IDENTIFICATION AND CHARACTERIZATION OF BENEFICIAL MICROBIOTA IN BEEF BILTONG FROM NAMIBIAN CENTRAL REGIONS

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ABSTRACT

Microbiota associated with beef biltong has not been assessed in Namibia and there is limited data in the literature describing the presence and role of lactic acid bacteria (LAB) in biltong products which is involved in the transformation of many aroma and flavour compounds and therefore, the quality of the final product. The aims of this study were to document the steps involved in beef biltong production, determine the physicochemical parameters that may influence microbial growth, characterize and identify LAB, yeast and moulds associated with beef biltong and, profiling LAB isolates for enzymatic and bacteriocin activities. Beef biltong is produced by a dry curing process. Its average pH ranged from 5.1±0.15 to 5.98±0.16, average sodium chloride content ranged from 3.16±0.62 to 3.98±0.86 g/100g, the average moisture content ranged from 7.56±7.56% to 18.86±8.96% and average water activity (a_w) ranged from 0.79±0.05 to 0.84±0.06. Beef biltong samples from Otjozondjupa, Omaheke and Khomas regions were characterized with an average total plate count ranging from 5.4±0.36 to 6.46±0.36 log CFU/g, average total LAB ranging from 5.66±0.41 to 7.02±0.35 log CFU/g while average total yeast and moulds ranged from 5.25±0.78 to 6.14±0.14 log CFU/g. Yeasts associated with beef biltong were identified as *Candida zeylanoides*, *C. guilliermondii*, *C. famata*, *C. krusei*, *C. tropicalis*, *Meyerozyma guiliermondii* and *Yarrowia lipolytica* using API 20 C AUX and sequencing of the 26S rRNA and ITS genes. The LAB were identified using API 50CHL and sequencing of the 16S rRNA as: *Lactobacillus brevis*, *L. graminis*, *L. plantarum*, *L. pentosus*, *L. paraplantarum*, *Lactococcus lactis* ssp. *lactis*, *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Pediococcus acidilactici*, *P. pentosaceus*, *Weisella cibaria*, and *W. confusa*, The cell free supernatant (CFS) of LAB was found to exhibit a variable degree of antimicrobial activity by agar well

diffusion method against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium perfringens*, *Candida albicans* and *Aspergillus niger*. After pH neutralization of the CFS, the antimicrobial activity due to bacteriocin or bacteriocin-like substances was detected in *L. plantarum* against *B. subtilis*, *C. albicans* and *A. niger*. Enzymatic activities detected in LAB using API ZYM included esterase, lipase, valine arylamidase, cysteine arylamidase, acid phosphatase, leucine arylamidase and β galactosidase. Beef biltong samples were associated with beneficial yeast with applications in food industries, as well as LAB with biotechnological properties such as enzymatic activity and bacteriocin production. Therefore, these strains may be important to be used as starter cultures to improve the quality and safety of meat products.

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ABBREVIATIONS

ACP African Caribbean and Pacific group

a_w Water activity

CFS Cell Free Supernatant

CFU Colony forming Units

DNA Deoxyribonucleic acid

et al et alia (and others)

EU European Union

FDA Food and Drugs Administration

GDP Gross Domestic Product

GRAS Generally recognised as safe

LAB Lactic acid bacteria

MC Moisture content

MRS Man Rogosa Sharpe

NaCl Sodium chloride

NCFS Neutralized Cell Free supernatant

PCR Polymerase Chain Reaction

pH Power of Hydrogen

rpm Round Per minute

SD	Standard deviations
sp.	Species (singular)
ssp.	Species (Plural)
TPC	Total Plate Count
USA	United States of America
USDA	United States Department of Agriculture
w/v	Percentage Weight/ Volume

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DEDICATION

This study is dedicated to the late David Angula, my mother Emilia Sheehama for her love, moral support and encouragement throughout my life. To my brothers and sisters; lastly, to nephew Bassanta, nieces Queen and Ndapandula, Aunty loves you!

DECLARATIONS

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

Signature …… Date……..

CHAPTER 1: INTRODUCTION

1.1 Orientation of the study

The shelf stability and safety of food products involves the creation of an antagonistic environment for undesirable pathogenic and spoilage microorganisms but at the same time capable of supporting the growth and metabolic activities of beneficial microbiota (Annalisa et al., 2016). Dry curing has been the oldest method employed for the preservation of meat products, whereby drying is achieved by the extraction or binding of moisture resulting in the reduction of water activity (a_w) within the commodity (Dzimba, Faria and Walter, 2007). Biltong is a ready to eat meat product comprising thin strips of dried meat which is spiced (Matsheka et al., 2014). The origin of the word biltong is thought to be derived from Dutch, "bil "reffering to buttock, rump or meat and "tong" to strips or tongue fillet shape (Petit et al., 2014; Attwell, 2003). Biltong is reported to anecdotally stem from the Dutch, who whilst escaping from British rule in South Africa some 200 years ago, preserved meat using vinegar and spices and hung it from back of Ox wagons for drying (Burfoot et al., 2010). The product can be made from beef or game by making thin slices of meat, dry-salting and then put into the mixture of apple vinegar, sugar, black pepper and coriander for curing (Engez, Baskan and Ergonul, 2012). Biltong is traditionally wind-dried in the shade during winter or produced in a drying box with a fan at an ambient temperature not exceeding 22^{o}C (Lindsay and Naidoo, 2010).

The product was popularized by the early local communities as a way of preserving meat. Currently biltong is a popular consumption in Namibia and most stores and service stations have biltong for sale. Additionally, biltong as a snack food is gaining international popularity and has found place in markets of Australia, Portugal, United States of America and United Kingdom (Attwell, 2003). Moreover, Attwell further

reported that, the biltong market value is increasing and in 2003, it was estimated to be between 40 and 70 million euros (2003). Biltong is generally considered as microbially stable due to the presence of curing salts, pH values ranging from 4.8-5.8 and low a_w values of 0.77 (Dzimba et al., 2007; Nortje, Buys and Minnar, 2005).

There are several dry cured meat products similar to biltong and produced in other countries. These include Jerky in North America (US), Pemmican in North American Arctic, Charque and Carne-de-sol in Brazil (Petit et al., 2014; Salva et al., 2012). Biltong differs from these dried cured products in that it is ready to eat and does not require rehydration or cooking treatment prior to consumption (Salva et al., 2012; Lindsay and Naidoo, 2010).

The final quality of dry cured meat product will depend on a large number of variables related to raw materials, enzyme activity, microbial population, type of starter culture and processing conditions (Mora et al., 2015). Lactic acid bacteria (LAB), yeasts and moulds play an important role in dry cured meat products. This is due to their halotolerant characteristics that may allow their presence during the manufacturing of the products (Dave and Ghaly, 2011; Wolter, Liang and Viljoen, 2000). These microorganisms contain relevant amount of enzymes which may be responsible for the enzymatic breakdown of carbohydrates, proteins and lipids which may contribute to the organoleptic and nutritional properties of the final product (Flores and Toldra, 2011). The proteolysis and hydrolysis processes in cured meat generally results in the release of free amino acids and peptides which contribute to the development of flavour and aroma of the product (Mora et al., 2015; Wolter, et al., 2000).

Previous studies revealed that fungi can be responsible for the development of specific aromas and flavours of dry meats, due to their lipolytic and proteolytic

activities as well as anti-oxidative properties (Ozturk, 2015; Ludemann et al., 2004). LAB is essential in carbohydrate fermentation and lactic acid generation with subsequent pH reduction which contribute to the flavour of the final product through the formation of noticeable acidic and acetic (vinegary) tastes (Adab, Essid and Hassouna, 2014). Moreover, LAB inhibits the growth of pathogenic and spoilage microbes through the production of antimicrobial compounds such as organic acids and bacteriocins (Annalisa et al., 2016). The aims of the present study were to determine the physicochemical parameters such as pH, a_w, moisture content (MC) and sodium chloride (NaCl) concentration in beef biltong samples from Omaheke, Otjozondjupa and Khomas regions; and to identify the LAB, yeasts and moulds associated with beef biltong, to study some of their biotechnological properties such as bacteriocins and enzyme production and to document the process involved in biltong production in Omaheke, Otjozondjupa and Khomas regions of Namibia.

1.2 Statement of the problem

Biltong may be a vehicle for beneficial microbiota, especially those belonging to LAB. In Namibia, microbiota associated with biltong has not been assessed and there is limited data in the literature describing the presence and role of LAB in biltong products which might indicate that LAB is involved in the transformation and quality of the final products. Furthermore, there is limited information in literature about the indigenous knowledge on beef biltong production in Namibia. It is then important to undertake biltong microbial studies in Namibia to assess the microbiota associated with it as well as to document the processing steps involved in beef biltong production.

1.3 Objectives

The main objectives of this study were:

1. To document the indigenous knowledge on the traditional methods of making beef biltong from the central regions (Omaheke, Otjozondjupa and Khomas regions).

2. To determine the main physicochemical parameters which affect microbial growth such as pH, water activity (a_w), moisture content (MC) and sodium chloride (NaCl) concentration of beef biltong samples from Omaheke, Otjozondjupa and Khomas regions.

3. To identify the LAB, yeast and moulds associated with beef biltong samples using culture dependent methods.

4. To profile the enzymatic activities of LAB isolates and screen them for bacteriocin production.

1.4 Research questions

1. What are the processing steps for beef biltong production in Omaheke, Otjozondjupa and Khomas regions?

2. Does a_w, MC, NaCl contents and pH have a relationship with microbial characteristics of biltong from Omaheke, Otjozondjupa and Khomas regions?
3. What are the dominant LAB, yeast and mould species associated with beef biltong from the Omaheke, Otjozondjupa and Khomas regions?
4. Does any of the microbiota isolates associated with beef biltong has a diversified enzymatic profile and bacteriocinogenic properties?

1.5 Significance of the study

The beef biltong microbial study is important to the contribution of indigenous food knowledge in Namibia. This study provides scientific information on microbiota associated with beef biltong from the central regions of Namibia. Secondly, production of bacteriocin is an important property as they are antimicrobial compounds inhibiting growth of food spoilage and foodborne pathogens and promising natural food preservatives which can prolong shelf-life of food (Cotter, Ross and Hill, 2013). On the other hand, enzymatic activity of food microbiota contributes to colour, taste and flavour in food. Thus, knowledge and understanding of the microbiota associated with the biltong can lead to further improvement in the biltong production process for its quality and safety and could be used to promote the product on the market.

1.6. Limitation of the study

Biltong is a diverse product affected by preparation methods and it may differ from one production point to another; therefore, for the microbiota study, a selection of four production points was done from each region and this may not fully reflect the situation for beef biltong in these regions. Furthermore, culture independent methods would allow the non-culturable microorganisms to be studied, but this could not be done since the budget could not allow the procurement of reagents, kits and buffers needed.

CHAPTER 2: LITERATURE REVIEW

2.1. Importance of beef and its industry to the Namibian economy

Agriculture sector is considered as an important part of the Namibian economy. The sector contributes at about 6% to the Namibian Gross Domestic Product (GDP), contributes to poverty alleviation and creates job opportunities (Mushendami, Biwa and Goamab, 2008). Beef industry is the most important part of the Agricultural sector. This is because of its contribution to the economy which is estimated at about 75% of the Agricultural economy (Emongor, 2007).

The beef industry creates an income for farmers when they sell their cattle to abattoirs and creates export earnings for the country through sale of meat products to other countries. Namibian beef is exported to South Africa and Europe, and the beef export value is reported at about US$ 200 million (Sattar, Diz and Franklin, 2003). According to Teweldemedhin and Mbai (2013), Namibia is granted a beef export quota of 13 000 tons to the Europen Union (EU) under the European Union / African Caribbean and Pacific group (EU/ACP) trade agreement. Therefore, this may be vital for Namibia to alleviate poverty through sustainable development and integration into world markets.

Biltong as a beef product is a wide consumption in Namibia. According to literature, some of the large producers of biltong are exporting part of their production to South Africa, However; there is no reported Namibian biltong producer selling to Europe (Sattar et al., 2003). This is because Namibia did not meet the United States Department of Agriculture (USDA) and the Food and Drugs Administration (FDA) regulations to export the products (Sattar et al., 2003). It is therefore important that biltong producers in Namibia develop an association to assist in meeting the export requirements and to promote the generic image of biltong among destinations in the

EU and the United States of America (USA), as this may contribute to the Namibian economy through export earnings.

