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INYUVESI YAKWAZULU-NATALI

The use of DNA barcoding in the forensic identification of animal species in processed meat products from KwaZulu-Natal, South Africa

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"In relinquishing the limited, the unlimited becomes yours" – Sadhguru

Abstract

The price of meat products in South Africa are exceptionally high, to the extent that these products have been categorized as a luxury for many South Africans. The estimated price of white meat per kg is R115,00 while the price of red meat can average at R190,00 per kg, this makes processed meat products an easy target for meat adulteration and substitution. This study aimed to use DNA barcoding to determine if samples collected from local meat markets around eThekwini, KwaZulu-Natal have been subject to food fraud by means of meat adulteration and mislabelling. Universal primers for the amplification of the mitochondrial gene, Cytochrome C oxidase subunit 1 (COI) coupled with bioinformatic analyses using Barcode Of Life Database was utilized in this study. This study has determined that 62% of the samples were indeed adulterated, lamb samples were reportedly substituted with mainly beef(64%), however species of duiker(12%), rat(1%), frog (1%) and even chimpanzee(2%) were also identified in the processed meat products. Chicken samples indicated substitution with fish, crab and even beef. Beef samples however reported the least substituted with only 1 samples showing substitution of beef to fish. Consumers have become increasingly aware of what they are consuming and for this reason this study serves to bring awareness to food fraud by means of meat adulteration and mislabelling. This study has provided valid information on the meat adulteration that is occurring in many local meat markets around Ethekwini, together with violations of the regulations pertaining to consumer protection and food labelling. The area of research is still in the novel stages in South Africa, for this reason it is recommended continued research is conducted not only looking at processed meat products but also fish products and even game meat available throughout South Africa.

Key Words: Cytochrome C oxidase subunit 1 (COI); Processed Meat; Food fraud; Meat adulteration; Mislabelling; Polymerase Chain Reaction; DNA barcoding; Bioinformatics;

PREFACE

The research data contained in this thesis was collected by the candidate, from February 2019 to November 2020, while based in the Discipline of Genetics, School of Life Sciences, University of KwaZulu-Natal, Westville Campus, under the supervision of Dr O.T Zishiri. The research was financially supported by South African National Research Foundation through Thuthuka Funding Instrument [grant no. TTK170411226583]. The contents of this study represent original work by the author, and has not been submitted in any form to another tertiary institution, except where the work of others is acknowledged in the text.

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Chapter 1: General Introduction

1.1 Background

In the last few years, food fraud has become a very big challenge worldwide (Shika et al., 2016). This impedes the interest of the consumer and undermines the confidence that should be found in the food market (Aung & Chang, 2014; Johnson, 2014; Wielogorska et al., 2018). Food fraud is a term which refers to purposeful substitution, tampering, addition or misinterpretation of ingredients, packaging, labelling and or information, this also includes any false statement about a product made for economic gain that can impact on the health of the consumer (Spink & Moyer, 2011). A popular example of this would be the mislabelling horsemeat saga that occurred in Europe during the year 2013, where horsemeat was being substituted for beef (NAO, 2013). In South Africa, Cawthorn. et al. (2013) reported that 68% of the samples investigated showed meat adulteration with plant protein such as soya and gluten, undeclared animal species of pork and chicken were detected as well. There are many detrimental consequences that accompany mislabelling of ingredients in food products (Ballin., 2010; Ali et al., 2012). These consequences are things such as guaranteeing compliance with prevailing legislation in terms of trade (Spink & Moyer, 2011; Nakyinsigeet al., 2012;), it can also be harmful to individuals who are allergic to specific meat products or animal species and it can pose a conflict in terms of certain religions that forbid the consumption of a specific animal species. (Hellberg et al., 2017). It has been reported in previous studies that 20-70% of various meat products are mislabelled (Cawthorn et al., 2013; Ozpinar et al., 2013; Mousavi et al., 2015; Okuma & Hellberg, 2015; Quinto et al., 2016). For this reason, food labelling no longer serves as a genuine source of authentic information for products and there is great concern about the product source and safety as well as production practices (Crandall et al., 2013; Walkera et al., 2013).

It is not a major challenge to identified meat products when they are sold as whole cuts but when taxonomic features have been removed because of processing technologies such as grinding, canning, curing and/or smoking, the way the meat product looks and the smell of the product can change (Perestam et al., 2017). This is when genetic traceability methods of profiles containing DNA or even protein are utilised at the species level, for the detection of specific products (Ballin, 2010; Hellberg & Morrissey., 2011). The reason being, DNA is not changed during the life cycle

of an animal and has been reported to be sturdy during procedures involved with processing (Fernández et al., 2013) methods used most commonly include Enzyme Linked Immunosorbent Assay (ELISA) (Ayaz et al., 2006), PCR-Restriction Length Polymorphism (RFLP) (Doosti et al., 2014) real time polymerase chain reaction (rtPCR) (Camma et al., 2012; Soares et al., 2013; Okuma & Hellberg, 2015) and DNA sequencing (Cawthorn et al., 2013; Kane & Hellberg, 2016; Quinto et al., 2016).

When considering the molecule which is to be used to identify undeclared animal species in meat products, protein methods have been found to have limitations (Farag et al., 2015). This is due to the conditions that the samples are subjected to, such as high temperatures, which will denature the protein (Farag et al., 2015). You. et al (2014) further explains that DNA based PCR techniques have significantly more advantages as compared to phenotypic based methods. This includes adaptability for many different types of products, an extremely rapid turnaround time, reliability, high sensitivity and scalability (You et al., 2014). Therefore, these techniques have been widely applied in investigations dealing with identification of specific species, food authentication and genetically modified organism quantification (You et al., 2014). The availability of equipment together with the cost of procedure determines which method of PCR (i.e., real time PCR, Restriction Fragment Length Polymorphism (RFLPs), conventional PCR, multiplex PCR, Simplex PCR) is likely to be used (Izadpanah et al., 2017). The gene amplified in the case of this study was the Cytochrome C Oxidase Subunit I (COI) for each animal species under investigation.

The method of DNA barcoding is set on principles of sequencing, this method has reportedly illustrated potential in identifying animal species (Hebert et al., 2003a,b; Shokralla et al., 2012). A genetic target, standardized in most animal species, is used. This gene of interest is found in the mitochondria and codes for cytochrome c oxidase subunit I (COI) (Herbert et al., 2003b). This gene, within species, is very well conserved and between species, high levels of divergence are shown, this enables the identification of samples at the species level (Hellberg et al., 2017). An unknown species is identified by being compared to a sequence database, in most cases Barcode of Life Database (BOLD), and the top species match is identified (Smith et al., 2008; Wong & Hanner, 2008; D'Amato et al., 2013). This nucleotide sequence is approximately 650 base pairs (bp) and is found in COI gene at the 5' end (Herbert et al., 2003a). Researchers have stated that

the introduction of this gene is due to it being an effective target for methods such as DNA barcoding together with multiplex PCR (Barcaccia et al., 2015; Hebert et al., 2003a,b).

1.2 Justification

The adulteration of processed meats is becoming increasingly evident throughout the world. The reason for this could be economic gain and sometimes contamination as a result of negligence under compromised hygiene in the meat processing plants. There is a paucity of published scientific literature from South Africa, let alone KwaZulu-Natal province concerning the specific animal species which are actually being used in processed meats products. The South African government does have regulations pertaining to food labelling however the issue of food adulteration and the non-compliance of the pertaining regulations could be due to poor auditing of local meat markets. Against this backdrop and in addition providing consumers with more information on what they are actually consuming, this study was conducted. There is a literature dearth on the specific animal species that are currently being incorporated into processed meats, this study will also serve to provide a greater understanding of what is truly found in processed meats in KwaZulu-Natal.

1.3 Hypothesis

It is hypothesized that processed meat products in KwaZulu-Natal do not contain any animal species that have not been declared on the packaging.

1.4 Aims and Objectives

1.4.1 To collect a representation of processed meats sold in KwaZulu-Natal from a variety of food outlets.

1.4.2 To isolate DNA from the processed meat samples using the DNeasy Blood & Tissue Kit.

1.4.3 To detect the presence of any undeclared animal species by amplifying the COI gene and then matching with the information deposited in the Barcode of Life Datasystems database (BOLD).

1.4.4 To confirm the undeclared animal species using DNA Barcoding.

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Chapter 2 : Literature Review

This chapter serves to review literature focussing on key areas that surround DNA barcoding in association with food fraud in processed meat products. The key elements of this review will include food fraud, processing of meat products, the Cytochrome C Oxidase I gene (COI), and methods, PCR and DNA barcoding. Previously used molecular techniques that have been investigated will be reviewed in this chapter to provide an understanding of where we are in forensic identification. There is a dearth of research surrounding this area of science, this literature review is therefore to aid in expanding forensic identification by means of DNA barcoding.

2.1 Food Fraud

According to Spink and Moyer (2011), the definition of food fraud is any intentional and or deliberate substitution, tampering, addition or misinterpretation of food, ingredients found in food products, packaging, mislabelling or statements that have been falsely made concerning a particular product mainly for economic gain. This is not a new problem, however in the recent years it has become more publicised due to incidences such as the horse meat saga that occurred in Europe in 2013 (NAO, 2013). Kane & Hellberg (2016) reported mislabelling occurred in 21% of the samples used in their research. Quinto et al. (2016) conducted an investigation of mislabelling in game meat having an occurrence rate of 18.5%. A much more recent study conducted by Tembe et al. (2018) in South Africa reported 65% of samples being adulterated. It is difficult to measure the economic or public health impact of food fraud (Spink & Moyer, 2011) however studies such as this one and the above mentioned make it possible. Consumer exploitation opportunities such as relabelling and selling meat products which are out of date are some of the economic incentives that result in food fraud (Calvin et al., 2006 ; Foster., 2011 ; Ma et al., 2014).

To detect the adulteration of processed meat with food ingredients that are undesired and undeclared is a very important food quality related issue. This is related to human health as certain ingredients might include toxins that cause allergies or are toxic to certain individuals (Ha et al., 2017). For individuals who present with allergies of certain food products, the avoidance of consuming those allergic ingredients is of paramount importance in order to prevent incidences of

allergic reactions (Druml & Cichna-Markl, 2014). It should also be considered that specific religious groups do not consume specific meats or have special preferences (Ortea et al., 2012), Islamic groups do not consume any meat or pork ingredients as well as animals with fangs or canine teeth, for example rats, dogs and cats (Ali et al., 2015), Jewish groups do not consume pork and shellfish and Hindu groups avoid the consumption of beef and pork products (Cawthorn et al., 2013; Premanandh, 2013). Schoeman (2017) conducted a study on the religious demographics in South Africa, this study has reported that KwaZulu-Natal has the highest population of Hindus (3.9%), the second highest population of Muslims (2.6%) and 0.1% of the population belong to the Jewish faith. These figures allow for an overview of which animal species are not consumed in large quantities in KwaZulu-Natal, thereby creating an oversupply in the meat market, triggering meat producers to fraudulently include the unsold meats in processed meat products against the wishes of consumers. These are just a few of the major religions in the world that are restricted to consuming certain animal species and food adulteration may pose a problem to these groups. It is in view of the previously alluded reasons that product labels should reflect exactly what the total food content is in terms of the ingredients used in order for consumers to safely choose what they can and cannot consume (Premanandh, 2013). It has been reported that products labelled as containing beef only are often adulterated with pork due to the economic advantage pork provides, i.e. in some countries such as Japan, Korea, China and South Africa, where pork is cheaper than beef and therefore this occurs (Singh and Neelam 2011; Soares et al, 2013). Many countries have food labelling regulations that require any meat species found in processed meat products to be declared to consumers for medical purposes, personal food preferences as well as religious food ethics (Doosti et al., 2014). However according to Tanabe et al. (2007) processed meat products are still being mislabelled for meat species, most especially pork, it should be noted that this can be intentional or purely accidental. Even though international and national food laws require that product labels contain the complete declaration of ingredients used, this is insufficient in preventing food that is adulterated from reaching food markets (Ballin, 2010; Druml & Cichna-Markl, 2014).

A study conducted by Cawthorn et al. (2013) reported meat products that were processed, where 68% of those products contained species that had not been declared on the food packaging label, this included animal proteins soya and gluten, as well as animal species of beef, water buffalo,

pork, sheep, goat, donkey and chicken. In Europe, at the beginning of 2013, there was a scandal in which the authenticity of processed beef products were in question due to the nondisclosure of horse meat and pork being added to processed products (Dept of Agriculture Food and the Marine, 2013). Tembe et al. (2018) reported that out of 40 processed meat samples collected from Durban, South Africa, 65% of those samples were contaminated with other undeclared meat species of sheep, beef, pork and chicken. Tembe et al. (2018) further reported that beef was the leading contaminant in the samples investigated with undeclared beef being detected in 60% of lamb sausages, 100% of chicken sausages and 28,6% in pork sausages. This study also found that sausages were more likely to be contaminated as compared to burger patties.

Food fraud impacts the quality of food that individuals are consuming on a daily basis and in some extreme cases it also affects the public health. Food fraud can affect public health in 3 different ways: indirectly, directly and technically (Spink & Moyer, 2011). The indirect effect of food fraud on public health involves consumers being at risk due to long term exposure, this results in a build-up of toxic contaminants in the body due to low doses of that contaminant being ingested (Spink & Moyer, 2011). Direct effects are the immediate or imminent risk of a consumer being exposed to a contaminant that is either acutely toxic or lethal (Spink & Mopyer, 2011). Lastly, the technical effect on public health as a result of food fraud involves a non-material nature, when there is deliberate misrepresentation of information such as mislabelling, omitting of specific ingredients or the origin of the animal species. (Spink & Moyer. 2011). For these reasons it is imperative that studies are conducted in this area of science to better the knowledge of the general public in terms of what they are feeding their bodies.

2.2 Food Processing

Meat is processed in order to extend the shelf life of the product and to allow for the production of a food product that is convenient to be used later (Casey et al., 2003). When meat is processed the enzyme activity is reduced and oxidation of fat in the meat does not occur, this essentially ensures that the meat cannot be spoilt by microorganisms (Casey et al., 2003). Meat can be processed by drying, curing with salts, and grinding to make sausages and patties (Casey et al., 2003).

The method of drying is one of the most inexpensive ways to preserve meat and can be tracked right back to prehistoric times (Ahmed et al., 2013). For this method basic equipment is needed. A dry or airtight storage area is all that is required (Faisal et al., 2009). Throughout history drying has been done using the sun, wind and fire to remove water from the meat products (Ahmed et al., 2013). When meat is dried, it essentially means the water content in the meat is lowered thereby minimising the rate of chemical reactions allowing for longer storage and even distribution of those products (Ahmed et al., 2013).

Curing started as a simple technique to preserve meat and it consisted of mixing salt into the meat. This later progressed to colour and flavour development being included in the curing process. Texture, juiciness and colour are the three qualities that are looked at in cured meat products (Mathur & Bhatia, 2014). Texture depends on the pre and post slaughter conditions as well as the cut of meat used, while juiciness and colour are mainly controlled by methods used in curing (Mathur & Bhatia, 2014).

