Pediococcus* stains. There was a 50% reduction for co-culture of *S. typhimurium* with *P. acidilactici* counts while co-culture of *S. typhimurium* with *P. pentosaceus* reduced with 40%.

The result of inhibition of co-culture *in vitro* impelled for further investigation of inhibition of *S. typhimurium* in co-culture system to analyse using CFS. The inhibitory aspect of CFS *Pediococcus* strain on *S. typhimurium* stored at 4°C for 15

days are shown in Table 7. The results did not show a steady increase or decrease, for example, *S. typhimurium* showed 16% reduction after nine days of storage. Thereafter, the twelfth days of storage, the population increased with a 37% for the population during storage. During storage at 4 °C, the populations of the pathogen in the control samples with *S. typhimurium* were relatively lower compared to the samples treated with *Pediococcus* strain. Table 7 further showed that there was no significant reduction ($P > 0.05$) of counts for both *S. typhimurium* and its co-cultures. Similarly, Amézquita et al. (2002) studied *L. monocytogenes* in beef, after seven days the population of *L. monocytogenes* in the control samples (with no LAB added) increased by approximately 2.5 log10. Their trend changed when *P. acidilactici* was co-cultured with *L. monocytogenes* on meat samples, there was a significant ($P < 0.05$) reduction in populations of *L. monocytogenes* during storage at 5 ^{o}C (Amézquita et al., 2002). The effects of probiotics on the growth of *S. typhimurium* was studied by Kadhim Isa & Hadi Razavi (2018). The minced meat was stored for 14 days at 4 °C, they reported a significant reduction *S. typhimurium* population within 14 days when it was co-culture with *L. acidophilus* and the average viable cell counts of *S. typhimurium* was reduced by 1.25 log cycle (Kadhim Isa & Hadi Razavi, 2018).

Moreover, Smith et al. (2005) demonstrated that more inhibitory effect was observed against *S. typhimurium* co-culture of LAB in beef samples, there was a reduction in all treatments of more than 2 logs as compared with the control as the storage days increased. The inhibitory effect for the first 9 days reduction in *S. typhimurium* may be due to production of lactic acid, pH decrease or perhaps the presence of hydrogen peroxide (Oliveira et al., 2008). In the absence of a satisfactory explanation to the

observed results, it can only be assumed that the increase in bacterial population after twelve days of storage could be attributed to an increase in pH or depletion of hydrogen peroxide. Another reason may be that both bacteria were detached from the meat, therefore no reaction occurred to cause the reduction in population as the first nine days. Alternatively, this may be because when *S. typhimurium* reached a stationary phase, after that, it went back to a growth phase, where both *P. acidilactici* and *P. pentosaceus* were not able to suppress the growth of *S. typhimurium.* In the current research, most inhibition of *S. typhimurium* in meat was obtained by its co-culture with *P. acidilactici* and then in co-culture with *P. pentosaceus*. This is in agreement with Kadhim Isa & Hadi Razavi (2018) where most of the inhibition of *S. typhimurium* was obtained by its co-culture with *L. acidophilus*, and then in co-culture with the mixture of *L. acidophilus* and *B. animalis*. On the contrary, co-culture of *S. typhimurium* with CFS *Pediococcus* did not yield any uniform significant increase or reduction in *S. typhimurium* population. İncili, et al. (2020) reported that involving chitosan shows strong antimicrobial effect against *P. acidilactici,* concluding that high inoculation level was preferred. This could have been applied with the curent research where higher inoculation level of *P. acidilactici* could be more effective than the inoculation level used in this study.

5.6. Inhibition of *E. coli* O157:H7 in meat samples

E. coli O157:H7 co-culture was inoculated on meat samples (Table 9). Meat inoculated with *E. coli* O157:H7 monoculture gave a recovery bacterial count of 7.95 ± 0.30 cfu/g. However, after three days of storage when *E. coli* O157:H7 combined with the CFS of *P. acidilactici* co-culture, the bacterial count reduced to 30%, and a slight reduction with the co-culture of *E. coli* O157:H7 with *P. pentosaceus* of a mere 11%.

Smith et al. (2005) reported that a combined co-culture of *E. coli* O157:H7 with LAB resulted in a significant reduction *E. coli* O157:H7 count than individual count which gave a lower reduction in *E. coli* O157:H7. Their research revealed that after three days there was a 2-log reduction as compared to 5 days which gave a five-day log reduction. Similarly, when meat samples were inoculated with LAB and *E. coli* O157:H7, to determine the effectiveness of the LAB treatment incubated at 4 ^{o}C up to 15 days. *E. coli* O157:H7 reaction was like that of *S. typhimurium.* Whereby, after the first nine days of refrigeration there was a significant reduction in *E. coli* O157:H7 bacterial count in both co-cultures but there was a sharp rise from day 12 to day 15. The explanation given under *S. typhimurium* may apply to the result reported in Table 9.