2.2. Overview of biltong processing methods

The methods of biltong processing are often passed down from generation to generation, therefore; several production methods exist (Naidoo, 2010). However, Burnham et al. (2008) reported that, there are several basic steps that are associated with biltong production. This includes selection of meat that will be used, marinating of meat with vinegar, spicing and drying (as shown in Figure 1). Biltong can be made from beef or game. However, beef is mostly preferred for biltong production (Lindsay and Naidoo, 2010). Meat from "silverside", "topside" or "eye of round" parts of beef are mostly preferred for biltong (Engez et al., 2012; Dzimba et al., 2007).

Furthermore, the selected meat is then cut into thin strips and fat is trimmed from the meat as it may go rancid during the processing and storage stages (Burfoot et al., 2010). The thin sliced meat is then soaked in apple vinegar and spice marinated. The different spices used for marinating include sodium chloride, sugar, black pepper and coriander for curing (Engez et al., 2012; Burfoot et al., 2010). The addition of chilli may be optional which is for producing chilli flavoured biltong. The steps of vinegar soaking and spice marinating are reported to contribute to flavouring, texture and colour of meat during production (Lindsay and Naidoo, 2010). Also, literature reported that, vinegar, salt and spice mixture exhibit antimicrobial effects, which may prevent the growth of pathogenic microbes on biltong (Engez et al., 2012; Burdock and Carabin, 2009). The meat is then refrigerated at 4^{o}C to allow saturation of spices into the meat and this will also reduce microbial growth due to cold shocking.

The last step in biltong production process is drying. This step involves the removal of water from the meat using drying techniques such as environmental controlled rooms or units with electrical devices that supply heat and air circulation (Engez et al., 2012; Burfoot et al., 2010). By decreasing water content in biltong, growth and multiplication of microorganisms may be prevented and this may contribute to the safety of the product. Then, after drying, biltong may be ready to eat.

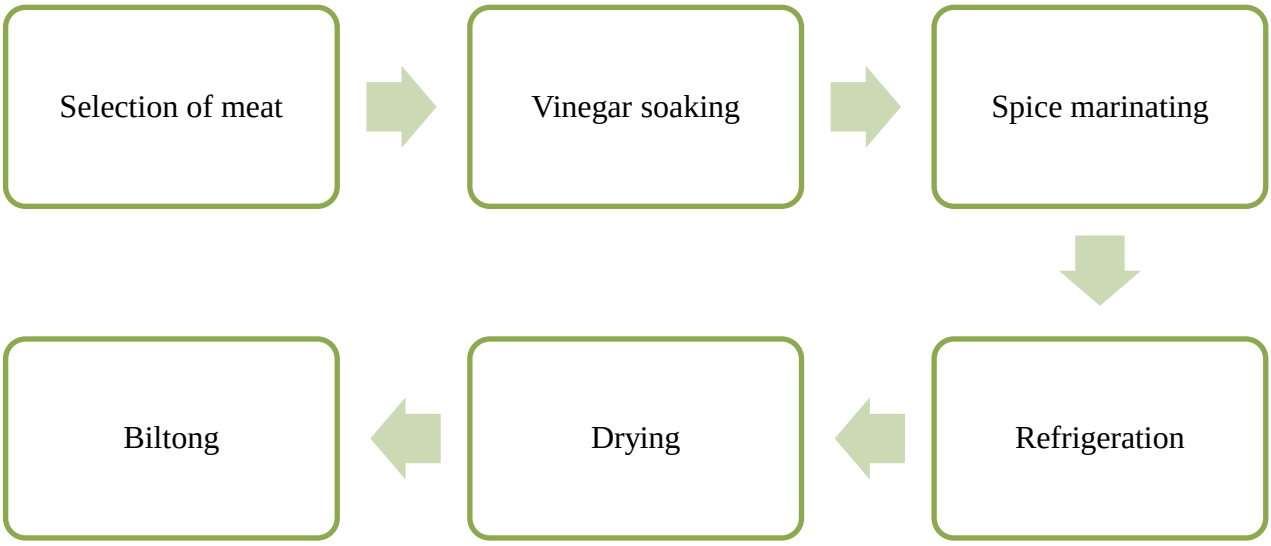

Figure 1: Flow diagram representation of biltong processing methods (Naidoo, 2010; Engez et al., 2012)

2.3. Methods of meat preservation

The diverse nutrient composition of meat makes it an ideal environment for the growth and proliferation of meat spoilage and food-borne pathogens. It is therefore essential that adequate preservation techniques are employed to maintain its stability and safety (Zhou, Xu and Liu, 2010). Meat preservation is important to control microbial growth. Therefore, preservation methods aim to inhibit the growth of spoilage and pathogenic microorganisms (Dave and Ghaly, 2011). Traditional methods of meat preservation are such as drying, smoking, brining and refrigeration

which are now reported to have been replaced by new preservation techniques such as chemical, biopreservative, non-thermal techniques or a combination of these techniques (Zhou et al., 2010).

2.3.1. Dry-curing preservative technique

Dry-curing has been the oldest method employed for the traditional preservation of meat products against the growth of spoilage and pathogenic organisms (Petit et al., 2014; Engez et al., 2012). Preservation through drying is achieved by binding or extraction of moisture resulting in the reduction of a_w within the product (Dzimba et al., 2007). The reduction of a_w ultimately results in the inhibition of microbial enzymes that are responsible for the spoilage of food. This means that, the reduction of a_w during drying of the product, may stabilize it by controlling the growth of undesirable microorganisms. According to Matsheka et al. (2014), effective drying to reduce a_w in meat products relies on drying time and other related process factors such as air temperature and relative humidity. The longer drying periods may be associated with safer products as longer periods may eliminate most pathogenic microorganisms than shorter drying periods depending on the low a_w associated with the final product.

2.3.2. Salting (sodium chloride)

Salting is one of the oldest methods of meat preservation which may results in semi-dehydrated product and prevents spoilage and shelf life extension of meat (Adeyinka et al., 2011). Sodium chloride inhibits microbial growth by increasing osmotic pressure as well as decreasing the a_w in the micro-environment (Dave and Ghaly, 2011). They further reported that, some bacterial growth can be inhibited by NaCl concentrations as low as 2% and concentrations of 20% can be high enough to inhibit many food spoilage yeasts including *Kloeckera apiculata*, *Zygosaccharomyces bailii*,

Zygosaccharomyces rouxii, *Kluyveromyces marxianus*, *Pichia membranaefaciens* and *Saccharomyces cerevisiae*. Bennani, Fraid and Bouseta (2000) reported that *Enterobacteriaceae* species were eliminated in kaddid (dry-salted meat product) as a result of reduced water a_w below 0.9 due to the subsequent actions of salting, spicing and drying. Furthermore, Wijnker, Koop and Lipman (2006) studied the antimicrobial properties of salt (NaCl) for the preservation of natural sheep casings at different a_w levels and found the activities of most spoilage and pathogenic bacteria; *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus* and *E. coli* O157:H7 stopped when a_w of 0.89 was reached. However, salt tolerant microorganisms such as lactic acid bacteria and yeasts could grow at these levels of a_w (Dave and Ghaly, 2011). Therefore achieving a low a_w is the main goal of creating a safer product.

2.3.3. Biopreservation

Another preservation method is biopreservation, the extension of shelf life and food safety using natural or controlled microbiota. The common example is fermentation, a process based on the growth of microorganisms in food. LAB represent the main microbiota in food biopreservation, as they are safe to consume due to their "generally recognized as safe" status (GRAS), as demonstrated by their long history of safe use in food and are reported to be the dominating microbiota during fermentation and, persisting during the storage of fermented foods (Corsetti, Perpetuini and Tofalo, 2015; Nuraida, 2015; Yam et al., 2015).

LAB are known to produce organic acids, diacetyl and acetoin, which can contribute to improvement of organoleptic properties of food. This is through the development of aroma and flavour of food products as well as producing compounds such as bacteriocins and hydrogen peroxide which possess antimicrobial effects, thereby

improving the quality and shelf-life extension of the food products (Nuraida, 2015; Gyawali and Ibrahim, 2014). In addition, lactic acid which is produced by LAB has shown antimicrobial activities against many pathogenic organisms such as *Clostridium botulinum* because of its abilities to reduce pH level, exert feedback inhibition and interfere with proton transfer across cell membranes (Doores, 2005). Further, lactic acid and acetic acid were shown to retard growth of microbes such as *Cladosporium* sp. and *Penicillium crustosum* (Crowley, Mahoney and van Sinderen, 2013).

Generally, LAB bacteriocins like the "producer" bacteria are considered GRAS compounds, being ingested with foods naturally containing bacteriocinogenic strains (Settanni and Corsetti, 2008). Particularly, bacteriocins were shown to be effective against foodborne pathogens and spoilage microorganisms. For example nisin was found to be effective against foodborne pathogens or spoilage bacteria such as *S. aureus*, *M. luteus*, *B. cereus* and *Listeria monocytogenes* (Rajendran, Nagappan and Ramamurthy, 2011; Galvez et al., 2010; Settanni and Corsetti, 2008). Therefore the application of LAB in the production of food products as starter or adjunct cultures or the use of bacteriocins as additives (shown on Figure 2) may improve the quality and safety of the products.

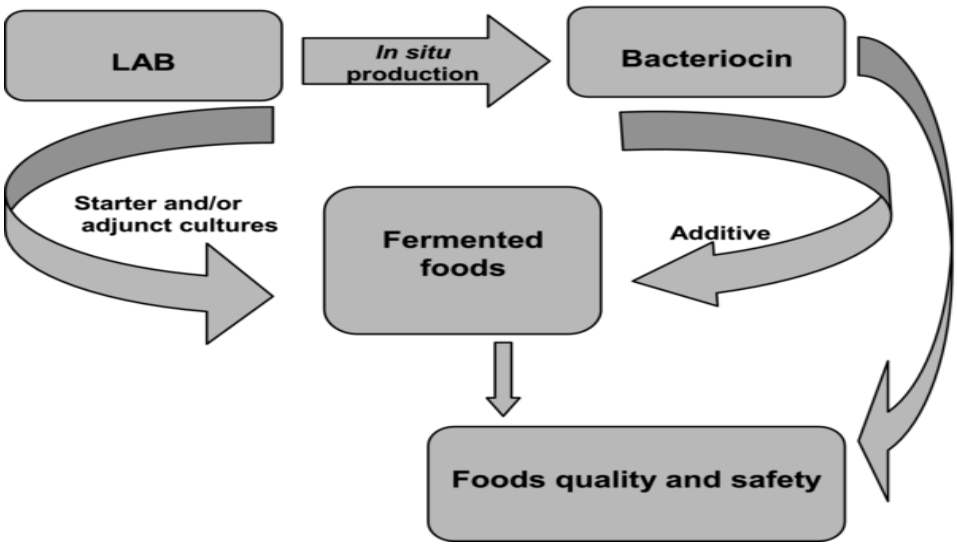

Figure 2: Overview of the application of LAB in food products (Beshkova and Frengova, 2012).

2.3.4. Chemical preservation

The use of chemical agents is one of the approaches used for meat preservation by inhibiting growth of undesirable microbes in meat (Dave and Ghaly, 2011). The types of preservatives, their levels and applications in foods are governed by legislation, which is regularly updated by the various regulatory bodies. For example the European Union derived legislation controls the use of additives in foodstuffs in the United Kingdom (Beales and Smith, 2004). This legislation provides legal definitions for the term food additive and also certain additive functions (example flavouring and preservative) and also provides a positive list of what is permitted in certain foods. The most legally permitted chemical preservative used in curing of meat is nitrite.

Nitrite which is used in meat preservation industry is usually in the form of salts such as sodium nitrite or potassium nitrite (Addis, 2015). Nitrite is an essential preservative and hurdle for growth of foodborne and spoilage microorganisms. For example, it has been used as antimicrobial compound inhibiting pathogenic and

spoilage microbes such as *Clostridium botulinum*, *Staphylococcus aureus* and *Yersinia enterocolitica* (Sindelar and Houser, 2009; Ray, 2004).

Nitrites affect the growth of microorganisms in food through several mechanisms including: reacting with alpha amino groups of the amino acids at low pH levels, blocking sulfhydryl groups which interferes with sulfur nutrition of the organism, reacting with iron-containing compounds which restricts the use of iron by bacteria and interfering with membrane permeability which limits the transport across cells (Ray, 2004). Despite the effectiveness of nitrites against food pathogens, the level of nitrites in meat products need to be kept as low as possible due to its toxicity, therefore; substances such as bacteriocins can be used as alternatives to chemical preservation of food products.