If one had to put meat into a grinder, the product that is obtained is not necessarily processed however the whole process of mincing or grinding meat can result in a lot of species contamination due to lack of proper equipment cleaning being a prominent reason for contamination. Processed meat is any meat product that was produced with the intention to extend the shelf life of the product or alter the taste of the product (Casey et al., 2003). Common ground processed meats include sausages and patties. Meat is put through a grinder, different spices are added and then it is either encased in animal intestine or patted down to form a patty.

2.3 Meat Adulteration

Generally when meat products are sold as whole cuts they can be identified easily. However once meat becomes processed through methods such as drying, smoking, canning and/or grinding, the appearance as well as sensory characteristics can change in the final product (Hellberg et al., 2017). This poses a problem as consumers are not able to identify species used in the products, this together with the combination of variation in retail prices of poultry and meat products, escalates the probability for species substitution (Perestam et al., 2017). There have been cases reported by Kane & Hellberg (2016) Perestam et al. (2017) as well as Tembe et al. (2018) which indicates that

processing meat products does increase the risk of secondary species additions that have not been declared on the label. In Ireland a study was conducted, where a number of ground beef products, beef burgers as well as salami was tested, and the findings reported adulteration of 37% of the products containing horsemeat that had not been declared, while 85% of the products contained pork meat that was undeclared (FSAI, 2013).

The intentional adulteration of food is mainly for economical gain but it can also be done to avoid import restrictions in countries such as America where only eligible products from approved countries can be imported provided no U.S.A regulations have been violated (Quinto et al., 2016). Meats which are cheaper such as chicken and pork are substituted for meat products that are high in value such as beef and lamb (Premanandh, 2013). An increasing demand for meat products throughout the world may also be the reason for intentional adulteration as again higher value meat products may be substituted with cheaper counterparts (Pinto et al., 2015). Cawthorn et al. (2013) reported that 12% of minced meat samples investigated had been substituted with soya, while pork was the most common undeclared species detected in minced meat (38%). Another similar South African study conducted by D'Amato et al. (2013) showed a high incidence of mislabelling occurring in game meat with meat products containing undeclared pork and lamb. There are some cases where unintentionally, undeclared animal species are found in meat products, this could be due to negligence while the meat product was being produced or even cross contamination in terms of the equipment used to process the meat not being cleaned thoroughly (Cawthorn et al., 2013).

Meat and meat products are part of the most highly priced food products as well as highly sought after all over the world, this does not differ in South Africa (Cawthorn et al., 2013). Beef, lamb, chicken and pork were found to be most consumed when focussing particularly on the South African population (Hoffman et al., 2005; Cawthorn et al., 2013). Since 2000, the price of beef and mutton together with their by-products has drastically increased in comparison to chicken and pork (NAMC, 2010; DAFF, 2011). A reason for this could be due to the rising costs of production of cattle and sheep (Cawthorn et al., 2013). With the world population rising, so is the demand for meat products (Cawthorn et al., 2013 ; Sumathi et al., 2015), this in turn means that meat prices, meat availability, meat quality as well as nutritional value will also increase, thereby allowing for the selection of meat products by consumers to be altered. (Mahajan et al., 2011; Santos et al.,

2012). With these changes comes an increase in cases of mislabelling as well as adulteration/replacement of more expensive meat with cheaper meat, and in some cases even the addition of a cheaper or less valued meat type in order to satisfy the growing demand of meat products, particularly processed meats (Aida et al., 2007; Ali et al., 2012; Kumar et al., 2015).

Against this backdrop, food labelling can no longer be considered a reliable source of information for products that individuals are consuming, there has been more concern, from consumers, about the practices, production, source of ingredients and safety of the food that they are being supplied with by retailers (Crandall et al., 2013; Walkera et al., 2013). It has also been reported that processed meats are more susceptible to fraudulent labelling due to economic gain which is a result of cheaper meats being sold as complete or partial replacements to higher value meats, as previously mentioned (Mafra et al., 2008; Singh & Neelam, 2011; Soares et al., 2013). While attempting to make informed food choices, for purposes of religion, health and or preference, consumers rely on the accuracy of food labelling (Ballin, 2010) however if food labels are not accurate it puts a consumer in a very vulnerable position, not knowing what is actually contained in the food they are consuming. Premanandh (2013) reported that food authentication has now become a routine monitoring parameter for food labelling verification and legislation issues, however in South Africa, new legislation has been published to promote proper description of food products as well as transparency when it comes to food. This includes regulations that relates to the Labelling and Advertising of Food items (R.146/2010) (DoH, 2010, pp 3-53) as well as the consumer Protection Act (R.467/2009) (DTI, 2009, 1-186), as stated by Cawthorn et al. (2013). The first legislation requires food labels with all food components being mandatorily declared, this includes certain designated common allergens. The second Act provides protection to individuals from being exploited in terms of the sale and marketing of consumer goods. Despite all of this food adulteration has been confirmed in the Durban metropole as recently as 2018 as reported Tembe et al. (2018). Therefore, it is essential that studies be conducted in this area in order to allow for consumers to be aware and knowledgeable.

2.3.1 Case Study Review on Meat adulteration

Kane and Hellberg (2016) conducted a study using full and mini barcoding to identify ground meat products sold in U.S.A. This study was able to report a mislabelling rate of 21%, with beef being the most common undeclared species found in meat products, undeclared pork, chicken and lamb were also reported as undeclared in the products that were investigated (Kane & Hellberg, 2016). Two of the investigated samples were reported to contain horsemeat. As reported by Kane & Hellberg (2016) trends for mislabelling possibly indicate meat products of higher cost species such as beef and lamb being intentionally mixed with lower-cost species such as chicken and pork, unintentional mixing is also a possibility due to the contamination that may occur in the processing facility (Kane & Hellberg, 2016).

Quinto et al. (2016) used DNA barcoding to identify mislabelling in game meat from U.S.A. As reported 18,5% of samples investigated were determined to be potentially mislabelled. Quinto et al. (2016) reported that the cause for mislabelling could be economic gain as four products were priced with a lower value than the actual identified species listed price value. In South Africa a similar study of game meat identification was conducted by D'Amato et al (2013). This study reported findings similar to that of Quinto et al. (2016) however the rate of mislabelling reported by D'Amato et al. (2013) is much greater (69,18%). Samples were reportedly substituted with beef, horse, kangaroo, pork and lamb. African wild species of kudu, nyala, bushbuck, zebra and giraffe were also reported as being used for substitution (D'Amato et al., 2013). Just as reported by Kane & Hellberg (2016) and Quinto et al. (2013), D'Amato et al. (2013) reported substitution could be both intentional, indicated by the presence of species not usually present on the market such as domestic animals, kangaroo and giraffe or unintentional by human error of contamination and misplacement of labels (D'Amato et al., 2013).

A similar South African study by Tembe et al. (2018) identifying undeclared meat species in processed meat products by means of species specific primers reported 65% of samples being contaminated with undeclared meat species. This study reported that 80% contaminated samples were sausages with only 50% of burger patties being contaminated. Tembe et al. (2018) further reported that animal species identified included lamb, chicken, beef and pork. This study also reported beef as the most common contaminant. Similar reasoning of economic gain by

substitution of higher value meat with lower value meat together with contamination due to human error in terms of improper cleaning of mincing machines or processing equipment have been reported by Tembe et al. (2018). A similar study conducted in South Africa by Cawthorn et al. (2013) reported undeclared animal and plant protein in 68% of investigated samples. Cawthorn et al (2013) also reported higher incidence of contamination in sausages and burger patties as compared to minced meat, dried meat and deli meats. Economic gain by meats of cheaper meat products being substituted more frequently in order to make processed products more accessible to consumers has been reported by Cawthorn et al. (2013) as a reason for the adulteration of processed meat products. Unlike the study conducted by Tembe et al. (2018) this study reported pork as the most common undeclared animal species detected in the products investigated (52%). Chicken was the second most frequently detected undeclared animal species reported by Cawthorn et al. (2013). A sample investigated by Cawthorn et al. (2013) detected donkey as an undeclared animal species in a sausage declared as beef. This indicates a high probability of intentional substitution for economic gain as donkey is not a commonly processed species for human consumption in South Africa.

2.4 Molecular techniques used for forensic identification of meat species

2.4.1 Meat Identification based on DNA methods

When trying to identify specific species in processed meats there are a number of detection methods which exist. These methods rely on protein or DNA analysis (Ha et al., 2017). Analytical methods that are protein based include immunological assays, such as Enzyme Linked Immunosorbent Assay (ELISA) (Chen and Hsieh, 2000), chromatography (Chou et al., 2007). It should, however, be considered that analytical methods based on proteins are not appropriate for the investigation of processed meat products as proteins are known to denature during processing due to the high temperatures used (Ha et al., 2017). For this reason, analyses based on DNA, such as polymerase chain reaction (PCR) using species-specific primers, real time PCR, and PCR restricted fragment length polymorphism are more frequently used to identify fraudulent meat products (Man et al., 2007; Murugaiah et al., 2009; Soares et al., 2010). This is because, PCR based methods are able to amplify billions of copies of a specific DNA fragment (Kadri, 2019). What makes PCR based techniques such a powerful molecular technique is the fact that it is capable of amplifying nucleotide sequences without the amount of DNA present becoming a limiting factor which therefore allows for sequences to be amplified with miniscule amounts of DNA extract present (Kadri, 2019). There are also numerous advantages of using DNA based analyses, such as, simplicity, sensibility, rapidity as well as specificity which is extremely important when trying to determine a specific species (Lockley & Bardsley, 2000).

The most appropriate molecule for identification and detection of specific species in foods most especially in this case processed meats, is considered to be DNA (Singh & Neelam, 2011). The relative stability of DNA at high temperatures, unlike proteins, allows for it to be analysed in fresh and frozen products, as well as degraded, mixed and processed products (Lenstra, 2003). In addition to this, the characteristics and presence of proteins is dependent on what type of tissue is being analysed, however, DNA is identical and is found in all cells. The genetic code also affords diversity which allows for even closely related species to be discriminated against (Lockley & Bardsley, 2000; Ballin, 2010). Despite the above and the fact that meat adulteration has been suspected of occurring in South Africa (Anonymous, 2011; Schroeder, 2011; Weiner, 2011), DNA

techniques have not been thoroughly utilized for meat products that are available commercially in South Africa to be authenticated (Cawthorn et al., 2013).

The PCR assay, on the basis of mitochondrial species-specific primers, has been shown to be a much cheaper and less time consuming technique for detecting adulterated and mislabelled processed meat products in comparison to other PCR based techniques previously mentioned (Dai et al., 2015). The use of this method does not require additional sequencing or further digestion of the products obtained from the PCR with restriction enzymes or endonucleases (Farag et al., 2015).

Multiplex PCR involves identification of several species simultaneously (Alikord et al., 2018). The use of this method allows for the hybridization of species specific primers in specific regions of the species under investigation (Alikord et al., 2018). Multiplex PCR allows for the use of both genomic and mitochondrial genes to be targeted for species identification (Ghovvati et al., 2009). Multiplex PCR advantages include efficiency pertaining to mixed samples, reliability and sample sensitivity (Alikord et al., 2018). A study conducted by Ghovvati et al. (2009) used multiplex PCR in the detection of poultry residuals and reported 40% of sausages and 30% cold cut samples to be contaminated with poultry residuals.

Another PCR based tool is Amplified Fragment Length Polymorphism (AFLP), which uses randomly amplified polymorphic DNA as well as restriction length polymorphisms (Muiru et al., 2008). In comparison to alternative DNA markers, this tool is characterised as being dense in information, repeatability is highly favourable and there is no need for prior sequence information in order to construct primers (Muiru et al., 2008). Additional advantages with AFLP methods include cost efficiency, user friendly operation and highly selective conditions that allow for results that are highly reliable (Muiru et al., 2008). AFLP methods have been used extensively in the analysis of genetic structures pertaining to populations, gene localizations and breed discrimination (Haase et al., 2014; Sabir et al., 2014). Chen et al. (2010) used this method for the authentication of commercial beef jerky. The study revealed the substitution of yak meat with cattle meat in jerky products (Chen et al., 2010). The method of PCR-AFLP has been reported to be inefficient in the identification of mixed samples purely due to the differential template amplification (Alikord et al., 2018). However, it should be stated that Murugaiah et al. (2009) used

this method for meat traceability and Halaal authentication in mixed meat products of pork, beef, quail, chicken, rabbit, goat and buffalo. Reported results of differences at the COI level among the RFLP types of species due to genetic variation indicated the method was an effective tool (Murugaiah et al., 2009). The limitation in this method arises due to the detection of mutations at the restriction sites of chosen enzymes even if several other regions may have differed throughout the gene (Murugaiah et al., 2009). This can be overcome by the inclusion of more potential enzymes in order for more mutation regions to be identified.

There are some disadvantages when it comes to qualitative PCR based techniques, these include sensitivity issues due to the amplification potential, that could result in target DNA becoming incapable of testing by means of any other method (Sawyer et al., 2003). Researchers have argued that techniques based on qualitative PCR methods are too sensitive and against this backdrop, infinitesimal traces of material may result in a positive result (Sawyer et al., 2003). Quantitative PCR tests are therefore adequately accurate in distinguishing samples that are contaminated with samples that have been intentionally adulterated (Sawyer et al., 2003). Real-time PCR is a quantitative PCR tool that efficiently and accurately amplifies target DNA (Kim et al., 2020). Real-time PCR with the use of mitochondrial DNA sequences are therefore able to amplify target DNA that has been degraded by the processing of food or even products that have been mixed with other animal species (Girish et al., 2004; Kim et al., 2019). Advantages of real-time PCR include measurements to be obtained at early stages of the PCR process which allows for more accuracy in comparison to the end point analysis that is commonly associated with qualitative PCR based methods and the gel based measurements at the end of the process (Sawyer et al., 2003). This method is rapid and can be used for heat-treated, mixed samples and raw samples (Alikord et al., 2018). The precision of real-time PCR is down to 0.1 pg and this makes it extremely reliable and accurate (Alikord et al., 2018). Okuma & Hellberg (2015) used real-time PCR to detect meat species in pet food. The study reported 38,4% of samples could have been potentially mislabelled.

When unknown samples are to be investigated DNA sequencing is the best applicable method due to obtained DNA sequences being entered and queried on either BOLD (http://www.barcodinglife.org) or NCBI (http://www.ncbi.nlm.nih.gov) thereby identifying the animal species of the unknown samples (Ballin, 2009). DNA based LCD-array may also be used

however this method will have to be followed by species-specific PCR and sequencing of COI, 12SrRNA and NADH Dehydrogenase 2 (ND2) for confirmation to detect any meat adulteration and mislabelling. This method was used by a study conducted by Cawthorn et al. (2013) and it revealed that 68% of samples contained undeclared species.