A similar study was done by Tshabalala et al. (2012), where beef samples were inoculated with similar levels of *E. coli* O157:H7 and *L. plantarum,* the growth of *E. coli* O157:H7 was only determined after 3 weeks of storage. In the present study, the growth of *E. coli* O157:H7 was determined after 15 days of storage, this was a short time to determine the effect that *Pediococcus* on the inoculated meat. Research by Dow et al. (2011) reported the effectiveness of LAB against *Salmonella* and *E. coli* O157:H7 in turkey products showed that the LAB was inhibitory to the pathogen and that during storage at increased temperatures and the rate of reduction was increased over time. Additionally, applying lactic acid solution at ambient temperature of 54 ^{o}C is more effective in killing pathogens (Buege et al., 2003). At Meatco, a lactic acid solution is prepared and allowed to equilibrate at room temperature before application, moving forward the above finding may be applied by maintaining the solution between

50-56 ^{o}C. It was reported that LAB were effective in reducing the growth of *Salmonella* in turkey products, coupled with a higher concentration of LA, which was significant in an overall greater reduction in the pathogen population (Dow et al., 2011). The reductions of *E. coli* O157:H7 were achieved when heavily inoculated cut muscle or fat surfaces were treated with 5 % lactic acid at 0.5 ml/cm^2 (Youssef et al., 2012). The results from both co-culture of *S. typhimurium* and *E. coli* O157:H7 did not give a satisfactory result, thus a LAB CFS was done to determine their effect.

Figure 12 shows OD measurements for CFS LAB, where *P. acidilactici* gave a high value of 2.11 while *P. pentosaceus* gave 2.09. The above results may be because most of the research on reducing the microbial risks associated with various foods has been theoretical studies conducted in laboratories and rare in food production facilities (Gao et al., 2019).

Another study by Turgis et al. (2008) which used a combination of essential oils, *E. coli* and *Salmonella typhi* were inactivated by essential oils or their major constituents with irradiation had a synergistic effect by irradiation in medium-fat ground beef. This was expected because studies involving *S. typhimurium* and *E. coli* O157:H7, *S. typhimurium* population is always less than *E. coli* O157:H7 population at the end of the study, *S. typhimurium* populations were always less with approximately 1 $\log_{10}$ cfu/ml than *E. coli* O157:H7 populations given the same treatments (Callaway et al., 2008).

CHAPTER SIX: CONCLUSION

From the research results, *P. acidilactici* had a better effect in the reduction of bacterial growth of *S. typhimurium* as compared to *P. pentosaceus.* Thus, *P. acidilactici* can be recommended for application in the meat industry such as Meatco, it can be applied to meat cuts in the production area just before the vacuum packaging stage. However, further research is still needed to determine the optimal bacterial concentration of *P. acidilactici*. Once *P. acidilactici* is enclosed in the meat packaging, it can reduce bacterial growth present during processing and handling. It is evident from the obtained results that research work in the laboratory yielded good results as compared to the application on the actual product (e.g., meat samples), where the effect of the tested *Pediococcus* strains on meat inoculated with pathogenic bacteria was not as good for their antimicrobial effects observed in the co-culture techniques. There was no significant ($P > 0.05$) reduction in populations of both *S. typhimurium* and *E. coli* O157:H7 during storage at 4^{o}C by the selected LAB strains. To date, no intervention can be guaranteed to eliminate all pathogens. This finding is supported and confirmed by the results of this research where there was no complete elimination or inhibition recorded in any of the co-cultures, but substantial reductions have been observed and lowering the risk of food-borne illness. Hence, *P. acidilactici* has better antimicrobial potential regarding the antibacterial compounds responsible for the inhibition of these two pathogens with a great interest for this antibacterial activity to be further investigated and characterized. From abattoir and slaughter level farmers supplying slaughter, ready to slaughter animals need to strengthen preventive strategies such as the addition of probiotics to animal feed to help eliminating the shedding of *E. coli* O157:H7 by slaughter animals (Uyeno et al., 2015). Equally important, slaughterhouses care must be taken during cattle handling to prevent contamination of

meat with *E. coli* O157:H7 and *Salmonella*. This research was aiming just at that, but though the pathogen population were very difficult to keep below the threshold, they were reduced to a low level. It can be concluded that LAB (*P. acidilactici* and *P. pentosaceus*) used in this research do not have a bactericidal effect, but the bacteriostatic effect as was observed on the first nine days during the inoculated meat sample during the storage. The use of LAB in food production to (*in-situ*) reduce microbial count is not common because most of the research were conducted in laboratories (*in-vitro*) and therefore, their potential application remains theoretical and need further confirmatory studies. In conclusion, the results presented in this study indicate that antimicrobial activity of *Pediococcus* strains used in this research were not able to inhibit growth of both *S. typhimurium* and *E. coli* O157:H7 as supernatant or as cell free supernatant. Therefore, the null hypothesis can be accepted that the antimicrobial activity of the selected commercial probiotic strains is not able to eliminate the growth of *S. typhimurium* and *E. coli* O157:H7 in single and co-culture design. Additionally, the level of *Pediococcus* strains used in the research and low storage temperature do not inhibit the survival of *S. typhimurium* and *E. coli* O157:H7 on beef.