2.3.5. Hurdle technology for meat preservation

According to Ananou et al. (2007) the hurdle technology states that, the microbial safety, stability as well as sensorial and nutritional qualities of food are based on the application of combined preservative factors (called hurdles) that microorganisms present in the food are unable to overcome. Thus, hurdle technology refers to the combination of different preservation methods and processes to inhibit microbial growth. The most important hurdles used in meat preservation are temperature (high or low), reduced a_w, acidity (pH), redox potential (Eh), preservatives (e.g. nitrite, sorbate, sulphate) and competitive microorganisms (e.g. LAB), (Zhou et al., 2010). Generally, bio-preservatives and natural antimicrobials provide an excellent opportunity for hurdle preservation systems. For example Friedly et al. (2009) reported that, low concentrations of citrus essential oils in combination with organic acids could be effective to control gram-positive bacteria, in this case, *Listeria*. Furthermore, in meat and poultry products, application of bacteriocins in

combination with high hydrostatic pressure (HHP) was found to reduce the growth and survival of pathogens which were *S. aureus* and *L. monocytogenes* (Jofre, Garriga and Aymerich, 2008).

2.4. Importance of physicochemical parameters

Meat serves as a good substrate for microbial growth because of its high nutritional value, especially proteins (Adeyinka et al., 2011). The relatively high water activity, partial acidity and presence of carbohydrates associated with meat, often provide favourable niches for the growth of spoilage and pathogenic organisms (Matsheka et al., 2014). pH; the acidity or alkalinity of an environment, has a profound effect on the activity and stability of macromolecules such as enzymes, therefore; growth and metabolism of microorganisms are influenced by pH (Adams and Moss, 2008). At low pH, the microbial growth is inhibited; therefore, few total viable counts are expected to be associated with the commodity. Removal of water by the drying process is an effective food preservation method used world-wide for years (Engez et al., 2012; Matsheka et al., 2014) which results in the reduction of water content of food products. By decreasing water content of the food (moisture content; MC) and increasing NaCl concentration, growth and multiplication of microorganisms can be reduced due to low a_w (the amount of unbound water molecules in food products that support the growth of microorganism). This means that, by decreasing the a_w of food products; the lag phase of microbial growth will increase while the growth rate and size of the population will decrease (Adams and Moss, 2008). This is due to the fact that reduction of a_w ultimately results in the inhibition of the microbial enzymes that are responsible for growth, degradation of food and generally metabolism as all chemical reactions of a cell may require an aqueous environment (Lindsay and Naidoo, 2010; Engez et al., 2012).

Dry-cured meats are classified as intermediate moisture foods having a_w values ranging from 0.60- 0.90 (Wolter et al., 2000). The minimum a_w value for active growth of LAB, yeasts and moulds is reported to range from 0.75-0.90 (Wolter et al., 2000; Adams and Moss, 2008; Dave and Ghaly, 2011). However, the growth of most pathogenic and spoilage microorganisms such as *Escherichia coli*, *Clostridium botulinum*, *Salmonella* spp., *Yersinia enterocolitica* and *Bacillus cereus* are reported to be inhibited at the a_w value of 0.90 (Wolter et al., 2000; Adams and Moss, 2010; Dave and Ghaly, 2011). Table 1 also show the minimum a_w required to support the growth of common pathogens reported in dry meat products. Dried cured meat products are generally associated with low pH and low a_w and therefore regarded as microbially stable (Dzimba et al., 2007).

Table 1: Showing the minimum a_w for growth of most common microorganisms associated with dried meat products.

Pathogen	Minimum a_w
Pseudomonas	0.97
Clostridium botulinum	0.96
Clostridium perfringens	0.94
Salmonella	0.93
E.coli 0157: H7	0.95
Listeria monocytogenes	0.92
Staphylococcus aureus	0.90
Aspergillus flavus	0.92

Source: USDA (2005)

2.5. Microbiology associated with biltong

Previous studies found that biltong carried microbial loads ranging from 5 - 9.7 log CFU/g of biltong (Petit et al., 2014; Lindsay and Naidoo, 2010; Wolter et al., 2000). Microorganisms that they found to be associated with biltong included LAB, yeast and moulds. Microbes such as *Debaromyces hansenii*, *Cryptococcus laurentii*, *Saccharomyces cerevisiae*, *Lactobacilli* sp., and *Lactococci* sp. were isolated from biltong in the study by Wolter et al. (2000). Furthermore, in the study of Petit et al. (2014) a high level of fermentative bacteria were found to be associated with biltong samples, with dry biltong meeting the standard hygienic quality with microbial count of 6.9 log CFU/g which they report to be in good agreement with the Food Standards Agency. In addition, LAB was also found to be the major component of biltong in the study of Wolter et al. (2000) with a count of 8 log CFU/g while yeasts were reported to indicate a count of 6.7 log CFU/g of biltong. Due to low pH, high salt content and low a_w of biltong, the halotorelant microorganisms were reported to be the dominant microbes associated with the commodity (Petit et al., 2014; Wolter et al., 2000).

2.6. Lactic acid bacteria

Lactic acid bacteria comprise of a heterogeneous group of gram-positive, non-spore forming, non-motile, aerotorelant, rod and coccus shaped organisms which produce lactic acid as a major end product during carbohydrate fermentation (Crowley et al., 2013). Early defined four main core genera involved in food fermentations are *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Khalid, 2011). However, reclassification has amended the original grouping and the LAB group is currently encompassing the following genera: *Aerococcus*, *Alloicoccus*,

Carnobacterium, *Dolosigranulum*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Ruas-Madiedo et al., 2012). The lactic acid bacteria are classified into two groups (homofermentative or heterofermentative lactic acid bacteria) based on the end-products formed during the fermentation of glucose (Figure 3). Homofermentative lactic acid bacteria such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some *Lactobacilli* produce lactic acid as the major end-product of glucose fermentation through the Embden-Meyerhof-Parnas pathway to generate two moles of lactate per mole of glucose while heterofermentative lactic acid bacteria such as *Weissella* and *Leuconostoc* and some *Lactobacillus* produce equimolar amounts of lactate, carbon dioxide and ethanol from glucose via the hexose monophosphate or pentose pathway.

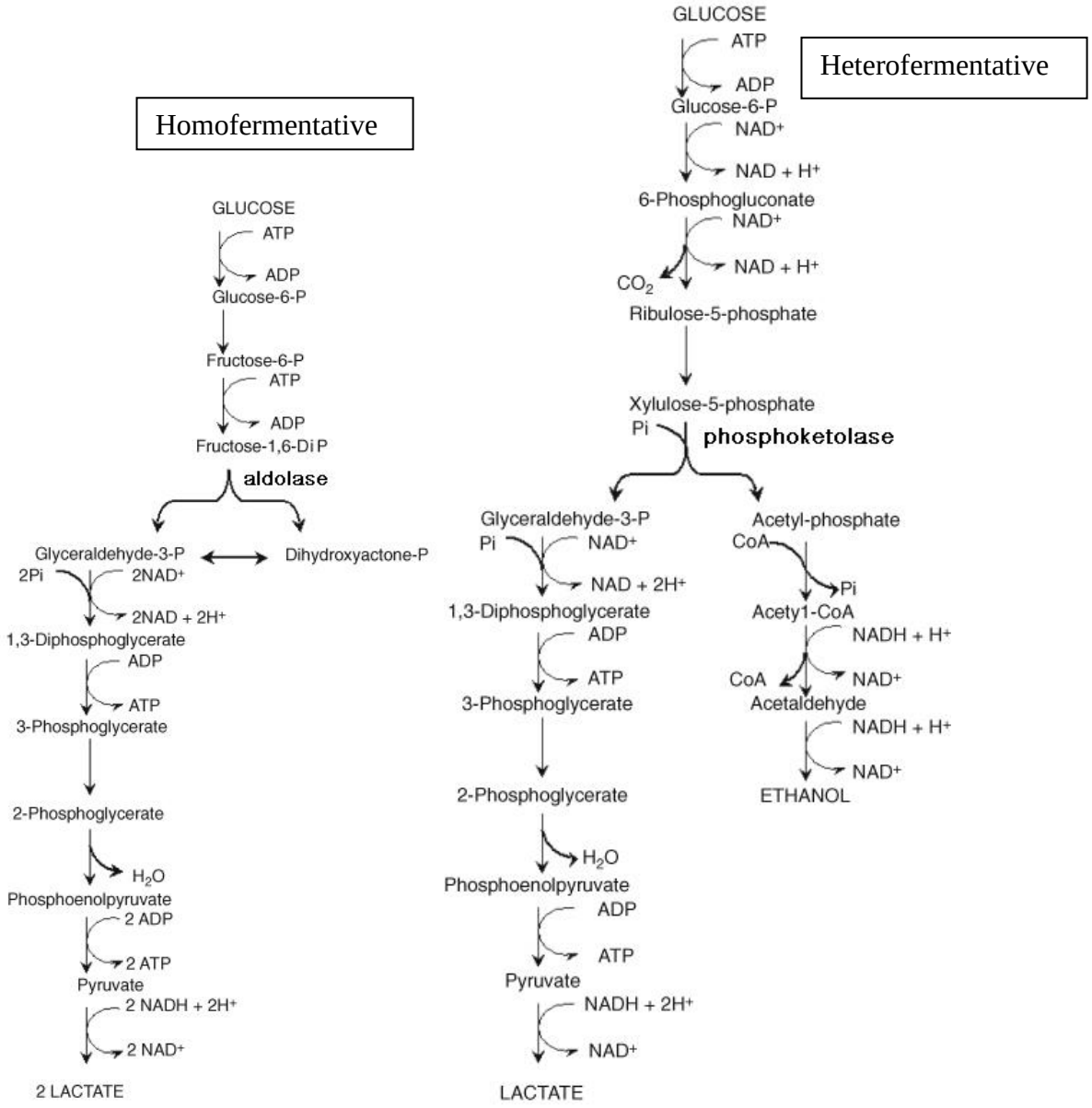

Figure 3: Glucose fermentation in lactic acid bacteria (Khalid, 2011)

Lactic acid bacteria are generally employed because they may significantly improve the nutritional value, organoleptic, technological and shelf-life characteristics in diverse fermented foods (Patel, Shah and Prajapti, 2013; Settanni and Corsetti, 2008). They initiate the rapid and adequate acidification of raw materials through production of various organic acids from carbohydrates, of which, lactic acid is the most abundant, followed by acetic acid, ethanol, bacteriocins, aroma compounds, exopolysaccharides and some enzymes within the food matrices (Patel et al., 2013;

Hui et al., 2012). With increasing pressure from consumers towards more natural food preservatives, LAB represents ideal candidates for commercial exploitation due to their "GRAS" status (Crowley et al., 2013). LAB bacteriocin producing strains have been successfully used in starter cultures in food in order to improve safety and quality example in reducing *Clostridium tyrobutyricum* (Lucera et al., 2012).

Lactic acid bacteria are known to produce several antimicrobial compounds. The antimicrobial compounds are substances used to preserve food by preventing the growth of microorganisms and subsequent spoilage of food. Therefore, food antimicrobials function to prolong shelf life and preserve the quality through inhibition of spoilage microorganisms. Several authors reported on the antimicrobial production by LAB, which are the organic acids, hydrogen peroxide and bacteriocins (Baek et al., 2012; Zacharof and Lovitt, 2012; Rattanachaikunsopon and Phumkhachorn, 2010).

2.6.1. Organic acids

Lactic acid bacteria are reported to produce organic acids such as lactic, acetic, propionic and benzoic acids as fermentation end products of carbohydrate metabolism. These weak acids will result in acidic environment which contributes to antimicrobial effects against the growth of microorganisms including spoilage or pathogenic microbes (Baek et al., 2012; Ross, Morgan and Hill, 2002), and this may contribute to the maintenance and preservation of nutritive qualities of food for an extended shelf life (Patel et al., 2013). Organic acids are generally thought to exert their antimicrobial activities by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Ross et al., 2002). According to Rattanachaikunsopon

and Phumkhachorn (2010) organic acids have a broad mode of action and inhibit the gram-positive and gram-negative bacteria as well as yeast and moulds. The inhibitory activity of lactic acid and acetic acid on moulds was studied by Pelaez et al. (2012) and they found out that both organic acids possess the inhibitory activities against different strains of *Aspergillus flavus*.

2.6.2. Hydrogen peroxide

According to Figueroa-Gonzalez, Cruz-Guerrero and Quijano (2011) the production of hydrogen peroxide by lactic acid bacteria can prevent growth of food-borne pathogens and are beneficial in food preservation. They further reported that, LAB that produce hydrogen peroxide have been shown to inhibit the growth of psychrotrophic and pathogenic microbes at refrigeration temperatures. The antimicrobial effects of hydrogen peroxide are reported to result from the oxidation of sulfhydryl groups causing denaturing of a number of bacterial enzymes and from the peroxidation of membrane lipids thus increasing its permeability (Figueroa-Gonzalez et al., 2011; Rattanachaikunsopon and Phumkhachorn, 2010; Suskovic et al., 2010).