2.4.2 Protein based methods for meat identification

Electrophoresis has been reported as the earliest method used for the identification of meat species in meat products, this method uses soluble proteins such as sarcoplasmic proteins and is implemented on raw meat samples (Hui & Sherkat, 2006). There are three types of electrophoresis that have been used for species identification in meat: Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE), Isoelectric focusing (IEF) and Capillary Electrophoresis (CE) (Alikord et al., 2018). SDS-PAGE is a method which separates proteins on the character patterns based on molecular size differences of the specific protein bands (Hui & Sherkat, 2006). This method however has several drawbacks such as low reliability, difficulty interpreting gels and lack of sample sensitivity (Hui & Sherkat, 2006). IEF is based on the differences of the isoelectric points present in proteins (Kim et al., 2004). This method allows for the identification of meat species in mixed meat products and has been reported to have an accuracy rate of 10% (Mackie et al., 2000). CE is a method that combines electrophoresis and chromatography (Hui & Sherkat, 2006). The sensitivity of CE allows for protein analysis, but it is not so sensitive to be used for routine identification pertaining to meat adulteration (Alikord et al., 2018). A disadvantage of this method is the expensive equipment that is required (Alikord et al., 2018).

Chromatography can be utilized for the identification, analysis and determination of proteins found in meat products (Ballin, 2009). This method has the advantage of being used efficiently for mixed and heated meat products however the protein pattern interpretation is relatively difficult (Alikord et al., 2018). There are two methods of chromatography that have been used for the identification of meat species: high performance liquid chromatography (HPLC) and gas chromatography (GC) (Alikord et al., 2018). In order for GC to have efficient product analysis the products need to be highly volatile, fatty acids are an example of what products can be used, however GC does not allow for the analysis of low volatility products such as amino acids, the use of these products may be incapable of being directly determined by GC (Tranchida et al., 2004). When HPLC methods

are used for the determination of meat species in products it is based on the differentiation of protein profiles, peptides and amino acids that are present within different meats (Alikord et al., 2018). This method is highly sensitive and reproducible which is best for routine analysis (Alikord et al., 2018). Major disadvantages of HPLC include extractions that can become tedious and time consuming analyses which may becoming a limiting factor in the widespread use of HPLC as a technique for the identification of meat species in meat products (Chou et al., 2007).

Methods involving spectroscopy are dependent on the absorption of light at wavelengths that are precise on the electromagnetic spectrum (Hui & Sherkat, 2006). A powerful spectroscopic tool is mass spectrometry (MS) which has become a vital part of peptide and protein analyses in food products (Alikord et al., 2018). This method is incredibly efficient in terms of sensitivity for structural identification (Alikord et al., 2018). MS however, is a very costly procedure and this method may yield too many signals to ensure efficient differentiation of meat sources (Alikord et al., 2018).

Enzyme-linked immunosorbent assay (ELISA) is a technique that was introduced in the early twentieth century (Alikord et al., 2018). This method is frequently used for the detection of animal proteins, and is based on the interactive nature of antibodies and antigens (Alikord et al., 2018). What makes this method advantageous is its simplicity, high sensitivity and most importantly the high level of accuracy (Alikord et al., 2018). ELISA is also used as a method that is efficient for the compulsory control of samples in order to provide safeness of food that is muscular such as meat (Hui & Sherkat, 2006). In terms of meat identification, two ELISA methods have been frequently used, the first is, Indirect ELISA in which two anitbodies are used, one attaches to the antigen and the other to the first antibody as an indicator for success (Alikord et al., 2018). Sandwich ELISA kits are the most frequently used for commercial purposes, due to the fact that the diluted extract can be directly added to the plate (Alikord et al., 2018). There are a number of commercial sandwich ELISA kits which are structured for the identification of cooked meats based on glycoproteins (Alikord et al., 2018). These kits however have a limiting factor which is the number and amount of animal proteins that can be processed for the identification of meat species (Van Raamsdonk et al., 2007). A study conducted by Giovannacci et al. (2004) confirmed that

ELISA kits used commercially are capable of detecting small quantities of lamb, pork, poultry and beef in canned meat products.

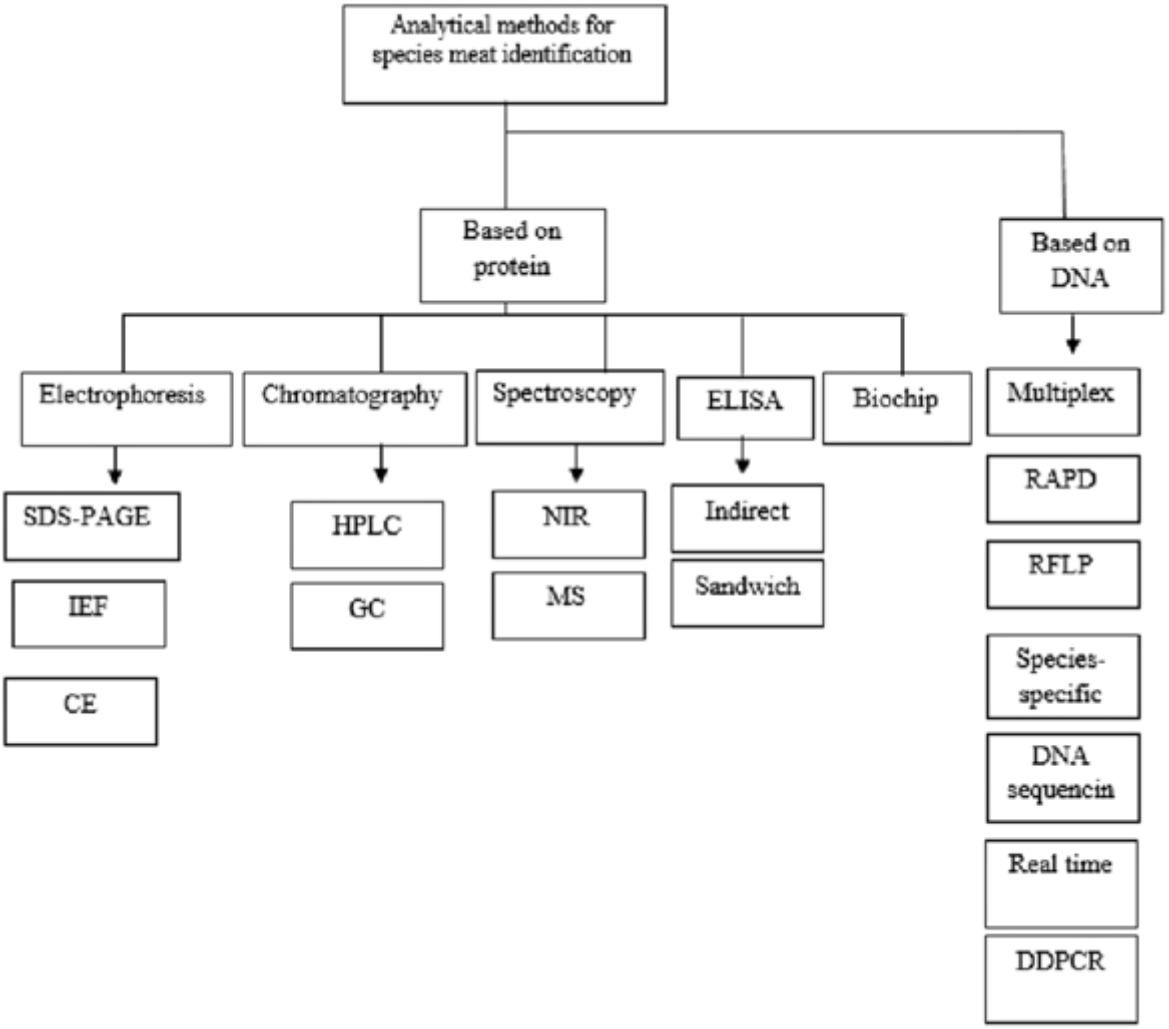

Figure 1: DNA and protein methods used for the identification of animal species used in meat products (Alikord et al., 2018).

2.5 DNA Barcoding

DNA barcoding is based on a molecular system with the use of a standardized genetic region which enables the identification of biological samples (Herbert et al., 2003a). Short DNA fragments that belong to the mitochondrial genome are amplified (Barcaccia et al., 2016). These regions, at the species level, are conserved and are also preserved in most processed products, this allows for the advantages of DNA barcoding in comparison to other genotyping and DNA fingerprinting approaches (Barcaccia et al., 2016). Successful species authentication, identification and delimitations can be conducted with the use of DNA barcoding where other methods would usually fail (Barcaccia et al., 2016). This method allows for proper labelling of species in processed products (Herbert et al., 2003a). DNA barcoding can be used for food products that are distinct and single or mixed species that produce DNA sequences that are species specific (Barcaccia et al., 2016). Against this backdrop, DNA barcoding has the potential to discover voluntary or accidental replacements occurring in food products (Barcaccia et al., 2016). In most animal species, the DNA barcode is approximately a 650 base pair (bp) region found in the mitochondrial gene. This region codes for cytochrome c oxidase subunit I (COI).

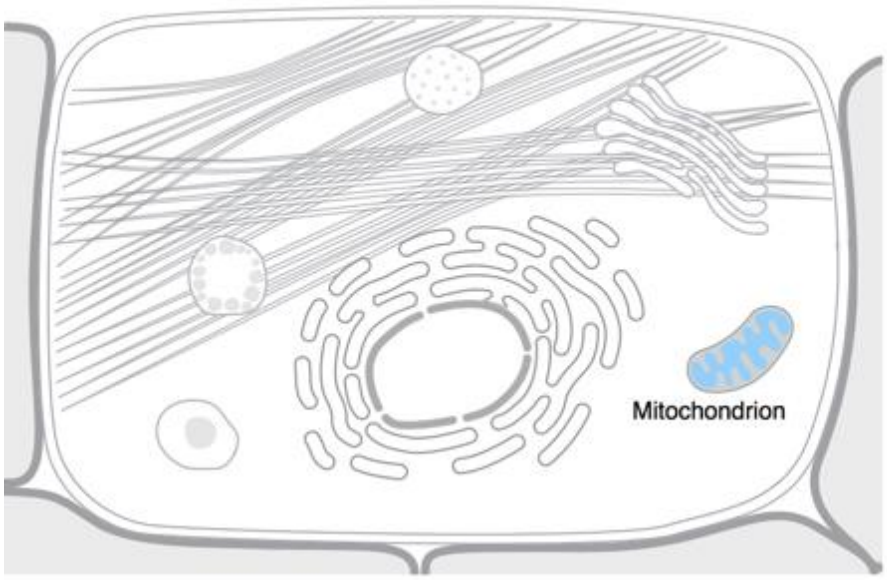

Figure 2: Illustrates an invertebrate cell with the mitochondrion highlighted in blue. (UniProt, Date Accessed 23 March 2019 https://www.uniprot.org/uniprot/L0NA81)

This method expands on the ability to identify species by looking at all life stages in an organism (Kress & Erickson, 2012). DNA barcoding can also be used as a discovery tool in terms of biodiversity, as it helps to identify species which are potentially new to science (Kress & Erickson, 2012). DNA barcoding consists of two basic steps, the first step is to build the DNA barcode library of the species of interest, which in the case of this study would be barcodes found in processed meat products. The second step is to match the barcodes obtained in the study against the barcode library in order to determine exactly which animal species are present in the processed meats (Kress & Erickson, 2012). The use of DNA barcoding has been in utilization for not more than a decade but there has experienced exponential growth with regards to the number of sequences that have been generated as well as the applications that can be used for barcoding (Kress & Erickson, 2012). DNA barcoding has been found to be more time consuming as compared to many techniques that are currently available for use but DNA barcoding is still advantageous as it enables an universal approach to the identification of species that is supported by genetic information of high level. (Hellberg et al., 2016). This study will make use of the database, Barcode of Life (BOLD) - www.barcodinglife.org – which allows for the integration of a bioinformatics platform, this supports all the analytical pathway phases, from the collection of specimens right up to a barcode library that is tightly validated (Ratnasingham & Herbert, 2007).

Eventually, this technique could have an impact in the scientific world when it comes to the development or even implementation of diagnostic molecular assays that are well suited for the genetic identification of meat products that are either fresh or processed with the use of DNA barcoding methods (Barcaccia et al., 2016). Against this backdrop, DNA barcoding is an extremely robust tool for diagnostic analyses, this is because of several features including evidence that the alteration of DNA is impossible to the common individual, DNA can be detected in every cell and it is resistant to heat treatments, and thus enables breed, individual or species identification. Despite the results of DNA barcoding being so promising, the techniques used are extremely expensive for utilization in tests occurring routinely, however they are molecular tools with a high level of reliability for identity checks, quality control and food fraud discovery in order to prevent the mislabelling of processed meat products (Barcaccia et al., 2016).

DNA barcoding advantages include features of this technique being testable and reliable on the assumption that the link between the barcodes and reference samples are supported (Barcaccia et al., 2016). DNA barcoding is a technique which can be reproduced at any given time by any given researcher (Barcaccia et al., 2016).