CHAPTER SEVEN: RECOMMENDATIONS

Based on the findings and conclusions presented, the following recommendations can be suggested:

1. It is recommended that senior management needs to invest in continuous training of slaughter personnel including both floor workers and to ensure that they take possession of hygiene practices throughout animal slaughter and during further processing.
2. Fast line speed also leads to cross-contamination during slaughter (especially between carcasses) as the floor workers are under pressure to finish the cattle number of the day.
3. Workers also need to be given training on the risk and effect of pathogens such as *S. typhimurium* and *E. coli* O157:H7 on human health, product quality and company revenue.
4. More *in-situ* applied research is needed on the conditions under which LAB inhibit pathogens in raw ground beef and on the mechanisms of action for this antimicrobial effect.
5. The result from this research indicates that *Pediococcus* species used can be an effective intervention for raw ground beef to control *E. coli* O157:H7 and *Salmonella.* Future research may include increasing the ratio of 1:2 or 1:4 (pathogens: LAB) to be applied in co-culture design whereby, in this research, both pathogens and LAB were at a 1:1 ratio.
6. Future research should consider the usage of selective agar that can differentiate between colonies of *Pediococcus* species against *E. coli* O157:H7 and *Salmonella* either by colour or colony shape, the current research used agar that was selective for pathogens only.

7. Future research can look at option where the working concentration from the laboratory can be applied to carcasses in the chillers at 2 - 4 ^{o}C, sampling can be done after 48 hours to measure the effectiveness of the *Pediococcus* solution.

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Appendices

Appendix 1 Ethical Clearance Certificate

UNAM
UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: **AREC/022/2020** **Date: 12/08/2020**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

Title of Project: **Antimicrobial effect of selected probiotic strains on Salmonella spp and E. coli 0157/H7 in a commercial slaughterhouse in Windhoek**

Nature/Level of Project: Master of Science in Microbiology

Researcher: **Kalihulu Martha Shapwa**

Student Number: 200417274

Faculty: Faculty of Science

Supervisor: Prof. A. Cheikhyoussef **Co-Supervisor:** Dr. J.D. Uzabakiriho

Take note of the following:

(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.

(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.

(c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.

(d) The UREC retains the right to:

(i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

(ii) Request for an ethical compliance report at any point during the course of the research.

AREC wishes you the best in your research.

Dr. Yvonne Hemberger

Dr. med. vet. M.Y. Hemberger
AREC Chairperson

Appendix 2 Official permit from Meatco

MEATCO Namibia

Head Office
Sheffield Street
P.O. Box 3881
Windhoek Namibia
Tel: + (264) 61 3216400
Fax: + (264) 61 3216401

To: Mr. Angus Claasen
Acting CEO Meatco

To: Dr. Adrianatus Maseke
Senior Manager: Quality Assurance, Health and Safety

Date: 19th December 2019

REQUEST TO USE MEATCO LABORATORY FOR MASTERS RESEARCH

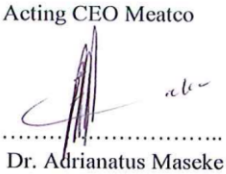
I am currently enrolled for a Masters in Microbiology by research through the University of Namibia (UNAM), specializing in Food Microbiology. I would like to ask for your permission to conduct a research study entitled "Antimicrobial effect of selected probiotic strains on *Salmonella* spp. and *E. coli* 0157:H7 in a commercial slaughter house in Windhoek, Namibia". Which will focus on ways to determine the antimicrobial activity of selected *commercial* probiotic strains in eliminating *E. coli* 0157 and *Salmonella* in beef in co-culture design and to evaluate the antimicrobial activity of locally isolated lactic acid bacteria and compare its potency in eliminating the growth of *Salmonella* spp. and *E. coli* O157:H7.

The reaserch will use strains of *Pediococcus pentosaceus*, *Pediococcus acidophilus, Lactobacillus, Lactococcus* and *Pediococcus,* these strain will be used in combination and their combination reduce the growth of most pathogen and bacteria, thus they will not contaminate the laboratory samples. The research will mainly focus on elimination the pathogen tested in Meatco Lab. The laboratory work for the research will be done after hours and weekend after normal laboratory testing is done to avoid contamination of sample.

I would like to appeal to your kind assistance to kindly provide us permission to use Meatco laboratory facility after hours and weekend and meat sample collection from production, the reagent and consumable will be provided by the University of Namibia. My main supervisor is Prof. Ahmad Cheikhyoussef (Head of Science and Technology division, Multidisciplinary Research Centre, UNAM) and cosupervised by Dr. Jean Uzabakiriho (Department of Biological Sciences, UNAM).

Namibia).

Mr. Angus Claasen
Acting CEO Meatco



Dr. Adrianatus Maseke
Senior Manager: Quality Assurance, Health and Safety

Kind Regards,
Kali Shapwa
Laboratory Manager
Meat Corporation of Namibia (MeatCo)
Tel : +264 (0)61 321 6008
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