2.6.3. Bacteriocins

Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity produced by many gram-positive and gram-negative bacteria; however, those produced by food grade LAB have received considerable attention due to their potential application in food industry as natural preservatives (Suskovic et al., 2010). The production of bacteriocins has been detected in several lactic acid bacteria isolated from meat products such as *Lactobacillus sakei*, *L. curvatus*, *L. plantarum*,

L. brevis and *L. casei* (Settani and Corsetti, 2008; Leroy, Verluyten and Vuyst, 2006).

Bacteriocins are classified into three major categories (Rodriguez et al., 2003). Class I, the lantibiotics, are a class of peptide substances that contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid. The most extensively characterized of these is nisin (the structure is shown on Figure 4) which has GRAS status for use as a direct human food ingredient. It is produced by strains of *Lactococcus lactis* ssp. *lactis* and has a broad inhibitory spectrum against gram-positive bacteria, including many pathogens and can prevent outgrowth of *Bacillus* and *Clostridium* spores (Rattanachaikunsopon and Phumkhachorn, 2010). Their modes of action are generally through pore formation, through membrane depolarization of the cytoplasmic membrane of the sensitive target species and interfere with cellular enzymatic reactions (Zacharof and Lovitt, 2012).

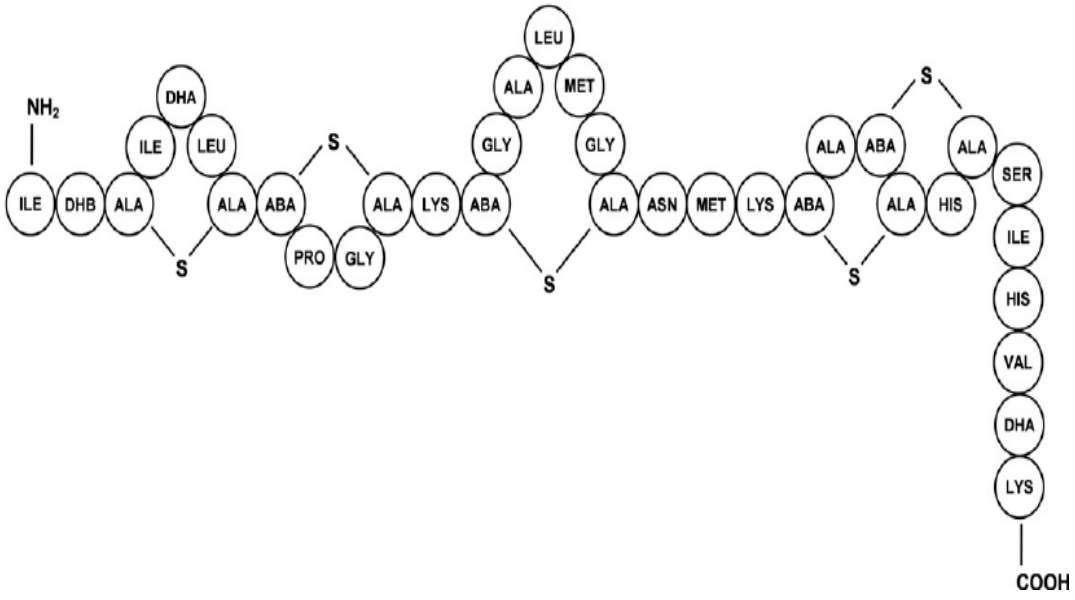

Figure 4: Structure of nisin, a lantibiotic (Annanou et al., 2007).

According to Beshkova and Frengova (2015) Class II encompasses the more common non-lanthionine-containing bacteriocins, which are non-modified, small (<10 kDa), heat stable peptides. Example is pediocin-like peptides which have attracted much attention due to their specific activity against food pathogens like *Listeria monocytogenes* (Zacharof and Lovitt, 2012). Moreover, class II bacteriocins; curvacins and sakacins have been reported in *L. curvatus* and *L. sakei* strains isolated from meat strains and they are also reported to exhibit a strong antilisterial activity (De Souza et al., 2015; Todorov et al., 2013; Cotter, Hill and Ross, 2005).

Class III: Bacteriocins (bacteriolysins) consists of heat labile proteins which have large molecular weight (>30 kDa). Their mechanism of action is distinct in function as they lyse the sensitive cells by catalysing cell-wall hydrolysis (Suskovic et al., 2010). Bacteriocins representing this group are helveticin I by *Lactobacillus helveticus* and enterolysin produced by *Enterococcus faecium* (Zacharof and Lovitt, 2012).

Lactic acid bacteria have traditionally been used in food production due to their potential ability to improve the safety of food stuffs which can be attributed to their bacteriocins production property. The detection of antimicrobial activity within LAB strains have been done in the previous studies using agar spot method or agar well diffusion method (Fatima and Mebrouk, 2013; Yang et al., 2012; Taheri et al., 2011). In their studies the cell free supernatant (CFS) was used to determine antimicrobial activity by LAB due to organic acid, hydrogen peroxide or bacteriocins, while; after neutralization of the pH (NCFS), antimicrobial activity was believed to be due to bacteriocins or bacteriocin-like substances. Inhibitory activity attributed to antimicrobial agents against pathogens such as *S. aureus*, *E. coli* and *Bacillus cereus* was reported in LAB strains isolated from fermented milk (Heita, 2014). In the study

of Yang et al. (2011) it was reported that 20% of LAB isolates from cheese and yogurts showed antimicrobial activity against *Listeria innocua* and *Lactobacillus sakei* which was attributed to bacteriocin production or bacteriocin-like substances. Furthermore, Fatima and Mebrouk (2013) reported that *L. plantarum* and *P. pentosaceus* strains isolated from milk products produced bacteriocins that displayed a wide spectrum of activity against *L. ivanovii* and food spoilage microorganisms.

Bio-protective effect of bacteriocins has been reported by several authors (Balciunas et al., 2013). In the study of Dal Bello et al. (2012) four strains of bacteriocinogenic *Lactococcus lactis* (nisin and lactacin producers) were used as starter cultures to control the growth of *Listeria monocytogenes* in cottage cheese production. The *Lactococcus* strains (producer of nisin) in combination with the high acidity reached during the manufacturing of cheese was found able to control and particularly reduce the growth of the *L. monocytogenes* inoculated in cheese at 3 log CFU/g (Corsetti et al., 2015). Lactic acid bacteria have been isolated in biltong samples in the study of Wolter et al. (2000) and Petit et al. (2014), however, the exhibition of antimicrobial activity by the LAB isolates was not investigated.

2.7. Enzymatic activity in LAB

LAB have been found to synthesise a diverse type of enzymes which may influence the compositional, processing and organoleptic properties as well as the overall quality of foods (Patel et al., 2013). Enzymatic activity of microflora including LAB may play a role in processes that occur during the ripening of meat products and result in formation of characteristic flavour, taste and colour of products (Tanasupawat, Phoottosavako and Keeratipibul, 2015; Stoyanovski et al., 2013).

The enzymatic profiling of LAB strains isolated from fermented meat, milk and beverages products have been reported in previous studies (Mechai, Debabza and Kirane, 2014; Arias et al., 2013; Stoyanovski et al., 2013). The API ZYM galleries (bioMerieux, France), was used to study several enzyme activities that were included in lipids, carbohydrates, proteins and phosphates metabolism (Tanasupawat et al., 2015). The detection of enzymatic activities such as esterase and leucine arylamidase, esterase lipase, α-galactosidase, valine arylamidase, cysteine arylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase, lipase, trypsin, alkaline phosphatase, α-chymotrypsin, β-galactosidase and α-galactosidase have been found to vary with LAB strains. For example in the study of Tamang et al. (2007) LAB strains isolated from Hamei and Marcha showed a strong arylamidase and glucosamidase activities and they showed no detectable esterase, lipase, phosphatase and proteinases (trypsin and chymotrypsin) activities. Furthermore, in the study of Arias et al. (2013) high β-galactosidase activity, aminopetidase and acid phosphatase have been reported in *Lactobacilli* and *Leuconostoc* strains while α-galactosidase, β-glucuronidase, α-mannodase and α-fuccosidase have not been detected in any strain. The exopeptidase and aminopeptidase are reported to degrade amino acids into volatile molecules that play a role in characteristic flavour and taste of products (Mora et al., 2015).

The exhibition of low proteinase, low esterase lipase and high peptidase activities may also be useful in improving the organoleptic properties of products (Arias et al. 2013; Georgiva et al., 2009). The activity of β-galactosidase is reported as an important feature for the strains to be used as probiotic due to that it is the main enzyme which is used by the homofermentative *Lactobacilli* to transform lactose to lactic acid. Therefore the activity may contribute to the acidification of products,

reduces lactose intolerance in human gut and can stimulate the growth and colonization of Bifidobacteria with probiotic effects in human intestines (Bassyouni et al., 2012). On the other hand LAB strains which do not exhibit β-glucuronidase activity could be considered as probiotic for humans since the enzyme is reported to be carcinogenic (Mechai et al., 2014; Bassyouni et al., 2012). Although enzymatic activities of LAB have been studied in several meat and dairy products, a gap in knowledge on the enzymatic profile of LAB strains from biltong still exists.

2.8. Yeasts and moulds associated with meat products

Yeasts are one of the groups of microorganisms most commonly used for food production in the food industries worldwide, with *Saccharomyces*, *Candida* and *Kluyveromyces* being the most representative genera (Diosma et al., 2014). The use of yeasts in the production of meat products is not well developed, however; the use of osmotolerant yeast *Debaromyces hansenii*, *Candinda zeylanoides*, *Pichia membranefaciens*, *Debaromyces polymorphus* and *Pichia guilliermondii* has been reported (Josephsen and Jespersen, 2004). Moreover, the use of moulds is also reported in meat products. Common moulds used in meat fermentation include *P. nalgiovense*, *P. chrysogenum*, *P. cameberti* and *Eurotium rubrumas* (Castellari et al., 2010; Josephsen and Jespersen, 2004).

Yeasts and moulds colonizing the surface layers of dry-cured meat products during maturation have been regarded as positively contributing to the chemosensory properties of final outcome of the products (Mora et al., 2015; Castellari et al., 2010; Sonjak et al., 2010). This may be through oxidation prevention and generation of volatile compounds enhancing aroma. An example is *D. hansenii* which is reported to possess proteolytic and lipolytic activities which may contribute to the

organoleptic properties of meat products (Ozturk, 2015; Lopez, 2014). Also, mould growth on the meat is reported to be desirable, as they may be responsible for the development of flavours and aromas of dry meats due to their production of extracellular proteinases and lipases which can be involved in proteolytic and lipolytic activities (Ozturk, 2015; Josephsen and Jespersen, 2004).

Furthermore, yeasts and moulds possess anti-oxidative effects which may be due to their oxygen consumption and barrier effects of their mycelium that reduces the penetration of oxygen and light (Bruna et al., 2003). This results in a stable colour and taste, and prevents the products from becoming rancid. Anti-oxidative effect is reported in *D. hansenii* and it has been shown to contribute to the colour-stabilizing effect on surface of meat products (Santos et al., 2001). Additionally, yeast and surface moulds can also have a protective role against pathogenic or spoilage microorganisms due to their antifungal and antibacterial activity (Roostita et al., 2011; Ludemann et al., 2004).

2.9. Background on the use of culture dependent methods for microbiota characterization and identification

Culture dependent methods are defined as those which consist of culturing and isolating microorganisms on synthetic media prior to their identification (Cocolin, Dolci and Rantsiou, 2011). The identification of microbiota can be based on either phenotypic or genotypic characteristics of microorganism (steps shown on Figure 5). For the phenotypic based methods; bacteria, yeast and moulds are identified using conventional microbiological methods. These may be based on morphological and physiological characteristics such as gram staining (bacteria only), cell shape, enzyme production and fermentation of different carbohydrates (Moraes et al., 2013).

The API system (bioMerieux, France) is reported to be widely used for microbiota identification based on carbohydrates fermentation (Abdelbasset et al., 2014; Temmerman, Huys and Swings, 2004). The API 50 CH is a standardized system containing 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. It is used in conjunction with API 50CHL medium and lactic acid bacteria are identified using the computer software; the API web. On the other hand, API 20 C AUX is used for yeast identification according to their sugar assimilation. Similar to API system is the Biolog (Biolog, Hayward, C.A) which is a unique plate used for identification of Gram positive and Gram negative bacteria with reference to the fermentation of 96 carbohydrates using MicroLog 3 software (Moraes et al., 2012).