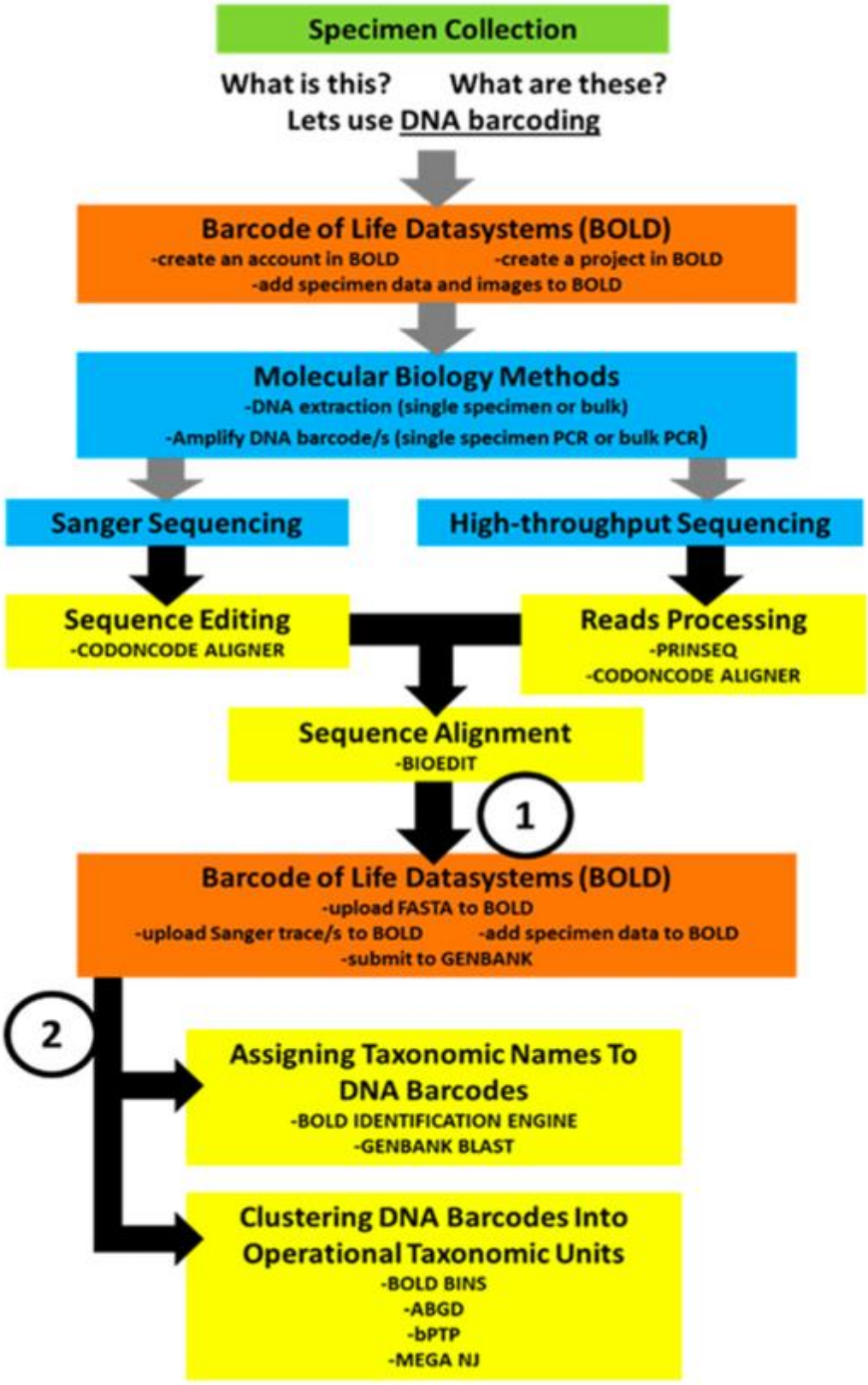

Figure 3: Steps involved in DNA Barcoding (Wilson et al., 2019)

The main stages in DNA barcoding involve DNA extraction, PCR amplification and DNA sequencing and analysis (Carmon et al., 2014). The isolation of DNA is a vital step in this process as without DNA that is high in quality, the amplification by means of PCR will not yield optimal results (Carmon et al., 2014). The step of amplification has to work and produce bands of good quality as the next step of DNA sequencing is dependent on the amplification results (Carmon et al., 2014). Lastly the final step of sequence analysis determines the identity of the unknown species and for this reason this step must be successful (Carmon et al., 2014). The accurate implementation of all three steps is vital for the success of DNA barcoding but it should be noted that modifications may sometimes be needed (Carmon et al., 2014). As illustrated in figure 3, the purpose of a DNA barcoding workflow is to firstly produce a digital representation of the DNA barcodes that is clean and reliable and to secondly to utilize the barcodes in order to obtain information pertaining to taxonomy of the unknown samples through algorithms that enable the comparison of DNA sequences (Wilson et al., 2019).

DNA barcoding targets the COI gene at a length of 658 base pairs to be precise. In well preserved and fresh specimens, this has been reportedly true. (Herbert et al., 2003a,b). There can be a reduction in the quality of DNA due to various conditions that are common to processed foods, these are things such as high temperatures and pressures as well as low pH levels (Hellberg & Morrissey, 2011). This in turn makes it difficult to obtain barcodes that are of full length for samples of food products that have undergone excessive processing, i.e. canned products, burger patties and sausages. Ultimately the processing of food leads to DNA fragmentation but the amplification of short DNA regions is still possible (Hellberg et al., 2017).

As mentioned above, the conventional barcoding method targets the COI gene sequence at approximately 650 base pairs to allow for species identification in well preserved fresh specimens (Herbert et al., 2003a,b). The quality of the DNA can however be reduced by conditions that are common to food processing such as high temperatures, high pressures and low pH levels (Hellberg & Morrissey, 2011). Fragmentation of DNA is often a result of food processing however, the amplification of the short DNA regions may still be a possibility (Hellberg et al., 2017). In order to overcome this challenge Muesnier et al. (2008) designed a universal primer set that targets a short region of DNA found within the full length barcode referred to as a mini-barcode. The mini

barcode universal primer is reportedly capable of amplifying the target DNA fragment in 92% of species which have been tested, this includes mammals, fish, insects and bird species (Hellberg et al., 2017). A study conducted by Shokralla et al. (2015) developed a mini barcoding system specifically for fish species identification in processed products, the study reported a success rate of 93,2% when tested against heavily processed fish products. If this is compared to Kane & Hellberg (2016) who conducted a study identifying ground meat products in U.S.A by means of full barcoding, the success rate reported here was only 21%. A similar study using full barcoding to identify game meat in U.S.A reported mislabelling occurring in 18,5% of the samples investigated (Quinto et al., 2016). As per the study conducted by Hellberg et al. (2017) full barcoding proved to be a robust method for the identification of poultry and meat species in several processed meat products that were declared as a single species on the label, with the exception being canned foods. Mini barcoding was able to outperform full barcoding when the analysis of turkey and duck products were performed but these mini barcoding primers did not perform well with many other species such as chicken, buffalo and beef.

In addition to the conventional full barcoding method and mini barcoding, there has also been research conducted by Dobrovolny et al. (2018) on a fairly recent method that combines DNA barcoding with next generation sequencing (NGS) known as metabarcoding. This method allows for multiplexing to occur and against this backdrop has become an even more powerful tool than traditional barcoding methods (Valentini et al., 2016). The process involves the use of short artificial oligonucleotides to index DNA molecules, thereafter amplicons are derived from different samples and are pooled and sequenced simultaneously in a single run (Valentini et al., 2016). Dobrovolny et al. (2018) reported Quality Scores (Q Scores) for both the forward and reverse reads at 89.9% and 87.8%. These results allow for the determination of how accurate the sequencing platform is, and is an indication of the probability that a particular base pair has been incorrectly called by the sequencer (Quality Scores for Next-Generation Sequencing, Pub. No. 770-2011-030 Current as of 31 October 2011). The study further indicated that all 21 meat species of interest were able to be identified and differentiated down to a proportion of 0,1%, against this backdrop, this method of DNA metabarcoding has great potential for application in the detection of meat species adulteration in routine analysis (Dobrovolny et al., 2018).

2.6 Cytochrome C Oxidase Subunit 1 (COI)

COI is a protein found in the mitochondria, with its location in the inner mitochondrial membrane (Strüder-Kype & Lynn, 2010). COI is a vital enzyme in the chain of electron transport (Strüder-Kype & Lynn, 2010). Against this backdrop COI is essential in the metabolism of organisms belonging to eukaryotic aerobic nature (Strüder-Kype & Lynn, 2010). The catalytic COI is encoded in the genome of the mitochondria and consist of several subunits as illustrated in figure 4. The vital role of COI in the metabolism was a vital aspect in this gene being chosen as a universal marker for DNA barcoding, as this characteristic ensures the presence of COI in all eukaryotes (Strüder-Kype & Lynn, 2010). Apart from the role in metabolism COI has, mitochondrial genes are naturally have higher evolutionary rates in comparison to genes encoded for by nucleotides (Herbert et al., 2003a,b).

Mitochondrial DNA has been selected as the source of markers for DNA barcoding of animal species as unlike nuclear genes, these genes are of haploid nature, mitochondrial genes have a high copy number , it lacks introns, there is low recombination in mitochondrial genes and these genes are exclusively inherited from maternal lineage (Barcaccia et al., 2016). Only in 2003 was it proposed that Cytochrome C oxidase subunit I gene (COI or *cox*1) be used as a standardized 658 base pair fragment universal marker for the identification of species (Herbert et al., 2003a). It would be used as a "DNA Barcode" being capable of tagging any taxon in the animal kingdom (Herbert et al., 2003a). After this idea was proposed, the availability of partial COI gene sequences in public databases grew exponentially (Pentinsaari et al., 2016). As of December 2020 there are approximately 8.1 million barcode sequences on the Barcode Of Life Datasystems database, (BOLD) (www.barcodinglife.org). There has been an extensive sequencing of the COI gene region recently (Pentinsaari et al., 2016). DNA barcoding studies that have been published have most commonly used this gene region as a tag for identification and species determination for the reason of the barcode being conveniently readable (Pentinsaari et al., 2016). The barcode fragment however is part of the fundamental energy production location within cells, in other words, COI is one of the building blocks for the Cytochrome C oxidase protein (COX) (Pentinsaari et al., 2016).

Of all the genes coding for proteins found on the animal mitochondrial genome, COI has been suggested for the use in animal species as a universal barcode marker due to two main reasons (Barcaccia et al., 2016). The first is that universal primers make for easy amplification of an approximately 658 base pair fragment situated at the 5' end of the COI gene in a broad spectrum of phyla (Barcaccia et al., 2016). The second reason is the nucleotide substitution rate in COI allows not only closely related species to be distinguished but also allows for the distinguishing of different races, biotypes or populations of the same species (Barcaccia et al., 2016). When barcoding is done, the DNA barcode which is commonly used in most animals is a region found in the mitochondrion that is approximately 650 base pairs (Hebert et al., 2003a,b). This region, as seen in figure 4 below, codes for COI. The use of COI to identify species of animals in meat products that have been processed is reportedly extremely effective due to there being genetic divergence that is low within species but high levels divergence between species thus allowing for undeclared meat to be detected. (Hebert et al., 2003a,b).

Researchers have found that COI has a better phylogenetic signal as compared to other mitochondrial genes, making it a good choice in terms of it being used in DNA barcoding (Strüder-Kypke & Lynn, 2010). It has been debated that the evolution of the COI gene is sufficiently rapid in order to discriminate between closely related species as well as to investigate intraspecific diversity (Herbert et al., 2003a,b; Lin et al., 2015).

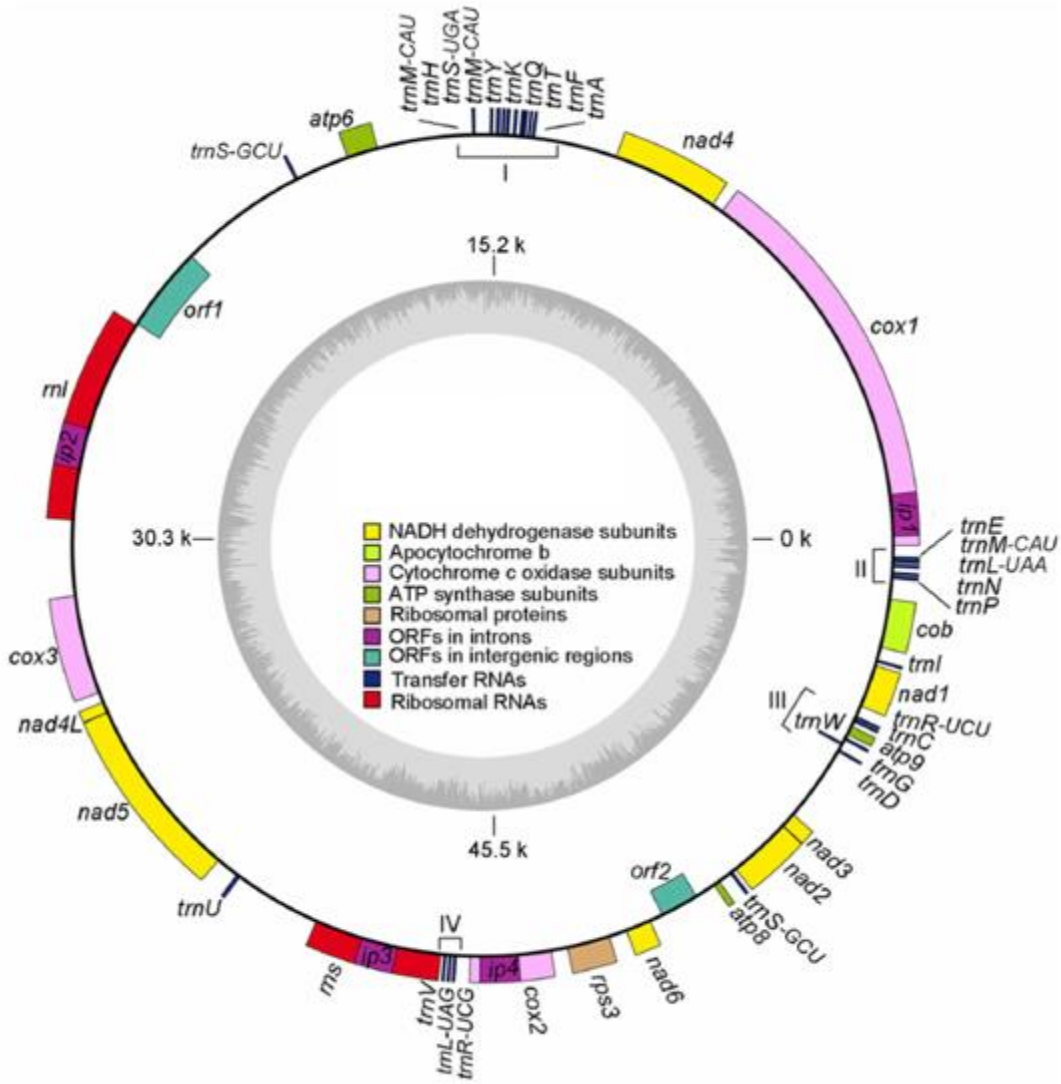

Figure 4: Mitochondrial DNA showing COI region referred to as cox1. (Li et al., 2013)

Figure 5 shows the location of the COI protein reconstructed from cattle, *Bos taurus*, found in the transmembrane of the COX protein. The barcode sequence is illustrated by the orange region (Pentinsaari et al., 2016). The complete COX molecule comprises of two subunits that are identical to each other. Figure 5 illustrates only the barcode area situated at the left side of the protein with (A) being a side view of the transmembrane surface and (B) a view from the matrix of the mitochondrion (Pentinsaari et al., 2016). As already stated, the COX protein is built of two identical units thus making this protein a dimer, these identical parts further comprise of several amino acids (Balsa et al., 2012). 11 of these amino acids are coded for by nucleotides and 3 are

coded for by the mitochondria in mammals (Balsa et al., 2012). The function of COX as the last enzyme in the electron transport chain is to reduce oxygen and pump protons across the mitochondrial inner membrane (Mathews et al., 2013). Against this backdrop any change that occurs in the amino acid sequence, modifies the protein and will ultimately affect energy metabolism (Mathews et al., 2013). Protein coding genes located in the mitochondria are constantly under purifying selection (Meiklejohn et al., 2007). Against this backdrop, amino acid substitutions are extremely rare especially in genes coding for cytochrome oxidase (Castoe et al., 2008). The constraints on the amino acid sequence are selective and reflection occurs at the sequence level of DNA, the sequence coding for the DNA barcode cannot vary freely and evolution is not neutral (Meiklejohn et al., 2007; Galtier et al., 2009). However, the evolutionary patterns of COI may vary between taxa, previous studies conducted by Pentinsaari et al. (2014) and Pentinsaari (2016) reported higher DNA barcode diversity between species of Coleoptera in comparison to Lepidoptera.

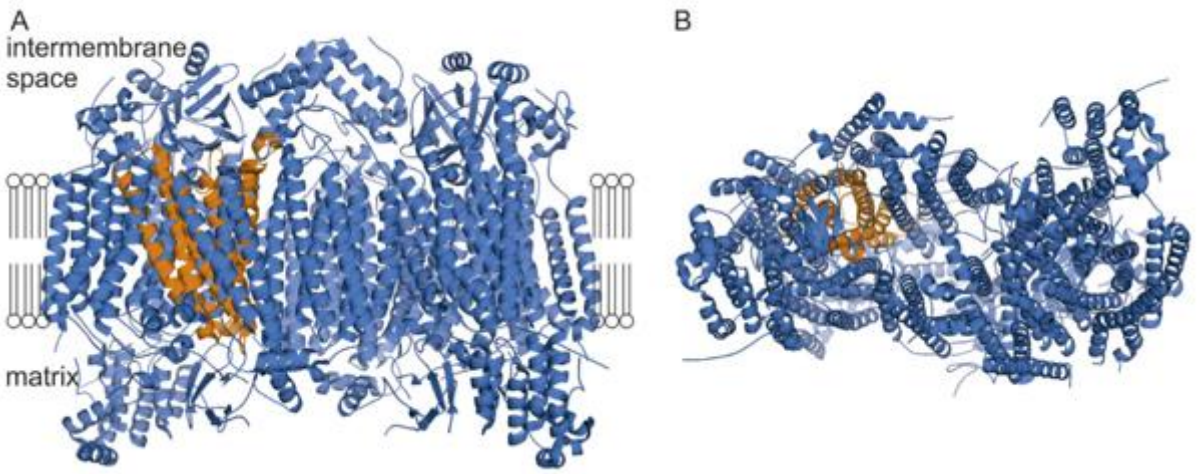

Figure 5: Protein Fragment Location coded by COI gene used in DNA barcoding (Pentisaari et al., 2016)

This literature review has identified the gap in literature in areas of research such as DNA barcoding, processes of mini barcoding and metabarcoding where extensive research needs to be done in this potentially routine analysis for species identification. Other areas highlighted in this literature review pertain to the extent of meat adulteration in processed and game meats in South Africa and mislabelling of those products. Molecular techniques highlighted can be used in the novel area of animal forensics to eradicate the occurrence of meat adulteration in the food industry by either DNA based methods which have been highly recommended or protein based methods. Extensive literature has been reviewed on the studies conducted on meat adulteration and species identification using different molecular techniques, with the focus being on conventional full length DNA barcoding. The gene of interest in DNA barcoding, COI with the protein COX have been reviewed in order to provide an understanding of the reason this gene was selected as the universal marker for DNA barcoding methods. The method of DNA barcoding has great potential in the identification of not only processed meat products but also fish products and game meat as stated in this review. The literature review in this study has indicated that significantly more research needs to be conducted in the area of animal forensics pertaining to DNA barcoding, most especially the developing method of metabarcoding.

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Chapter 3 : Detection of species substitution in Processed Meat Products from Ethekwini, KwaZulu-Natal, South Africa

Abstract

Meat adulteration is an enormous challenge world-wide. Processed meat products are highly susceptible to adulteration due to the physical nature of the products, unlike whole cuts of meat processed meat products cannot be distinguished by appearance solely. Economically processed meat products such as sausages and patties in South Africa are much less expensive as compared to whole cuts of meat, as lower value meat species can be substituted for higher value meat species, in this sense local meat processors gain a profit when selling these processed products. The above mentioned two factors are major contributors of food fraud in processed meat products. This study aimed to determine if there is an extent of meat adulteration occurring within local meat processors around the Durban metropole in South Africa by using DNA barcoding. Adulteration occurred in 62% of samples investigated. This study reports findings of processed meat products being adulterated with different species of Duiker (12%), Deer (2%), Fish (7%) and even Chimpanzee (2%). Majority of lamb samples were found to be adulterated with beef and it is interesting to note that only one beef sample was found to be adulterated. Sausages were found to be more suspectable to adulteration with 66% of sausages being found to contain undeclared animal species as compared to 34% of burger patties. Meat adulteration poses a serious threat to individuals who have allergic reactions to specific meat species and even to certain religious groups who are not permitted to consume specific meat species. Not many studies have been conducted in South Africa pertaining to the identification of meat species in meat products. Against this backdrop, there is still a dearth of research in this area of science and future studies are highly recommended in order to prevent the extent of meat adulteration occurring in local meat processing factories.

Key words: Cytochrome C oxidase subunit 1 (COI); Food fraud; Economic Gain; Processed Meat Products; DNA barcoding; Meat Adulteration; Mislabelling; Barcode of Life Datasystems (BOLD)

3.1 Introduction

On average the South African annual per capita meat consumption is estimated to be 33,9 kg in retail weight equivalent, this figure is expected to increase by 1,5kg to 35,4kg per capita in retail weight equivalent by 2024 according to the OECD-FAO agricultural outlook, 2015 edition. In South Africa, the price of food commodities varies, however meat ranks among the more highly priced products, making this a huge financial strain for a population were on average 50% of the citizens live below the poverty datum line of R561,00 (using 2019 prices) per person per month (Hagen-Zanker et al, 2011; StatsSA, 2019). This makes the consumption of meat a luxury in South Africa (NAMC, 2010 ; DAFF, 2011). There has been an increased awareness of health and consumers have become particularly concerned about what they are feeding their bodies, this has led to a demand for scientific information that is comprehensive regarding the safety and origin of foods that are being consumed (Grunert, 2002; Taljaard et al., 2006; Verbeke & Ward, 2006;) As a response to the growing concern, bodies that govern regulations in South Africa have published legislation on the proper description and transparency of food products (Cawthorn et al., 2013). This includes the regulations pertaining to the labelling and advertising of Food products (R.146/2010) (DoH, 2010) that require a declaration that is mandatory for the stating of all food components on the food labels, as well as the Consumer Protection Act (R.467/2009) (DTI, 2009) that protects consumers from any kind of exploitation in the sale and marketing of consumer goods (Cawthorn et al., 2013). Nevertheless food labelling regulations have become more stringent, misrepresentation pertaining to the adulteration of food products for unlawful financial gain persists as a recurrent feature of society (Shears, 2010; Singh & Neelam, 2011).

In recent years, food fraud has become an enormous challenge worldwide (Shika et al., 2016) This harms the interest of the consumer and undermines the confidence that should be found in the food market (Smith et al., 2005; Aung & Yoon, 2014; Johnson, 2014; Wielogorska et al., 2018). A study conducted by Tembe et al. (2018) investigated meat adulteration in processed meat samples with the use of simplex and multiplex PCR, this study reported an occurrence rate of 65% which is very similar to the reporting of Cawthorn et al. (2013) with adulteration occuring in 68% of meat products available on the South African meat market. A similar study conducted in America by Kane & Hellberg (2016) reported adulteration occurring in only 21% which is significantly low as

compared to the South African studies. It has been reported in previous studies that 20-70% of various meat products are mislabelled (Flores-Munguia et al., 2000; Pascoal et al., 2004; Ayaz et al., 2006; Cawthorn et al., 2013; Ozpinar et al., 2013; Mousavi et al., 2015; Okuma & Hellberg, 2015; Quinto et al., 2016).

This study aims to collect a representation of processed meat products sold in eThekwini from a variety of local meat processors, thereafter isolate genomic DNA, amplify the COI mitochondrial region by means of PCR and use those PCR products to identify any undeclared animal species by means of bioinformatic analyses with the information deposited onto the Barcode Of Life Datasystems database. In the last decade or so, society has become increasingly concerned with fitness, eating healthily and consuming only the most nutritional foods. Against this backdrop, this study aims to make consumers aware of adulteration occurring in processed meat products.

3.2 Materials and Methods

3.2.1 Sample collection

The study was approved by the Animal Research Ethics Committee of the University of Kwa-Zulu Natal (Reference numbers AREC/051/017M, AREC 071/017, AREC 014/018). The field sampling protocols, samples collected from animals, and the research were conducted in full compliance with Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984) and were approved by the South African Department of Agriculture, Forestry and Fisheries DAFF (Section 20 approval reference number 12/11/1/5).

In total, 100 samples were obtained from 31 local meat markets in the eThekwini municipality of KwaZulu-Natal, South Africa. 67% of the samples were processed sausages, 33% were processed burger patties. A further breakdown of the sample collection shows that 43% of the samples were lamb, 29% were chicken, 22% were beef, 5% were pork and 1% of the samples were a mix of both beef and chicken.

The sampling method that was used was purposive sampling, this method is commonly used in qualitative research for the purpose of identification and selection of samples that are information rich in relation to the study of interest (Palinkas et al, 2015).

Figure 6: Map showing KwaZulu-Natal outlined in red (http://gis.durban.gov.za/cmv-cgis/viewer/?config=cgisPublicViewer Date Accessed: 10/12/2020)

Figure 7: Map showing eThekwini Municipality, yellow highlighted areas show meat market locations where meat products were obtained. (https://municipalities.co.za/map/5/ethekwini-metropolitan-municipality Date Accessed: 10/12/2020)

3.2.2 Genomic DNA Extraction

The extraction of genomic DNA was done using the Quick DNA Miniprep Plus Kit from ZymoResearch. According to instructions provided by the manufacturer, 0.25 g of the fresh processed meat products were sampled. To the micro centrifuge tube containing the 0.25g samples, 95 µl of nuclease free water, 95µl of solid tissue digestion buffer and 10µl of Proteinase K was added. The samples were thoroughly mixed using a vortex for 15 seconds and then incubated in a heating block for 3 hours at 55°C, until the solid tissue was fully solubilized. 400 µl of Genomic Buffer was added to the supernatant and the samples then vortexed for 15 seconds. The mixture was then transferred into a Zymo-spin IIC™ column in a collection tube and centrifuged at 12000 × *g* for 1 minute. The Zymo spin column was transferred into a new collection tube and 400 µl of DNA Prewash Buffer was added, the mixture was then centrifuged at 12000 × *g* for 1 minute. 700 µl of g-DNA Wash Buffer was added to the Zymo spin column, the column was centrifuged at 12000 × *g* for 1 minute. 200 µl of g-DNA Wash buffer was added to the Zymo spin column and then centrifuged at 12000 × *g* for 1 minute. Thereafter, the Zymo spin column was transferred to a clean microcentrifuge tube and 100 µl of DNA elution Buffer was added into the spin column. The solution was incubated for 5 minutes at room temperature and then centrifuged at 16160 × *g* for 1 minute in order to elute the DNA from the mixture. The DNA was eluted a second time to improve the yield by adding 100 µl of DNA elution Buffer, followed by incubation at room temperature for 3 mins and thereafter centrifugation 16160 × *g* for 1 minute. The purity of concentration of the DNA was verified using a spectrophotometer (Nano-Drop, at UKZN, Durban, South Africa) and the DNA was used thereafter for PCR analysis.

3.2.3 PCR amplification and confirmation gel electrophoresis

The amplification of the CO1 gene in the extracted DNA was done using a PCR assay and universal primers specified in Table 1. The PCR amplification was conducted in 25 µl reactions. Each reaction contained 5 µl of genomic DNA.

Table 1: Universal Primers utilized for the confirmation of Mitochondrial Gene, Cytochrome Oxidase Subunit 1

Primer Name	**Direction**	**Target Gene**	**Sequence (5'-3')**	**Band Size (bp)**	**Reference**
LCO 1490	Forward	COI	5'GGTCAACAAATCATAAAGATATTGG3'	650	Folmer et al, 1994
HCO2198	Reverse	COI	5'TAAACTTCAGGGTGACCAAAAAATCA3'	650	Folmer et al, 1994

The PCR reaction mixture composed of 12,5 µl of Thermo Scientific DreamTaq Green PCR mix (2X), 5,5 µl nuclease free water and 5 µl of genomic DNA template 1 ul of the forward Universal primer LCO1490 and 1 ul of the reverse primer HCO2198 by Folmer et al, 1994. The total volume of the PCR reaction was 25 µl.

Thermal cycling was conducted using BIORAD T100 Thermal Cycler. Thermal cycling conditions were adapted from conditions described by (T Tinago, 2014) to allow for better amplification: Initial Denaturation at 94°C for 2 minutes; 48 cycles of Denaturation at 94°C for 30 seconds; Annealing at 50 °C for 30 seconds; extension at 72°C for 1 minute, and a final extension at 72°C for 10 mins.

The confirmation of the presence of the COI gene was done by the electrophoresis of 5 µl of each PCR amplicon, in a 2% suspension gel stained with 5 µl SafeView™ Classic, at 80 V for 60 minutes. A Uvitec UV transilluminator was used to visualise the DNA bands and each gel image was captured using an Uvitec digital camera. Positive samples were identified using Thermo Scientific Generuler 100 base pair plus DNA ladder molecular weight marker.

3.2.4 Sequencing

DNA sequencing was done on confirmed amplicons by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The amplicons were sequenced in the forward direction using primers from the initial amplification process. In a nutshell, the PCR products were cleaned by mixing 10 µl of the PCR product with 2.5 µl of Exo/SAP mix ((exonuclease 1 (NEB M0293) 20U/µl; Shrimp alkaline phosphatase (NEB M0371) 1U/ µl)). The mixture was incubated at 37 °C for 30 minutes then the reaction was stopped by heating the mixture at 95 °C for 5 minutes. DNA sequencing was then conducted using the ABI V3.1 Big dye kit in accordance with the manufacturer's instructions on an ABI 377 automated sequencer. The Zymo Seq clean-up kit was used to clean the labelled products thereafter, the products were injected in the ABI 3500XL Genetic Analyzer (with a 50 cm array) using POP7.

3.2.5 Sequencing Results and Bioinformatic Analyses

Using Geneious Prime (Biomatters Ltd., Auckland, New Zealand, Geneious Prime 2020.2.4 https://www.geneious.com) raw data was edited by identifying ambiguous nucleotides with the correct nucleotides of G,C,T and A by peak inspection. Successful sequences were those that had less than 2% ambiguities, these sequences were then queried using the Barcode of Life Database (BOLD) Animal Identification Request Engine (http://www.boldsystems.org/), Species Level Barcode Records. Top species matches that indicated ≥ 98% similarity to the query sequences were recorded. Sequences that were not able to yield a species match on BOLD were then queried in Genbank using the Mega Basic Alignment Search Tool (megaBLAST) and top species matches showing ≥ 74% similarities were recorded. The Basic Local Alignment Search Tool (BLAST) in NCBI (National Centre for Biotechnology; www.ncbi.nlm.nih.gov/) was used to confirm the identity of those specific samples. The IUCN website (http://www.iucnredlist.org/) was used to verify the common name of top matches as well as to determine the stability of those species' populations.

3.3 Results

The PCR assay showed that out of 100 samples, 72 were able to amplify using the universal primer shown in table 1.