Although the application of phenotypic techniques has been useful for microbiota identification, there is a general awareness that they exhibit several limitations. These include poor reproducibility, logistic difficulties for large scale applications, poor discriminatory power and that similar phenotypes displayed by strains do not always correspond to similar or even closely related genotypes (Mohania et al., 2008; Temmerman et al., 2004). As a result, a shift exists towards the use of genotypic characterization methods for the provision of a better classification and differentiation (McCartney, 2002).

The genotypic methods of microbiota identification are based on the principle of Polymerase Chain Reaction (PCR). This technique enables the specifically targeted DNA fragments to be selectively amplified through the use of oligonucleotide primers under controlled reaction conditions (Ndoye et al., 2011; Temmerman et al., 2004). The sequencing of the 16S rRNA 1500 bp fragment or 250-500 bp fragment of the D1/D2 region of 26S rRNA is the most widely used technique for bacteria or

yeast identification, respectively. The genomes of all bacteria or yeast contain these conserved genes and the small variability is specific to each species (Mohania et al., 2008). The microbiota are identified to the genus or species level by comparing the nucleotide sequence of the 16S rRNA or 26S rRNA genes to other sequences of known organisms in electronic databases such as SILVA, Ribosomal Database Project (RDP), Greengenes and GenBank in National Centre for Biotechnological Information (NCBI) (Chaudhary et al., 2015)

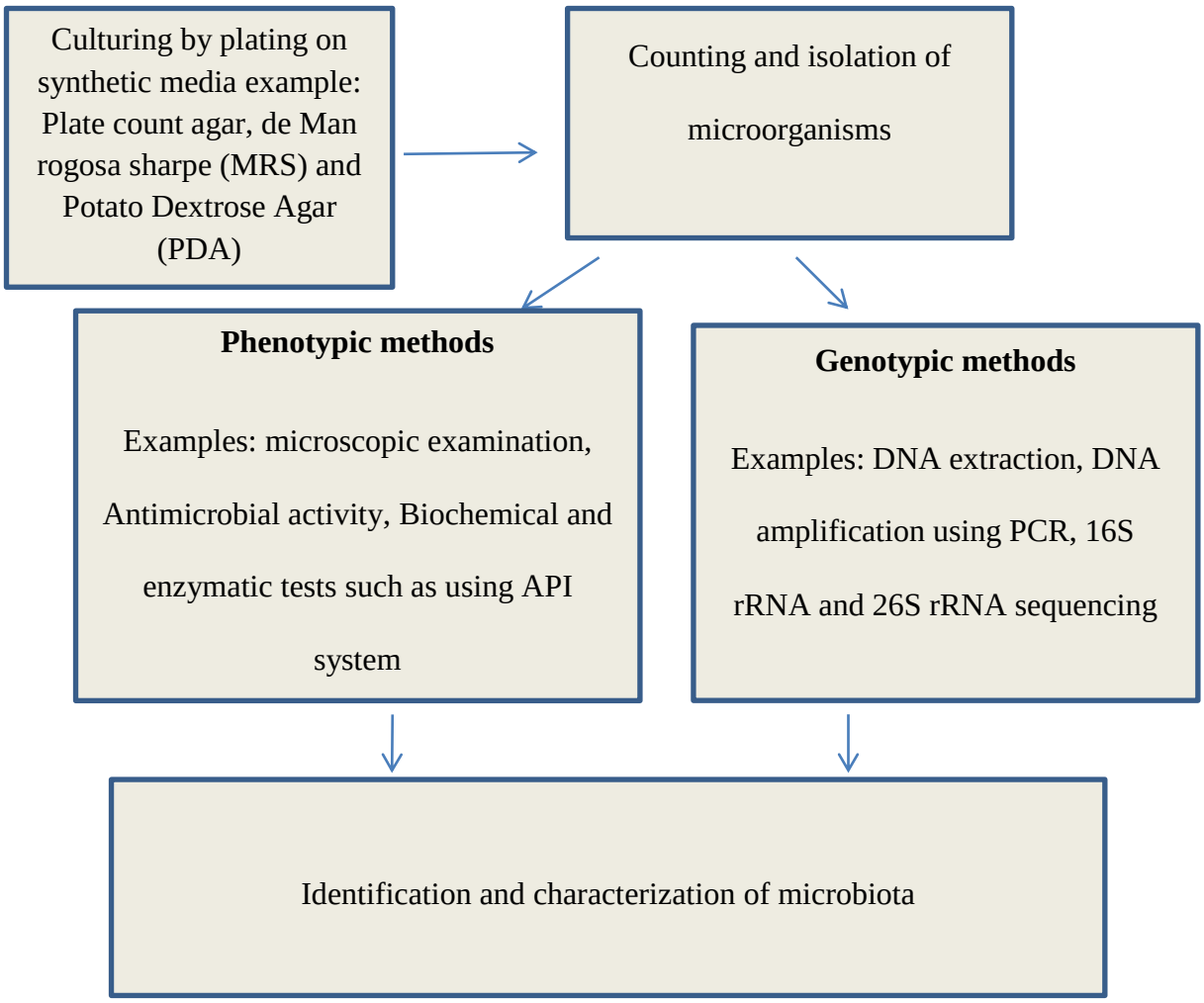

Figure 5: Flow diagram of culture dependent methods of studying microbiota (Cocolin et al., 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1. Research design

The research design used in the study consisted of both quantitative and qualitative approaches. The qualitative data produced in the study were the indigenous knowledge of making beef biltong from Omaheke, Otjozondjupa and Khomas regions. Microbial counts, antimicrobial assays, enzymatic activities and DNA related experiments produced quantitative data.

Beef biltong samples (36) were purchased from the Namibian central regions; Omaheke, Otjozondjupa and Khomas regions (Figure 6). The managers of the beef biltong production points from Omaheke, Otjozondjupa and Khomas regions were interviewed using questionnaires (see Appendix 4) on the processing methods of beef biltong production. The samples were then transported under cool conditions to the University of Namibia. After that, a_w, pH, MC and NaCl were determined. Microbiota associated with beef biltong was then isolated, identified and characterized using culture dependent methods. Finally, LAB were screened for bacteriocin production and enzymatic activities.

3.2. Sample collection

A total of 36 beef biltong samples were purchased from Omaheke, Otjozondjupa and Khomas regions (Figure 6). Twelve samples were obtained from 4 production points which are known as main biltong suppliers in each region. The biltong samples were transported under sterile and cool conditions in wrapped plastic bags to the Department of Biological Sciences of the University of Namibia. The beef biltong samples were then processed, and beef biltong microbiota were then identified and characterized using culture dependent methods. Enzymatic activity and bacteriocins

production were screened using commercial API-zym (bioMérieux, France) and agar well diffusion method (Rehaiem et al., 2016) respectively.

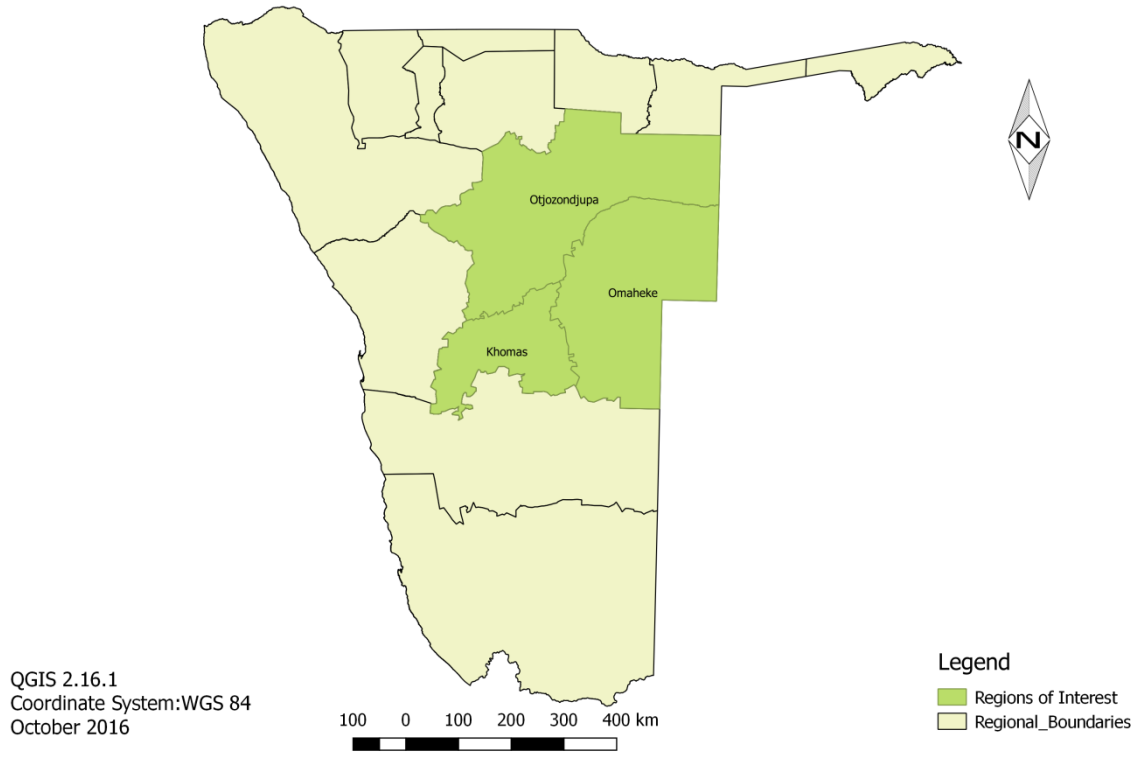

Figure 6: Study areas; Omaheke, Otjozondjupa and Khomas regions.

3.3. Physico-chemical parameters determination

3.3.1. pH

Beef biltong samples (5g) were homogenized in 45 ml distilled water using a stomacher blender (Seward, UK) at 360 rpm for 1 minute. The homogenized samples were left to stand for 30 minutes before measurements with a pH meter (Petit et al., 2014). The measurements were done in triplicate.

3.3.2. Water activity

Using aseptic measures, beef biltong samples were cut into thin slices and placed inside the water activity meter (Deurotherm, Germany). Water activity was directly measured by means of a hygrometer built into the lid of the water activity meter

(Deurotherm, Germany). The measurements were done in triplicate (Petit et al., 2014).

3.3.3. Moisture content

The beef biltong samples (2g) were cut into small thin slices and placed in a dish inside the moisture content analyser (Mettler Toledo, Switzerland). The samples were then oven dried at 106^{o}C for 30 minutes. The percentages of moisture content were then read on the moisture content analyser. The beef biltong samples were analysed in triplicate (Petit et al., 2014).

3.3.4. Sodium chloride by titration

The Volhard's method was used for NaCl determination (Rajkovic, Sredovic and Miradovic, 2010). The beef biltong samples were cut into small pieces and 6g of the samples was weighed into a 500ml conical flask. Then 50ml of 0.1M silver nitrate (0.1M $AgNO_3$), 20ml concentrated nitric acid (HNO_3) and 100ml distilled water were added and heated. As the solution boiled 30ml of 5% potassium permanganate ($KMnO_4$) was added until the biltong particles were completely digested. The solutions were cooled down, filtered using filter papers and diluted to 500ml. Excess $AgNO_3$ was then back titrated with potassium thiocyanate (KSCN) solution, employing 1ml ferric ammonium sulphate solution as an indicator. The appearance of the orange-red colour indicated the titration end point. The number of moles and mass of NaCl were calculated. The results were expressed as mass of NaCl/ 100g of biltong. The experiments were done in triplicate.

3.4. Microbiological analysis

3.4.1. Total plate count

The beef biltong samples (25g) were aseptically transferred into a sterile stomacher bag followed by the addition of 225 ml of buffered peptone water (1:10dillution).

The sample in the stomacher bag and the diluent were then blended for 1 minute at 360 rpm using a stomacher blender (Seward, UK). The homogenized beef biltong samples were serially diluted in buffered peptone water to 10^{-5}. After that 0.1 ml of the diluted samples was spread plated on total plate count agar (TPC) using a spreader for total bacteria count and incubated at 30^{o}C for 24 hours (Petit et al., 2014; Wolter et al., 2000). The experiments were done in triplicate and results were expressed as Log CFU/g (log colony forming units per gram).

3.4.2. Enumeration of LAB, yeasts and moulds

de-Man-Rogosa-Sharpe (MRS) agar was used for enumeration of total LAB while yeasts and moulds were enumerated on Sabouraud Dextrose Agar (SDA) (Victor et al., 2013; De Man, Rogosa and Sharpe, 1960). The homogenized beef biltong samples were diluted from 10^{-1} to 10^{-5} by transferring 1 ml of the homogenized biltong sample into 9ml buffered peptone water. An amount of 0.1ml of the diluted beef biltong samples was inoculated on MRS agar and SDA followed by incubation at 37^{o}C for 24 hours and 25^{o}C for 3 days, respectively. The experiments were done in triplicate and results were expressed as Log CFU/g.