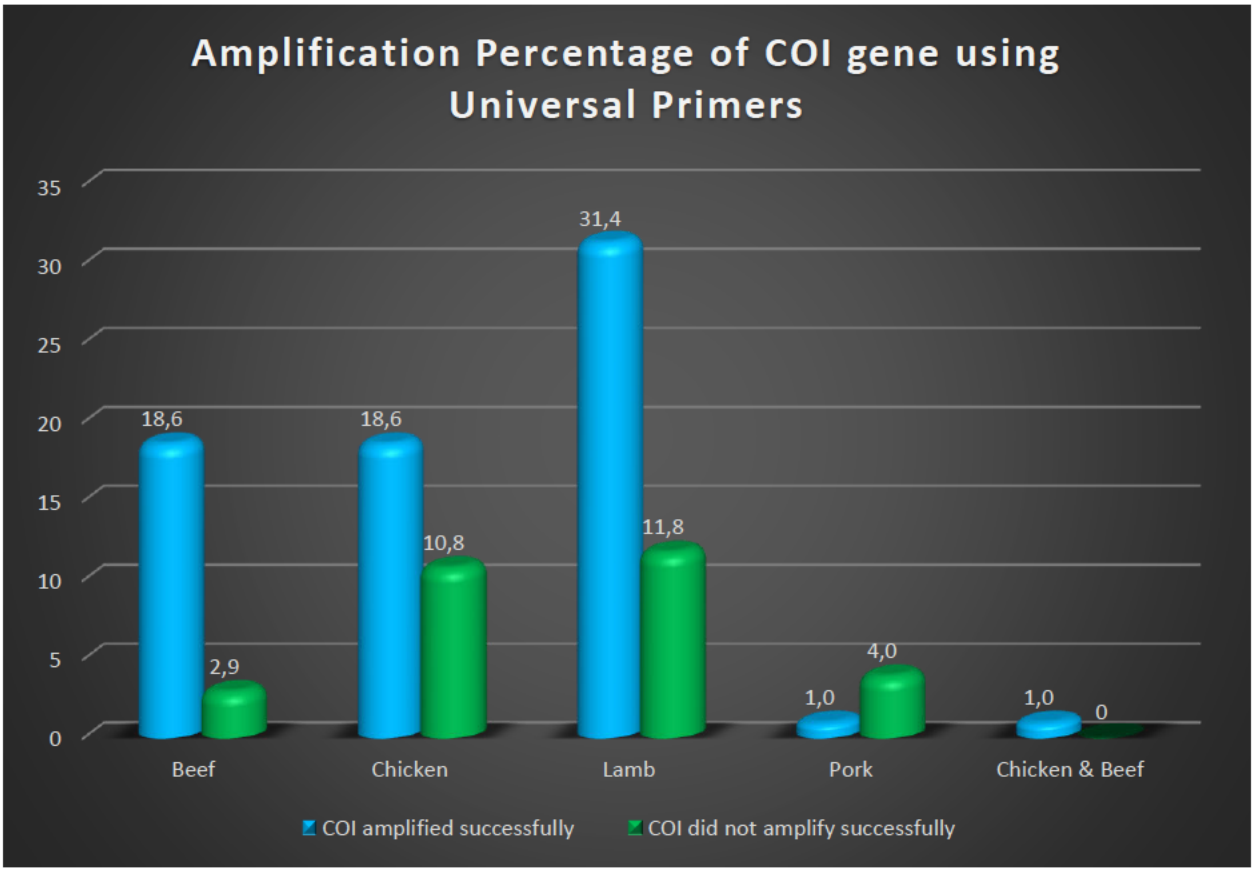

Figure 8: Successful amplification of the COI gene occurring in the 100 samples collected.

Figure 8 shows that out of 100 samples, 71% amplified positively with the universal primer. Positively amplified beef samples were 19%, chicken samples that amplified successfully were 19%, 31% of the lamb samples amplified, 1% of the pork samples amplified and 1% of the chicken and beef combination sample amplified as well. 29% of samples did not amplify. 3 % were beef samples, 11% were chicken samples, 11% were lamb samples and 4% were pork samples.

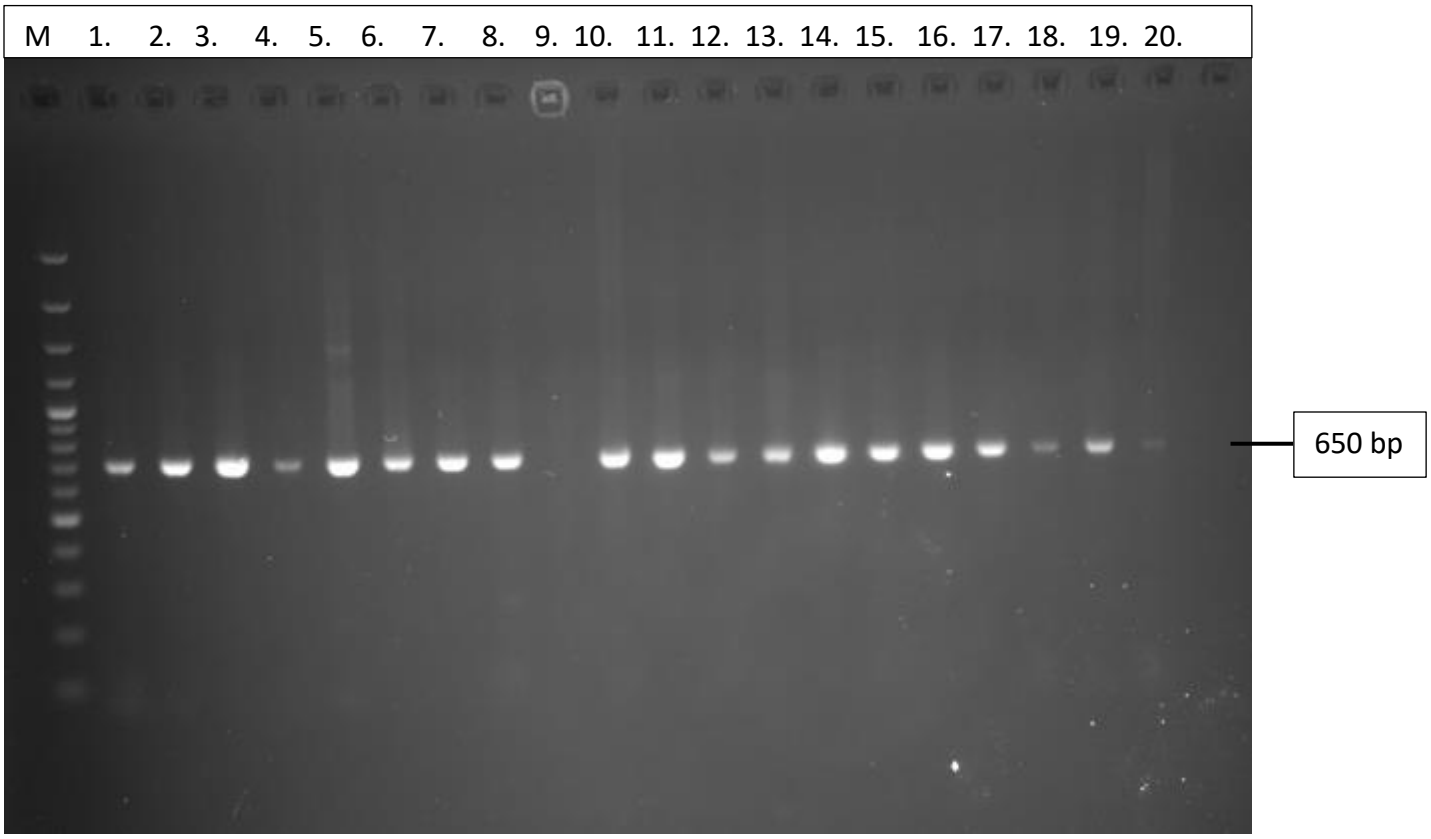

Figure 9: Representative Agarose Gel Electrophoretic analysis (2%) of Beef samples amplified with the universal primers. Lane 1: Molecular weight Marker (M). Lane 2-20: Beef sausage and patty samples.

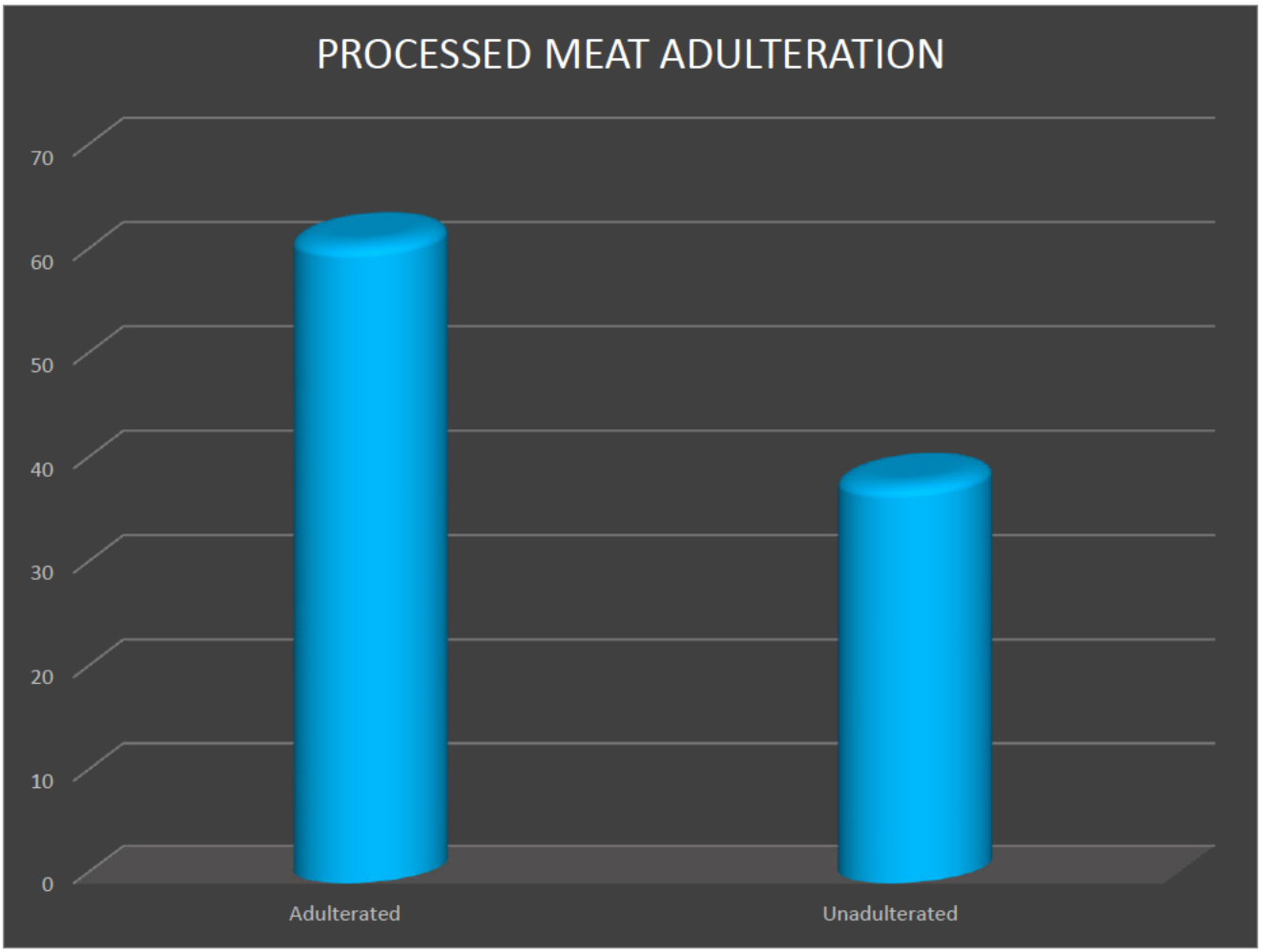

Figure 10: Percentage of processed meat samples that are adulterated and unadulterated.

Figure 10 shows that 62% of all samples investigated where adulterated and the packaging was mislabelled while only 38% were correctly labelled and contained the meat species that had been declared on the packaging.

Table 2: DNA Barcoding Results obtained from BOLD or using MegaBLAST. Organisms highlighted in yellow indicate endangered species.

SEQUENCE NUMBER	PRODUCT LABEL	SAUSAGE/PATTY	TOP SPECIES MATCH	GENETIC SIMILARITY (%)	DATABASE
3	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	99.84	BOLD
4	LAMB	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
5	LAMB	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
6	LAMB	SAUSAGE	Cattle (*Bos taurus)*	100	BOLD
8	LAMB	SAUSAGE	Blue duiker (*Cephalophus monticola),* Northern Treeshrew *(Tapaia belangeri)*	77 - 82	NCBI
9	LAMB	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
10	LAMB	SAUSAGE	*Cotton Leaf Roller(Haritalodes derogate),* Large Emerald Moth*(Geometra papilionaria)*	83 - 97	NCBI
11	LAMB	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*		BOLD
13	LAMB	PATTY	Ray finned fish *(Actinopterygii sp.)*	89	NCBI
14	LAMB	PATTY	Cattle *(Bos taurus)*	83	NCBI
15	LAMB	PATTY	Cattle *(Bos taurus)*	100	BOLD
17	LAMB	SAUSAGE	Red Junglefowl *(Gallus gallus)*	100	BOLD
18	LAMB	SAUSAGE	Cattle *(Bos taurus)*	89	NCBI
22	LAMB	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	99.84	BOLD

23	LAMB	SAUSAGE	Blue duiker (*Cephalophus monticola),* Northern Treeshrew *(Tapaia belangeri)*	77 - 82	NCBI
25	LAMB	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
26	LAMB	PATTY	Aders's duiker(*Cephalophus adersi*) Northern Treeshrew *(Tapaia belangeri)*	77 - 81	NCBI
31	LAMB	PATTY	Aders's duiker(*Cephalophus adersi*), Copper Pheasant (*Syrmaticus soemmerringii)*	76 - 79	NCBI
32	LAMB	SAUSAGE	Aders's duiker(*Cephalophus adersi*) Northern Treeshrew *(Tapaia belangeri)*	78 - 81	NCBI
33	LAMB	SAUSAGE	Bighorn sheep*(Ovis canadensis canadensi)*	79	NCBI
34	LAMB	SAUSAGE	Cattle *(Bos taurus)*	100	BOLD
35	LAMB	PATTY	Cattle *(Bos taurus)*	100	BOLD
36	LAMB	SAUSAGE	Blue duiker (*Cephalophus monticola),* Aders's duiker(*Cephalophus adersi*)	81	NCBI
37	LAMB	SAUSAGE	Ray finned fish *(Actinopterygii sp.),* Lyngbye *(Halosiphon tomentosus)*	80 - 90	NCBI
38	LAMB	PATTY	Fallow Deer (*Dama dama),* Northern Treeshrew *(Tapaia belangeri),* Giant Pikehead *(Luciocephalus pulcher), Green Hairstreak (Callophrys rubi),* Chimpanzee *(Pan troglodytes)*	77 - 82	NCBI
39	LAMB	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD

40	LAMB	SAUSAGE	Cattle *(Bos taurus)*	100	BOLD
41	LAMB	SAUSAGE	Cattle *(Bos taurus)*	100	BOLD
42	LAMB	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
43	LAMB	SAUSAGE	Cattle *(Bos taurus)*	100	BOLD
44	LAMB	SAUSAGE	*Weyns's Duiker(Cephalophus weynsi), Peters's duiker (Cephalophus callipygus), Common Treeshrew (Tupaia glis), Short-tailed Spiny-Rat (Proechimys gularis), Black-striped Frog (Sylvirana nigrovittata)*	74 - 79	NCBI
45	LAMB	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
48	CHICKEN	PATTY	Red Junglefowl *(Gallus gallus)*	88	NCBI
51	CHICKEN	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	99.52	BOLD
56	CHICKEN	PATTY	Ray finned fish *(Actinopterygii sp.)*, Crab (*Paralomis cristulata)*	93, 84	NCBI
61	CHICKEN & BEEF	PATTY	Cattle *(Bos taurus)*	100	BOLD
64	CHICKEN	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
71	CHICKEN	SAUSAGE	Ray finned fish *(Actinopterygii sp.)*, Mediterranean mussel *(Mytilus galloprovincialis)*, White ermine *(Spilosoma lubricipeda)*	85 - 89	NCBI
75	CHICKEN	SAUSAGE	*Oomycete Plant Pathogen (Phytophthora capsici)*	98.03	BOLD

76	CHICKEN	SAUSAGE	Ray finned fish *(Actinopterygii sp.)*, Mediterranean mussel *(Mytilus galloprovincialis*	84 - 90	NCBI
86	BEEF	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
87	BEEF	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
88	BEEF	PATTY	Cattle *(Bos taurus)*	100	BOLD
89	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	99.81	BOLD
90	BEEF	SAUSAGE	*Cattle (Bos taurus)*	87	NCBI
91	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	99.70	BOLD
92	BEEF	PATTY	Cattle *(Bos taurus)*	100	BOLD
93	BEEF	SAUSAGE	Cattle *(Bos taurus)*	98.93	BOLD
95	BEEF	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
96	BEEF	SAUSAGE	Cattle *(Bos taurus)*	82	NCBI
97	BEEF	SAUSAGE	Cattle *(Bos taurus)*	100	BOLD
98	BEEF	SAUSAGE	Ray finned fish *(Actinopterygii sp.)*	88	NCBI
99	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
100	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
101	BEEF	PATTY	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
102	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	99.84	BOLD
103	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD
104	BEEF	SAUSAGE	Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*	100	BOLD

105	BEEF	SAUSAGE	Cattle *(Bos taurus)*	99.30	BOLD
108	CHICKEN	SAUSAGE	Red Junglefowl *(Gallus gallus)*	96	NCBI
113	CHICKEN	SAUSAGE	Cattle *(Bos taurus)*	98	BOLD
116	CHICKEN	PATTY	Red Junglefowl *(Gallus gallus)*	99.44	BOLD
117	CHICKEN	SAUSAGE	Red Junglefowl *(Gallus gallus)*	94	NCBI
118	CHICKEN	PATTY	Cattle *(Bos taurus)*	97.90	BOLD
121	CHICKEN	SAUSAGE	Ray finned fish *(Actinopterygii sp.)*	83	NCBI

Table 3: Representative Accession Numbers obtained by submission of sequences to NCBI:

Sequence Number	**Accession Number**
4	MW475447
5	MW575448
6	MW575449
25	MW575450
34	MW575451
35	MW575452
39	MW575453
45	MW575454
87	MW575455
103	MW575456
113	MW575457

Table 4: Population Stability of Species found in processed Meat Products

SPECIES	POPULATION STABILITY	AREA OF ORIGIN	LAST ASSESSED
Blue Duiker (*Cephalophus monticola)*	Decreasing	Central and South Africa	07 January 2016
Northern Treeshrew *(Tapaia belangeri)*	Stable	Thailand	10 June 2016
Ray finned fish *(Actinopterygii sp.)*	Stable	Global	28 February 2018
Aders's duiker(Cephalophus adersi)	Vulnerable	Central Africa	28 September 2016
Bighorn sheep(Ovis canadensis canadensi)	Stable	Central America	03 December 2019
Fallow Deer (*Dama dama)*	Stable	Mediterranean	10 December 2006
Giant Pikehead *(Luciocephalus pulcher*	Stable	Indonesia	18 January 2019
Chimpanzee *(Pan troglodytes)*	Endangered	North and Central Africa	24 March 2016
Weyns's Duiker(Cephalophus weynsi)	Decreasing	Central Africa	07 January 2016
Peters's duiker (Cephalophus callipygus)	Decreasing	Central Africa	07 January 2016
Common Treeshrew (Tupaia glis)	Decreasing	Malaysia	09 June 2017
Short-tailed Spiny-Rat (Proechimys gularis)	Stable	South America	10 June 2016
Black-striped Frog (Sylvirana nigrovittata)	Decreasing	Thailand and Malaysia	30 April 2004
Red Junglefowl (*Gallus gallus)*	Decreasing	Myanmar, Indonesia and Japan	01 October 2016
Copper Pheasant (Syrmaticus soemmerringii)	Decreasing	Japan	01 October 2016
Mediterranean mussel *(Mytilus galloprovincialis)*		NO DATA	
White ermin *(Spilosoma lubricipeda)*		NO DATA	
Cattle *(Bos taurus)*/ Zebu Cattle *(Bos indicus)*		NO DATA	
Crab (*Paralomis cristulata)*		NO DATA	

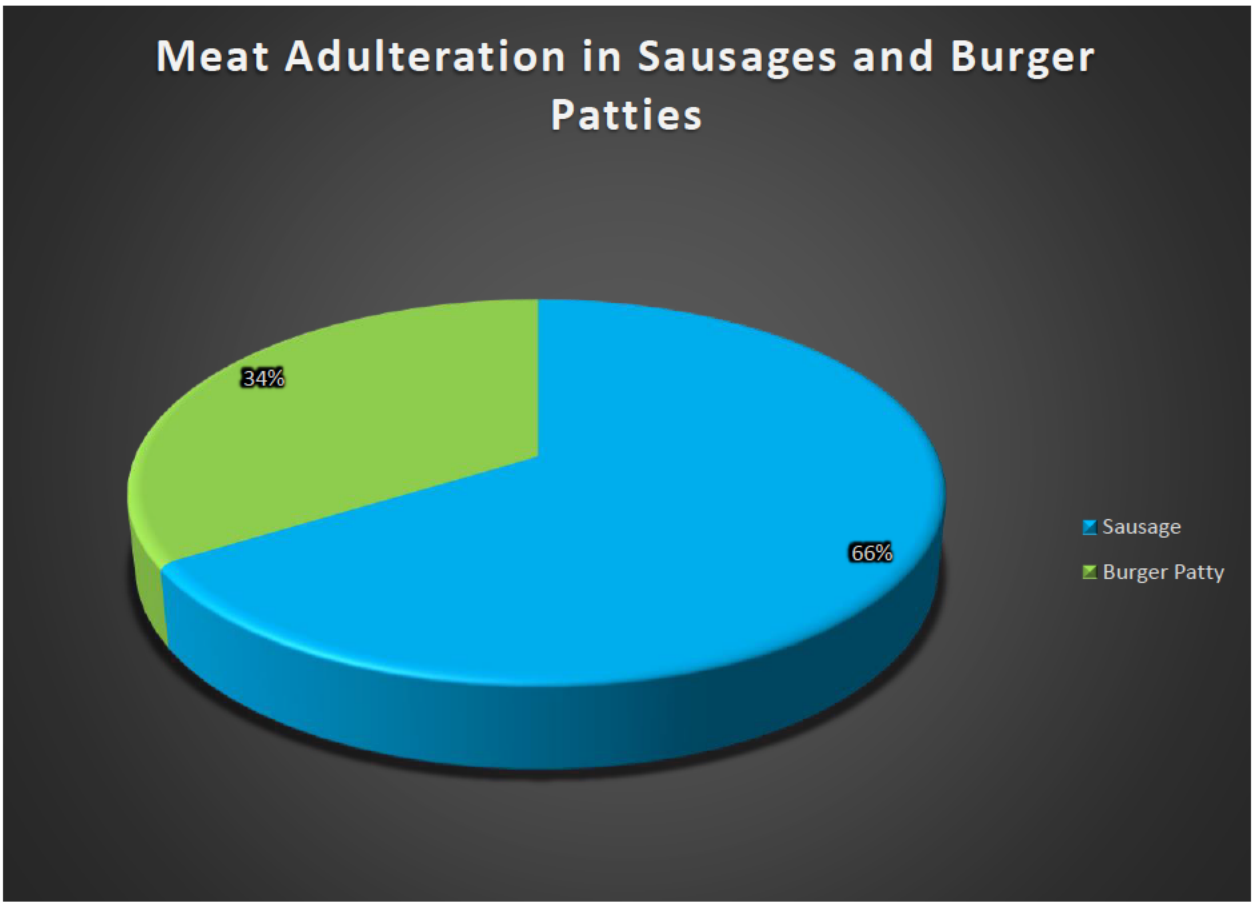

Figure 11: Percentage of Sausages and Burger Patties that were adulterated. The graph indicates that 66% of adulterated samples were sausages and 33% were burger patties.

Table 5: Percentage of samples queried using either BOLD or Genbank MegaBlast. Majority of the samples (63%) were identified using BOLD and 36% did not find a match in BOLD and had to be queried using GenBank MegaBLAST.

Samples Queried using BOLD	Samples Queried using GenBank MegaBLAST due to no hit found on BOLD
63%	37%

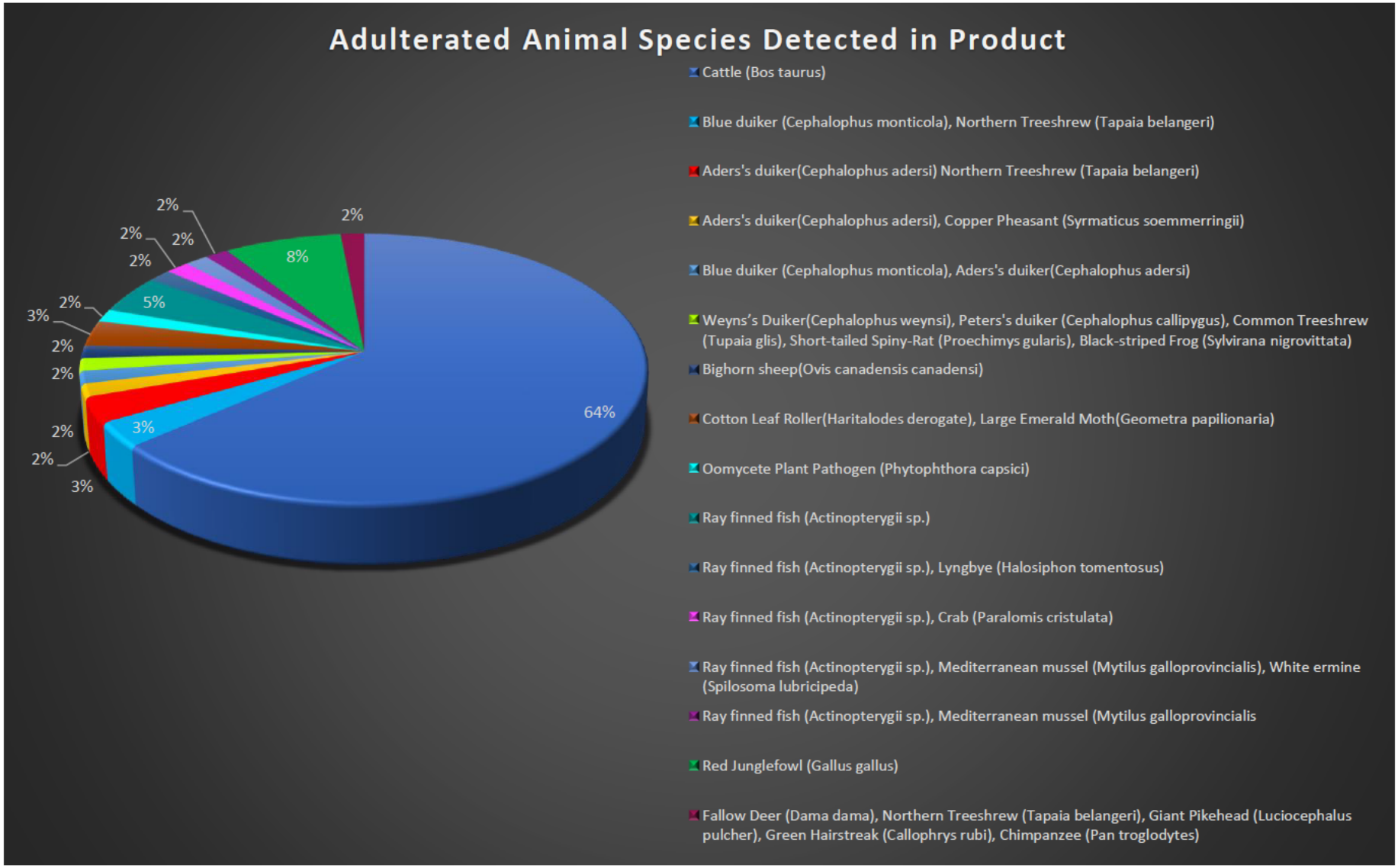

Figure 14: Animal species detected as a form of adulteration in processed meat products.

3.4 Discussion

The PCR technique used in this study involved universal primers that amplify the COI gene. PCR analyses of genes that are homologous has become one of the most powerful approaches in detecting and identifying species (Leray et al., 2013). In the past, COI was thought to be a marker that was not suitable for use due to its large amplicon size (Jarmen et al., 2002; Jarmen et al., 2004; Deagle et al., 2006) however the universal primers LCO1490 and HCO2198 have become a powerful assets in understanding many different ecological processes as well as biomonitoring programs (Leray et al., 2013). The mitochondrial COI gene has become the most readily available sequence region in many public reference libraries, due to the versatile or in other words universal COI primers that target the 658 bp barcoding region (Leray et al., 2013).

In this study, as indicated in figure 8, 71% of extracted DNA samples were able to yield a positive amplification. This indicates that the overall amplification process was successful as a majority of the samples were able to amplify positively. The positively amplified products will be further analysed by means of DNA barcoding.

Figure 9, confirms by means of an agarose gel, the amplification of samples at 650 base pairs which is the target region of the COI gene (Leray et al., 2013). The amplification of the COI gene has been successful as single clear bands at approximately 660 base pairs can be identified.

According to results from a study conducted by Ayaz et al. (2006) substitution of meat species occurs quite frequently in meat products that are processed, examples of this are cured meat and ground meat, such as sausages and patties used in this study. This could be due to deliberate substitution with readily available and cheaper species that is rather challenging to recognise visually as compared to fresh, whole cuts of meat such as fillets (Cawthorn et al., 2013). The techniques involved in processing leads to many physical changes of the meat species used with regards to the appearance, colour, texture and even flavour, which could be due to all the added spices and flavouring, this allows for the origins of the meat to be disguised easily in the mixture of meat (Flores-Munguia et al., 2000).