3.4.3. Isolation and purification of LAB, yeast and moulds

LAB strains were isolated from 36 beef biltong samples by aseptically transferring 10ml of the homogenized samples into 90ml of MRS broth and incubated at 37^{o}C for 24 hours. Ten-fold serial dilutions were done in buffered peptone water to 10^{-5} and 0.1ml aliquots of appropriate dilution were inoculated on MRS agar plates for LAB isolation, on M17 agar for *Lactococcus* spp. and Rogosa agar for *Lactobacillus* spp. isolation (Terzaghi and Sandine, 1975; De Man et al., 1960). The plates were then incubated at 37^{o}C for 48 hours. The representative colonies were obtained from each

agar plates and sub-culturing was done on the same media to obtain a pure culture. The LAB isolates were then grown in MRS broth at 37^{o}C for 24 hours and preserved in 25% glycerol.

Furthermore, yeasts and moulds were isolated from 36 beef biltong samples by aseptically transferring 10ml of the homogenized samples into 90ml of nutrient broth and incubated at 25^{o}C for 24 hours. Serial dilutions were done from 10^{-1} to 10^{-5}. Aliquots (100µl) of appropriate dilutions were inoculated on yeast glucose chloramphenicol agar (YGC) for yeasts and potato dextrose agar (PDA) for moulds. The plates were then incubated at 25^{o}C for 3 days. After incubation, representative colonies were picked from each agar plate. The yeast and moulds were purified by sub-culturing on the same media (YGC and PDA) (Wolter et al., 2000). The isolates were transferred into nutrient broth, incubated at 25^{o}C for 3 days and preserved in 25% glycerol.

3.4.4. Biochemical identification of LAB

A total of 10 isolates that were gram positive and catalase negative (gram staining and catalase test protocols on appendix 1) were picked from MRS, M17 and Rogosa agar plates for carbohydrate test using the API 50 CH stripe/ CHL medium for identification (Ozgun and Vural, 2011). The selection of isolates was made from each region according to the shapes and colour of isolates. The identification of LAB was made according to the manufacturer's instructions (bioMerieux, France). The API LAB PLUS software (bioMerieux, France) was used according to the manufacturer's instruction for the interpretation of results.

3.4.5. Biochemical identification of yeast

A total of 30 isolates from YGC agar and PDA were selected for API 20 C AUX identification. The yeasts isolates were selected from the three regions according to the colour and shapes of colonies. API 20 C AUX identification of yeasts was done according to the manufacturer's instructions (bioMerieux, France). The results were interpreted using API LAB PLUS software (bioMerieux, France) according to the manufacturer's instructions.

3.5. Genetic identification and characterization of LAB, yeast and moulds

3.5.1. Genomic DNA extraction

DNA was extracted from pure cultures whereby the isolated LAB colonies were grown in MRS broth, while yeasts and moulds were grown in nutrient broth at 37^{o}C for 48 hours and 25^{o}C for 3 days, respectively. The grown cultures were pelleted by centrifugation at 16000g for 5 minutes and washed with double distilled water. DNA was then extracted using the ZYMO Bacterial/ Fungal DNA Extraction Kit (ZYMO Research Corp, USA) according to the manufacturer's instructions with a few modifications (see appendix 1).

3.5.2. Polymerase Chain reaction (PCR) DNA amplification and sequencing of the 16S rRNA, ITS and 26S rRNA genes

The extracted DNA from pure cultures was used for PCR amplification in the thermocycler (BIO RAD, Singapore). The 1500bp segment of the 16S rRNA bacterial gene was amplified using the universal primers 27F (5' AGAGTTTGATCTGGCTCAG 3') and 1492R (5' CGGTTACCTTGTTACGACTT 3') as described by Weisburg et al. (1991). The primers ITS1(5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'

TCCTCCGCTTATTATTGATATGC 3') as well as NL1 (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G 3') and LS2 (5' ATT CCC AAA CAA CTC GAC TC 3') were used to amplify the 500bp of ITS1-5.8S-ITS2 region and 250bp D1/D2 region of the 26S rRNA in yeasts as reported by Jespersen et al. (2005) and Cocolin et al. (2002), respectively. The amplification of the genes was performed in a 25 µl PCR reaction mixture containing: 5µl of DNA template, each primer concentration of 0.8mM, 12.5µl of master mix (Thermo Scientific, USA) and 3.5µl of RNase free water (Thermo Scientific, USA).

Bacteria PCR conditions consisted of 30 cycles (initial denaturation at 94 ^{o}C for 3 minutes, second denaturation step at 94^{o}C for 30 seconds, annealing at 55 ^{o}C for 1 minute, extension at 72 ^{o}C for 1 minute and final extension at 72 ^{o}C for 10 minutes (Mukisa et al., 2012). Yeast and moulds PCR conditions consisted of 34 cycles, denaturation at 94^{o}C for 4 minutes, second denaturation at 94 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 1 minute 30 seconds, extension at 72 ^{o}C for 1 minute and final extension at 72 ^{o}C for 10 minutes (Greppi et al., 2013). The LAB and yeast strains identified from previous studies were used as positive controls while a negative control did not contain any DNA template. The PCR amplicons together with a 1kb DNA ladder (Thermo Scientific, USA) were separated by electrophoresis in 1.0% (w/v) agarose gel and Tris borate-EDTA (1XTBE) buffer stained with Gel green (Thermo Scientific, USA), for 60 minutes. The DNA bands were viewed under UV light and the un-purified PCR products were sent to Inqaba Biotechnical Industries, South Africa for purification and sequencing. The sequences were cleaned and aligned to generate a consensus using BioEdit software (Tom Hall, Carlsbad). The

aligned sequences were then used to search GenBank using Basic Local Alignment search tool (BLAST).

3.6. Screening for bacteriocin activity

The agar well diffusion assay was used to screen for antimicrobial activity. Overnight cultures of indicator strains; *E. coli*, *B. subtilis*, *S. aureus*, *C. perfringens*, *C. albicans* and *A. niger* were grown in nutrient broth at 37 ^{o}C. A lawn of an indicator strain was made by spreading the cell suspension over the surface of Muller Hinton agar (MHA) plates with a cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 6 mm was used to make wells in the agar plates. LAB strains were grown in MRS broth at 37 ^{o}C for 48 hours. Cultures were centrifuged at 13000g for 10 minutes to enable the collection of cell-free supernatant (CFS).

The CFS was used to test for the antimicrobial properties of LAB and was used as a control for bacteriocin production, while the neutralized CFS in which sodium hydroxide (1M NaOH) was added to adjust the pH was used to determine the bacteriocin production by LAB. The CFS and neutralized CFS were filtered through a 0.22 µm pore-size membrane filters and then 80µl of filtered CFS and the neutralized CFS were loaded into the wells of the MHA plates. The plates were kept at 4 ^{o}C for 2 hours, to ensure diffusion of the supernatant fluid into the agar and then incubated at 37 ^{o}C for 24 hours. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. The presence of the inhibition zone by the neutralized CFS indicated the production of bacteriocins or bacteriocins-like compounds (Rehaiem et al., 2016).

3.7. Enzymatic characterization of LAB

The enzymatic profile of LAB were assayed using commercial API-zym (bioMérieux, France) galleries by testing for the activity of the 19 enzymes according to the manufacturers' instructions. The fresh 24h grown culture of each strain was centrifuged at 5000xg for 5 minutes. The pellet was washed with double distilled water and suspended in API suspension medium. The API zym strips were placed in the incubation boxes and the wells were inoculated with the cell suspension. The samples were then incubated at 37 ^{o}C for 4 hours. Then a drop of reagent A and B was added to each well. The enzymatic activity was expressed as number scores, graded from 1 to 5 according to the color reaction chart. When there is no activity, the score was 0, 1-2; low activity, 3; average activity, 4; good activity and 5; strong activity (Stoyanovski et al., 2013).

3.8. Data analysis

All experiments were done in triplicates; means and standard deviation (±SD) of physicochemical properties (pH, a_w, MC and NaCl), microbial counts and bacteriocins inhibition zones from each region were calculated using Microsoft excel 2010 (Microsoft, USA). Kolmogorov-Smirnov test was used for normality test before one way analysis of variance (ANOVA) was used to compare means in Statistical Package for Social Sciences (SPSS) statistics version 22 software. The differences between the means were identified by post hoc test using Fisher's Least Significant Difference (LSD). Pearson correlation was used to asses if there was a relationship between physicochemical properties and microbiota in beef biltong. All analyses were tested at 5% level of significance.

The sequences generated in the study were cleaned and aligned using Bioedit (Tom Hall, Carlsbad), a sequence alignment editor. The aligned sequences were then used to search GenBank using BLAST tool for microbiota identification. The BLAST outputs were presented on a table showing the accession numbers which are unique identifiers for sequences; E values representing statistical indicators of how significant particular matches were (the smaller the E value, the more significant the matches are); Scores which describes the overall quality of an alignment (the higher number corresponds to the highest similarity) and Identities which is an extent to which sequences match in an alignment, expressed as percentages (Donkor, Dayie and Adiku, 2014).

3.9. Research ethics

A written consent was obtained from the butcheries which stated the confidentiality of the traditional methods used for making biltong which was used for educational purpose only. Secondly, the butcheries were coded in numbers for confidentiality. This research was done following the guidelines and research ethics policy of the University of Namibia (2013).

CHAPTER 4: RESULTS

4.1. Processing steps for beef biltong production

According to the description of beef biltong manufacturer's from the Omaheke, Otjozondjupa and Khomas regions, biltong is a ready to eat meat product, produced by dry curing process. The first step in beef biltong production involves the meat selection. The meat from the "silver side" and the "top side" (meat from the buttock or hips) of beef is the mostly preferred for making biltong. Excess fat is trimmed from the meat and then cut into long thin strips (about 2cm thick) to speed up the drying process. After that meat is cured using coriander, black or white pepper, nitrite, salt or vinegar and mixed in a tray. The meat is then refrigerated overnight for the spice to marinade which can then be placed on plastic hooks, air dried using a fan, then biltong will be ready to eat and can be packaged in plastic bags. Variation exists in the spices composition used during marinating which are for beef biltong flavour differentiation by consumers. Further, there is a slight difference in the drying process of beef biltong production, since the drying period may take 2-4 days depending on the type of product the manufacturer would like to achieve. The process involved in beef biltong production from the central regions is represented in Figure 7.

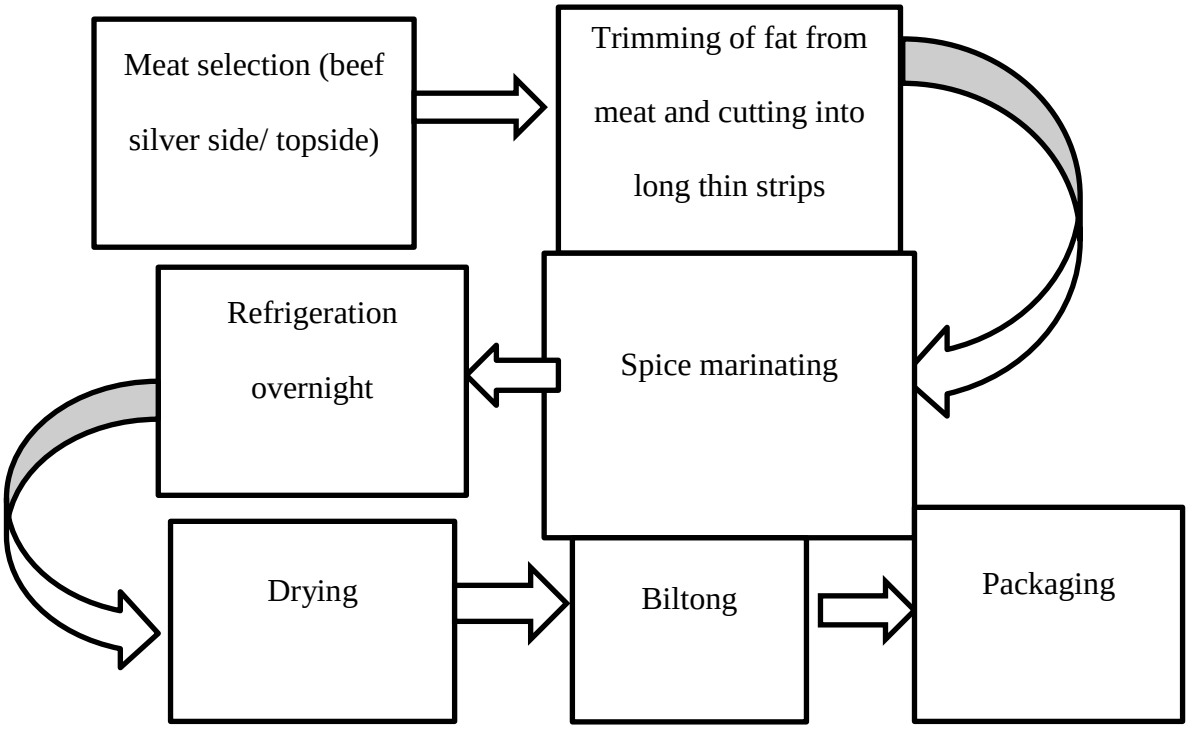

Figure 7: Flow diagram representing the traditional methods of beef biltong production from Otjozondjupa, Omaheke and Khomas regions.