Figure 10 shows that a large percentage of samples, 62%, that were tested in this study showed meat adulteration once the bioinformatic analysis of DNA barcoding was conducted, this means that 38% of the samples was not adulterated in any manner. A similar study by Cawthorn et al. (2013) showed that undeclared meat/plant species were identified in 68% of samples tested. This study has recorded a lower percentage in undeclared meat products from the samples tested however it should be noted that the sample size used in the study by Cawthorn et al. (2013) was 139 whereas this study only used 100 samples. Both studies recorded more than 50% of samples containing undeclared meat species. This is concerning given that the food labelling legislation in South Africa (DoH, 2010, pages 3-53) requires all product constituents to be clearly declared on the labels on items of food. This study focused mainly on local meat markets in KwaZulu-Natal (KZN), the study conducted by Cawthorn et al. (2013) makes mention of the fact that out of the 4 provinces sampled, KZN and the Eastern Cape had approximately 90% of samples containing one or more undeclared species in the meat products, an interesting fact here is that in South Africa, KZN and the Eastern Cape are the provinces that have the highest number of individuals belonging to low income groups (van Aardt & Coetzee, 2009). Against this backdrop, it is therefore possible that lower priced meat components could be added more frequently to processed meat products in such areas, as the population may be more concerned with the cost of the product as compared to its composition (Cawthorn et al., 2013). In 2012, Cawthorn et al. conducted a study that investigated the extent of fish mislabelling in South Africa, this reported that KZN was the province with the lowest level of compliance in terms of the correctly describing the commercial marine species available.

Table 2 describes the different species that have been used in the sausages and patties sampled, it's interesting to note that many of these species would normally not be consumed. Analyses done on BOLD and NCBI MegaBLAST indicates substitution of lamb with species such as beef, fish, duiker, tree shrew, pheasant, deer, rat, frog, traces of moth and even chimpanzee. Chicken showed to be substituted with beef, fish, crab, mussel, traces of moth and a plant pathogen. Interestingly the only substitution found in the beef samples was fish and this was only in one sample. This could be due to the fact that KZN is abundant in cattle as compared to other species of livestock. According to the Department of Agriculture, Land Reform and Rural Development (DALRRD, 2020) as of the most recent livestock count that occurred in May 2018, KZN has approximately

2.5 million cattle, 700 000 sheep, 150 000 pigs and 700 000 goats. These numbers indicate that because of this abundance of cattle in KZN it could be a reason as to why majority of the chicken and lamb samples have been substituted with Beef. This causes great concern because according to the 2019 mid-year population estimates from the Department of Statistics South Africa (StatsSA) KZN has the second highest population in South Africa with approximately 11.3 million people and according to figures obtained from the official consensus held in 2011 the second highest population group in KZN is South African Indians, majority belonging to the Hindu faith which prohibits the consumption of beef. Another possibility for these findings could be due to accidental cross contamination where mincing equipment may not have been thoroughly cleaned or even improper handling of the meat samples. (Owusu-Apenten, 2002). The concern with cross contamination are those individuals who are allergic to specific meat species. These people believe that they are safe by consuming a certain product however due to cross contamination that product could contain traces of the meat species they are actually allergic to.

The trend with these results illustrate red meat samples of Lamb are more often than not substituted with animals that contain red meat such as beef, different species of duiker, deer and even chimpanzee. An article released by SANBI (2019) states that the bushmeat hunting of blue duiker for protein and trade is the main threat for this species of duiker. A study done by Danson et al. (2016) highlights the health concerns that arise from the consumption of bush meat, comprising of duiker, deer and chimpanzee. This includes the transmission of various diseases to humans due to the consumption of bushmeat, some of the health problems that were reported in the study are diarrhoea and brucellosis. The white meat samples of chicken show substitution with animals that also contain white meat such as fish and crab, however beef was also found in these sample, the presence of beef could be due to cross contamination or it could be that beef fat was used in these samples as well. Vatila et al. (2018) conducted a study reporting that the consumption of ray finned fish provides nutrients that is critical for human nutrition such as protein, fat, omega- 3 and 6, fatty acids, iron, zinc as well as Vitamins A,B12 and D however despite the advantages of fish consumption a lot of individuals are highly allergic to seafood therefore the consumption of products that contain these undeclared meat species can pose severe health problems for these individuals (Madsen et al., 2012). Symptoms of an allergic reaction range from mild to severe with mild symptoms being itchy skin, stomach cramps and the tingling of the throat and mouth while

severe symptoms include difficulty breathing, tightening or swelling of throat, persistent cough and consistent dizziness. (Betterhealth, Victoria State Government, 2017. Date Accessed 27/20/2020)

Many cultures around the world are known for their acquired taste to species such as frog and rat such as European countries where frog legs are considered a delicacy (Onadeko et al., 2011). Some regions in Indonesia and Nigeria refer to frogs as "jumping chicken" purely because the taste is said to be very similar to that of chicken. (Altherr et al., 2011). The availability of frogs, the fact that frogs are palatable to humans as well as the abundance could be a reason why traces of frog can be found in processed meat samples.

Rats have been a staple in countries like Myanmar and Indonesia for years, in South and Central America several species are seen as culinary items and in some areas are even farmed just as livestock (Gruber, 2016). It is interesting to note that in table 4 which shows population stability of the unfamiliar meat species found in samples of this study the origin of the rat species identified is South America. Describing the results illustrated in Table 4, Aders's duiker which was identified in a number of samples is currently a vulnerable species and the sample which was identified as Chimpanzee has been shown as an endangered species according to IUCN. All animal species in Table 4 have been used as a substitute to beef, lamb or chicken. 6 out of 19 (32%) species identified in 102 samples have a decreasing population size, 1 out of 19 (0,5%) of the 102 samples belongs to a vulnerable population and 1 out of 19 (0,5%) belongs to an endangered population, so the need for conversation is critical to ensure a healthy population. These findings of the vulnerable and endangered species identified is slightly lower as compared to studies conducted by Quinto et al. (2016) that reported a 1,85% observance of threatened species in game products analysed and 1,10% of threatened seafood and fish species reported by Wong and Hanner (2008) examining mislabelled seafood. However a similar finding was reported by Warner et al. (2013) where 0,58% of 1215 seafood products was identified as endangered.

Figure 11 shows that majority of the samples that were adulterated and contained undeclared meat species were processed sausages which is similar to finding that have been reported by Tembe et

al. (2018), which reported 80% contamination in sausages, and Cawthorn et al. (2013) reporting 68%. It should be noted that 63% of sequences were identified using BOLD and only 37% were identified using BOLD as indicated in Table 5.

Figure 12 is a comparative pie chart that illustrates the adulteration that occurred in the study conducted. After the bioinformatic analyses was conducted it reported that there were many other species contained in the processed meat products. The highest percentage of undeclared species was found to be beef at 64%, this is a much larger percentage than that which was actually declared as beef products on the labels of processed meat products, with the percentage being twice as high as that which was declared. These findings concur with studies conducted by Tembe et al. (2018) and Kane & Hellberg (2016) that reported beef as the leading contaminant in processed meat products. 48% of the samples investigated were declared as lamb species however after the bioinformatic analyses was done it confirmed that only 2% of the processed products were actually lamb. Chicken was declared to be in 20% of processed meat products according to the label on the packaging however this study reported that 8% of the products contained chicken. Against this backdrop it can be reported that adulteration either intentional for economic gain or unintentionally by means of contamination, did occur in the investigated samples.

The overall findings in this study report that 62% of samples were either mislabelled or were contaminated in some form. As compared to studies previously investigating the sale of game and meat products in South Africa that showed mislabelling percentages between 68 % – 69 % (Cawthorn et al., 2013; D'Amato et al., 2013), this is slightly lower. If the findings in South Africa are compared to studies that have been done in the United States of America and Turkey, these countries have a much lower rate of mislabelling, with an average of 19,3% (Ayaz et al., 2006). This could be due to that fact that South Africa has much stricter regulations pertaining to the complete disclosure of all ingredients in food products. Economic gain could also be a likely cause for mislabelling as majority of the lamb samples in this study have been identified as Cattle (Bos taurus)/ Zebu Cattle (Bos indicus) and on average local meat markets in Ethekwini sell lamb for R165,00/kg whereas beef is sold on average at R100,00/kg. Many previous studies such as Cawthorn et al. (2013), Kane & Hellberg (2016) and even Quinto et al. (2016) have reported

substitution of livestock however this study has reported substitution of animal species such as rat, frog, deer, duiker and even chimpanzee, which consumers would normally not be eating.

3.5 Conclusion

The results in this study show that there is definitely food fraud occurring in the local meat markets around Ethekwini, KwaZulu-Natal. More than half (62%) of the samples were mislabelled and adulterated with meat from duiker, chimpanzee and fish, just to name a few. This is despite the strict regulations that South Africa has put in place to prevent this very thing from occurring.

Considering all the information and research obtained from previous studies together with the findings in this study, it is apparent that the mislabelling and adulteration of processed meat products is due to the fact that these meat species are not easily identifiable by consumers. However, it is unsure if this adulteration has been intentional or unintentional. The abundance of beef in KZN coupled with the fairly reasonable selling price as compared to lamb indicates that economic gain could be an intentional driving force for meat adulteration in these local meat markets. Apart from economic gain, the marketing of a cheaper meat product, especially in a province where approximately 50% of the population lives below the poverty datum line of R561,00 could be a contributing factor to meat adulteration in sausages and patties. Cross contamination due to equipment not being cleaned in between uses or even mishandling of these processed meat products could be due to human error which would indicate the unintentional adulteration of processed meat samples.

There however is still not enough research that has been done in South Africa pertaining to meat adulteration, against this backdrop it is recommended that more knowledge and similar studies are done around KwaZulu-Natal and even South Africa to accurately determine if processed meat samples are being adulterated intentionally or unintentionally. Studies on the mislabelling of processed seafood and even game meat are very few in South Africa and research in this area is in great demand. Further investigation should be conducted to determine if the source of the adulteration is the local meat markets and retailers that are selling the processed products or if it is in fact the suppliers of those local meat markets who are the cause behind the adulteration.

This study highlights the importance for close monitoring of local meat markets in South Africa in order to ensure that regulations pertaining to the declaration and transparency of all products

used on food labels are met together with providing consumers with products that are safe and healthy for consumption.

Ethical Approval

This study has been approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (UKZN), Durban, South Africa [reference nos. AREC/051/017M, AREC 071/017 and AREC 014/018; Act No. 35 of 1984 Section 20 approval reference no. 12/11/1/5].

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Chapter 4: General Discussion, Conclusion and Recommendations

4.1 General Discussion

The detection of meat adulteration is possible by means of DNA and protein analyses, specific breeds can also be identified by means of DNA analysis (Marson et al., 2005) such as the one that has been used in this study. This type of detection is extremely important for several reasons, with individuals allergic to specific animal species and religious beliefs being two of the main aspects (Ballin et al., 2009). Against this backdrop, the proper labelling of meat products is not only important but also stated in the regulations pertaining to labelling and advertising of food products (R.146/2010) (DoH, 2010). A previous study conducted by Tembe et al. (2018) detected that 65% of processed meat samples were contaminated with another meat source that had not been declared on the label, further analyses indicated that 80% of sausages contained undeclared meat species while only 50% of burger patties showed adulteration. A similar outcome was obtained by Cawthorn et al. (2013) reporting the detection of undeclared meat species in 68% of samples with the highest species substitution being found in raw sausages followed by burger patties. This study reported similar findings of adulteration occurring in 62% of investigated samples with two thirds (66%) of the contaminated samples presenting as processed sausages.

The studies mentioned however did not use DNA barcoding as a means of analyses, both studies made use of species specific primers. This study has used the method of DNA barcoding to determine any meat species in processed meat products that have been undeclared and the findings have been shocking. Species such as duiker, deer, rat, fish and chimpanzee have been reported after the bioinformatic analyses. These species pose a great threat to humans upon consumption as bushmeat is extremely susceptible to various diseases that can easily be transmitted to humans if these species are consumed (Danson et al., 2016). The technique of DNA barcoding relies on the amplification of a region in the mitochondrial gene, known as Cytochrome Oxidase Subunit 1 (COI) that is similar within species however shows great diversity between species (Herbert et al., 2003). The evolution of the COI gene is adequately rapid in order to allow for this discrimination between closely related species and also to investigate intraspecific diversity (Herbert et al, 2003; Lin et al., 2015). DNA barcoding is able to correct field misidentification, it allows for the

reduction of ambiguity in terms of species identification and it also allows for this identification to be more exact (Stoeckle at el., 2004).

This study has shown that majority of the lamb samples have been identified as beef, this indicates that economic gain could be a factor contributing to the adulteration of processed meat products. On average in local meat markets around KZN a kg of lamb is sold for approximately R165,00 whereas a kg of beef is sold for approximately R100,00. KZN also contains a larger number of cattle as compared to sheep as reported by the latest livestock count conducted in May 2018, with cattle numbers at approximately 2.5 million and sheep at a much lower count of 700 000 according to the Department of Agriculture, Land Reform and Rural Development (DALRRD, 2020). However it cannot be reported if the adulteration of these processed meat products have been intentional in the form of economic gain as mentioned previously or purely as a result of human error by means of equipment contamination or mishandling of different meat species which would be unintentional. It should be reported that species such as rat, chimpanzee, deer and duiker are not usual types of animal species that would be found in local meat markets or even the meat market suppliers and the detection of these animal species in the investigated processed meat products raises great concern pertaining to the safety of consumers as well as the hygiene of local meat markets. Against this backdrop, research in this area of animal forensic is of dire importance.

4.2 General Conclusion and Recommendations

The overall results obtained in this study have proven that there is indeed meat adulteration occurring across local meat markets in eThekwini, KwaZulu-Natal. The extent of the adulteration however can still not yet be determined due to the fact that there is a dearth of research in South Africa with regards to mislabelling of processed meat products and meat adulteration. Potential reasons could be either unintentional where products have been cross contaminated due to human error or even intentional where processed meat products have been adulterated for economic gain. The latter is a much more serious scenario as this is in complete violation of South Africa regulations around the declaration of all ingredients used in a product sold for human consumption.

DNA barcoding has been able to identified animal species in processed meat products that have not been declared, however, more research will have to be conducted to determine the extent of the adulteration as well as if it was accidental or intentional. It is highly recommended that further investigations should be conducted to determine if these local meat markets are purchasing these processed meat products with these undeclared species already in the products or if the adulteration is occurring within these local meat markets itself. This study only sampled local meat markets around eThekwini, for this reason more studies in this field should be conducted around South Africa and each municipality in our country to bring awareness and allow for consumers to feel safe about what they are actually consuming.

Ethical Approval

This study has been approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (UKZN), Durban, South Africa [reference nos. AREC/051/017M, AREC 071/017 and AREC 014/018; Act No. 35 of 1984 Section 20 approval reference no. 12/11/1/5].

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