4.2. Physicochemical parameters

The average MC, a_w, NaCl content and pH values of beef biltong samples from Omaheke, Otjozondjupa and Khomas regions are presented on Table 2. The average NaCl content in beef biltong from Omaheke region ranged from 2.6g/100g to 4.8g/100g and from Otjozondjupa ranged from 1.4g/100g to 4.3g/100g, while NaCl content in beef biltong from Khomas region ranged from 2g/ 100g to 3.8g/ 100g (see appendix 2). According to statistics, there was no significant difference between the average NaCl content in beef biltong from all the regions ($P > 0.005$).

The average pH in beef biltong samples from Omaheke region ranged from 4.94 to 5.35 while from Otjozondjupa region, pH ranged from 5.82 to 6.15 and that of Khomas region was found in the range of 5.24 to 5.95. Statistically, the average pH

in beef biltong samples from all the regions significantly differs from each other as ($P < 0.005$).

The average a_w in beef biltong samples from Omaheke region ranged from 0.74-0.9 and the moisture content ranged from 6.32% to 29.87%. On the other hand, the average a_w in the beef biltong samples from Otjozondjupa region ranged from 0.68-0.84 and the MC ranged from 5.88% - 25.47%. The average a_w in beef biltong samples from Khomas region ranged from 0.76-0.83 and the MC was in the range of 8.54% to 33.74%. Statistically, there was no significant difference between the a_w values ($P > 0.005$) in beef biltong samples as well as MC ($P > 0.005$) in beef biltong samples from Omaheke, Otjozondjupa and Khomas regions.

Table 2: Average physicochemical properties ± SD of beef biltong from Omaheke, Otjozondjupa and Khomas regions.

Physicochemical parameters / Regions	**Omaheke**	**Otjozondjupa**	**Khomas**
Average MC (%)	16.46 ±6.7[a]	17.65 ± 7.56[a]	18.86 ± 8.96[a]
Average a_w	0.84± 0.06[a]	0.79 ± 0.05[a]	0.81 ± 0.02[a]
Average NaCl (g/100g)	3.98±0.86[a]	3.16 ± 0.98[a]	3.11 ± 0.62[a]
Average pH	5.14± 0.15[a]	5.98 ± 0.16[b]	5.52 ± 0.27[c]

Means followed from different superscript letters in a same row differ significantly by LSD test at $P<0.005$.

4.3. The microbiology of beef biltong samples

4.3.1. Microbial enumeration in beef biltong samples

Beef biltong samples from Omaheke, Otjozondjupa and Khomas regions were characterized by LAB, yeast and mould isolates. The average total plate counts, average LAB counts and average yeast and moulds counts in beef biltong samples are presented on Table 3. The TPC of beef biltong samples from Omaheke region ranged from 5.4 log CFU/g to 6.4 log CFU/g while the TPC from Otjozondjupa

region ranged from 6. 04 log CFU/g to 7.02 log CFU/g and that of Khomas region ranged from 5.24 log CFU/g to 5.41 log CFU/g. The highest TPC was observed in beef biltong samples from Otjozondjupa region while the lowest was from Khomas region. Statistically TPC differs significantly within the regions ($P < 0.005$).

The LAB counts observed in beef biltong samples from Omaheke, Otjozondjupa and Khomas regions fell in the following ranges respectively: 5.4 log CFU/g to 6.4 log CFU/g, 6.45 log CFU/g to 7.39 log CFU/g and; 5.35 log CFU/g to 6.45 log CFU/g. LAB counts from the central regions significantly differ from each other ($P<0.005$) with a difference being between Otjozondjupa and Khomas as well as between Omaheke and Otjozondjupa (See appendix 2). On the other hand, the yeast and mould counts in beef biltong samples ranged from 5.5 log CFU/g to 6.4 log CFU/g, 5.93 log CFU/g to 6.35 log CFU/g and 4.03 log CFU/g to 6.38 log CFU/g from Omaheke, Otjozondjupa and Khomas regions respectively. Statistically, there was a significant difference ($P<0.005$) between Khomas and Omaheke, as well as between Otjozondjupa and Khomas regions.

Table 3: Microbial characteristics of beef biltong samples from the central regions

Microbial characteristics /Regions	**Omaheke**	**Otjozondjupa**	**Khomas**
Average TPC	5.4 log CFU/g± 0.36	6.46 log CFU/g ± 0.33	5.41 log CFU/g ± 0.06
Average LAB	6. 01 log CFU/g ±0.42^{a*}	7.02 log CFU/g± 0.35	5.66 log CFU/g± 0.41^{a*}
Average Yeast and moulds	5.99 log CFU/g ± 0.35^{a*}	6.14 log CFU/ g± 0.14^{a*}	5.25 log CFU/g ± 0.78

Average microbial count expressed as log CFU/g ± SD, Means followed from same superscript letters (^{a*}) in a same row do not differ significantly by LSD test at $P<0.005$.

4.4. Relationship between microbial counts and physicochemical parameters

Pearson correlation coefficient analysis was calculated to determine if the physicochemical properties had a relationship with the TPC, total LAB as well as total yeast and moulds in beef biltong samples. Total plate count had a positive correlation with pH (r = 0.415), a weak positive correlation with salt content (r = 0.009) as well as MC (r = 0.06). However, a_w had a weak negative correlation (r = -0.030) with TPC. The correlation between TPC and physicochemical parameters was not statistically significant at (P>0.005).

Furthermore, total LAB had a strong positive correlation with pH (r = 0.559) which was significant (P<0.005), a weak positive correlation with moisture content (r = 0.272) and a weak negative correlation with salt content and water activity (r = -0.188 and r = -0.33 respectively) which were all not statistically significant (P>0.005). Moreover, there was a weak positive correlation between total yeast and moulds with salt content and pH (r = 0.075 and r = 0.350 respectively) while a_w and MC had a weak negative correlation with total yeast and moulds counts (r = -0.075 and r = -0.322) respectively. The correlation was not statistically significant (P>0.005).

4.5. Biochemical identification of yeast

A total of 30 yeast isolates from beef biltong samples were selected for identification using API 20 C AUX (bioMerieux, France). The results are shown on Figure 8. Four beneficial yeast species were identified at the genus level as *Candida famata* (5.3%), *Candida guilliermondii* (42.1%), *Candida krusei* (10.5%) and *Candida zeylanoides* (15.8%). However, outliers were also identified as *Trichosporon mucoides* (15.8%) and *Cryptococcus laurentii* (10.5%). A total of six isolates could not be identified by API 20 C AUX.

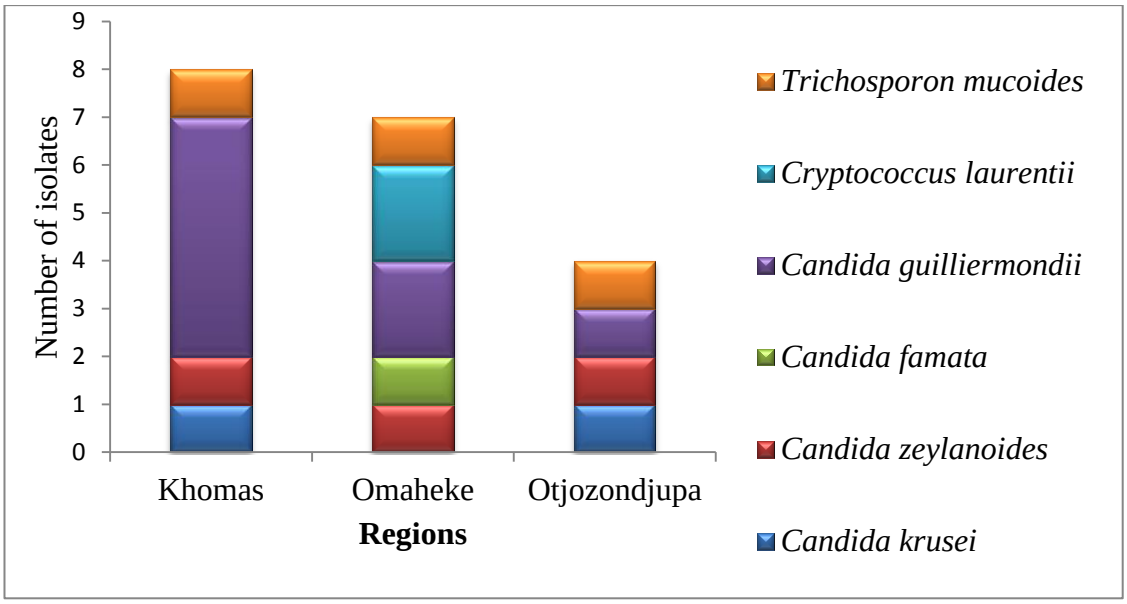

Figure 8: Yeasts identified in beef biltong using API 20 C AUX

4.6. Genetic identification of Yeast and moulds

Fourty (40) beef biltong yeast and mould isolates from Otjozondjupa, Omaheke and Khomas regions were identified via blasting sequenced data from amplified 250bp (Figure 9) and 500bp fragments of the 26S rRNA and ITS genes respectively against nucleotide databases, and identifying each isolate as the organism with the highest similarity sequence (BLAST results shown in table 4). The beneficial yeasts (4 species) were identified as *Candida zeylanoides* (2.5%), *Candida tropicalis* (2.5%), *Meyerozyma guilliermondii* (7.5%) and *Yarrowia lipolytica* (57.5%). Outliers were also identified as *Rhizopus stolonifer* (12.5%), *Candida parapsilosis* (5%) and *Filobasidium unigutulattum* (12.5%). The number of strains identified from each region is represented on Figure 10.

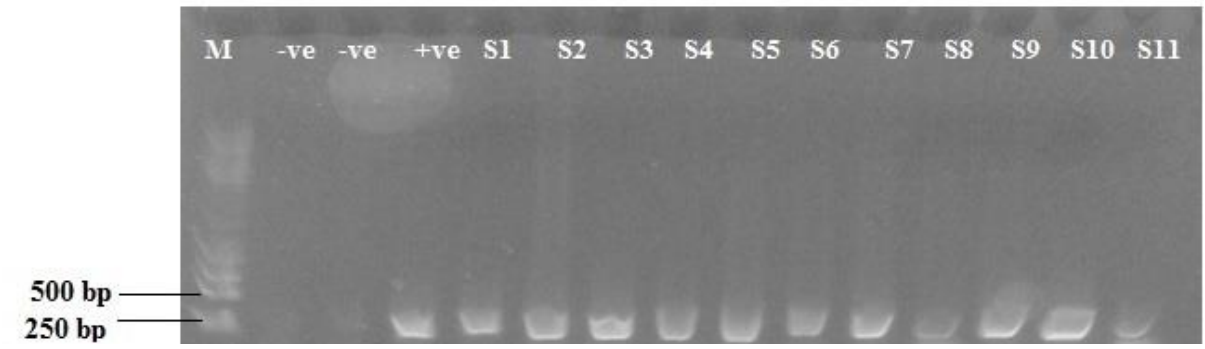

Figure 9: Example of amplified 26S rRNA fragments from yeast and moulds isolates on 1% agarose gel. Lane M: Molecular weight marker 1kb, -ve: negative control, +ve: positive control, S1-S11: yeast and moulds samples 1-11.

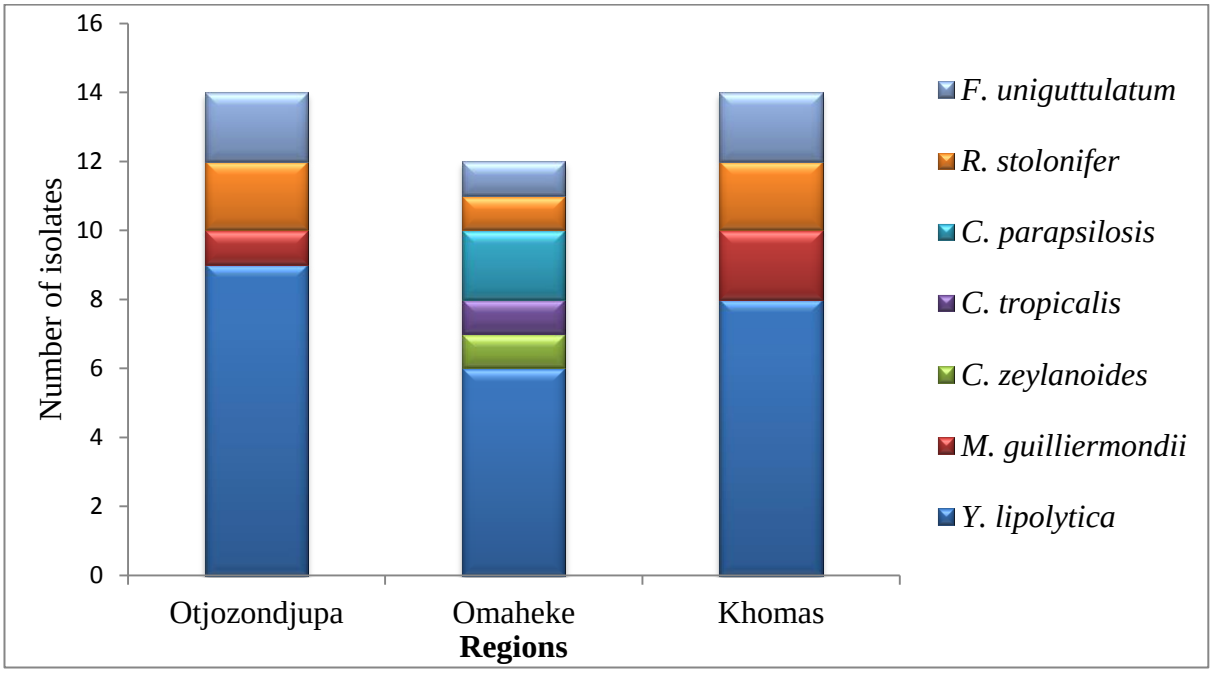

Figure 10: Yeasts and moulds identified in beef biltong from the central regions

Table 4: BLAST results for 26S rRNA and ITS genes sequencing of yeast isolates from beef biltong.

Sequence origin	Most related reference	Scores	E value	Identities	Accession number
1B	*Yarrowia lipolytica* MR	324	5e-85	97%	HM627078.2
2B	*Yarrowia lipolytica* S-1-15	346	1e-91	99%	KP195091.1
4B	*Yarrowia lipolytica* KKUY 0101	348	3e-92	100%	JQ690257.1
5B	*Yarrowia lipolytica* TJY17b	342	1e-90	98%	EU346755.1
7B	*Yarrowia lipolytica* s-3-03	322	2e-84	100%	KP195103.1
11B	*Yarrowia lipolytica* TJY 9b	239	2e-59	93%	EU327112.1
13B	*Yarrowia lipolytica* KKUY 0101	348	3e-92	100%	JQ690257.1
15B	*Meyerozyma guilliermondii*	335	2e-88	95%	KM609507.1
16B	*Yarrowia lipolytica* S-3-03	3111	3e-81	100%	KP195103.1
18C	*Yarrowia lipolytica* S-3-03	409	2e-110	100%	KP21948.1
19B	*Yarrowia lipolytica* KKUY 0101	348	3e-92	100%	JQ690257.1
20B	*Yarrowia lipolytica* WSYC 781	340	5e-90	100%	FR856618.1
26B	*Meyerozyma guilliermondii* EXF-6111	387	7e-104	100%	JF766630.1
26C	*Yarrowia lipolytica* Y-D1P	387	7e-104	100%	KX353828.1
27B	*Candida zeylanoides* W37	372	2e-99	99%	EU131538.1
46B	*Yarrowia lipolytica* KKUY0101	346	1e-91	100%	JQ690257.1
32B	*Yarrowia lipolytica* S-1-15	351	2e-93	100%	KP195091.1
34B	*Meyerozyma guilliermondii* TM3-27	387	7e-104	100%	AB304757.1
35B	*Yarrowia lipolytica* Y-D1P	353	6e-94	100%	KX347554.1
17B	*Yarrowia lipolytica* WSYC	340	5e-90	100%	FR856610.1
21B	*Yarrowia lipolytica* MS29	302	2e-78	99%	JN021559.1
22B	*Yarrowia lipolytica* WSYC 781	350	8e-93	100%	FR856618.1
23B, 24B	*Yarrowia lipolytica* WSYC 781	346	1e-91	100%	FR856618.1
30B	*Yarrowia lipolytica* S-3-03	99	3e-17	91%	KP195103.1

4.7. Biochemical identification of Lactic acid bacteria

The API 50CHL was used to identify 10 LAB isolates from beef biltong samples which were gram positive and catalase negative. They were identified to the genus level using API web following the manufacturer's instruction (bioMerieux, France). Four species of lactic acid bacteria were identified as *Lactobacillus plantarum* 1 (60%), *Lactococcus lactis ssp. lactis* (20%), *Lactobacillus brevis* (1%) and *Leuconostoc mesenteroides ssp. mesenteroides* (1%). Their gram staining results are

represented on Figure 11. The results showed that beef biltong was dominated by *Lactobacillus* species. The numbers of isolated strains from each region are presented on Figure 12.

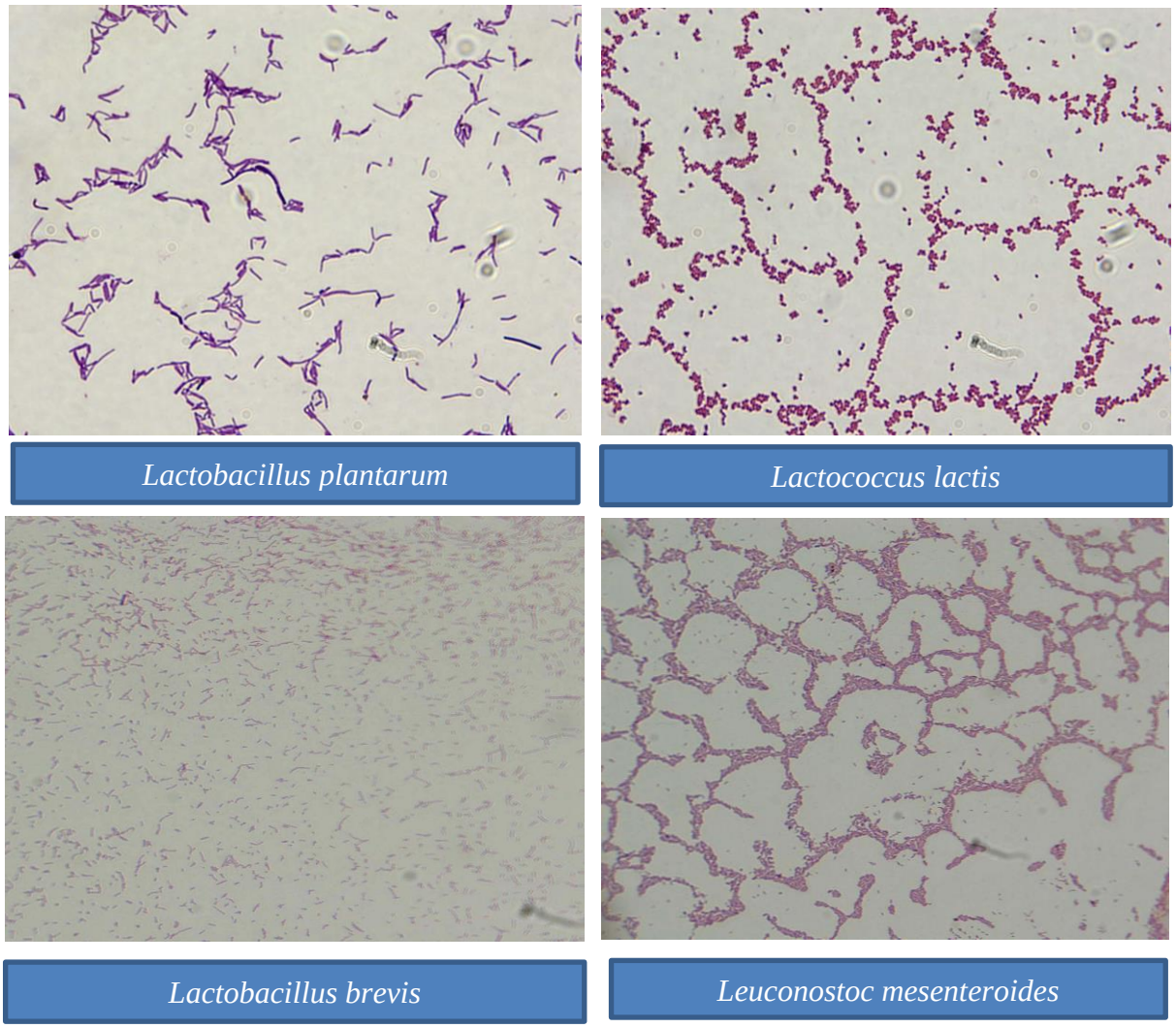

Figure 11: Representation of LAB isolates characterization by gram staining reaction

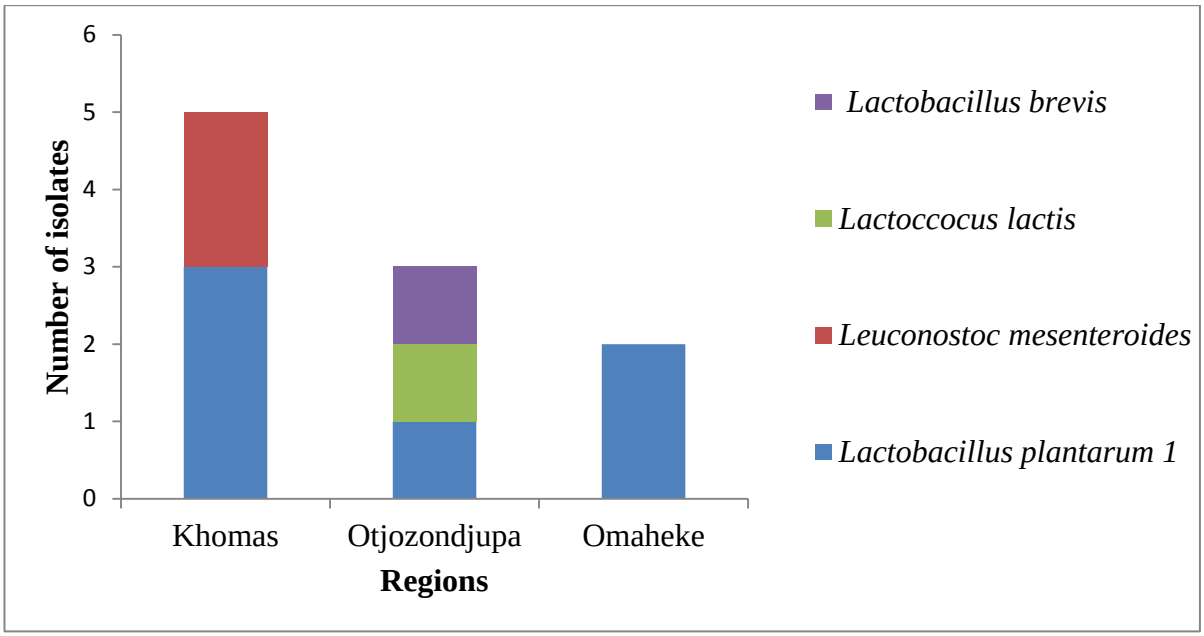

Figure 12: Representation of LAB species identified by API 50CHL

4.8. Genetic identification of LAB

A total of 38 LAB strains were identified to the genus level by sequencing 1500 bp segment of 16S rRNA gene (Figure 13). The sequences were compared to strains in the National Center for Biotechnology Information (NCBI) BLAST library. BLAST sequencing results (sequence similarities Table 5) showed that beneficial LAB strains were *Lactobacillus plantarum* (34.2%), *Weisella cibaria* (13.2%) *Pediococcus pentosaceus* (10.5%), *Pediococcus acidilactici* (13.5%), *Weisella confusa* (2.6%), *Lactobacillus pentosus* (5.3%), *Lactobacillus paraplantarum* (5.3%) and *Lactobacillus graminis* (2.6%). Beef biltong samples were dominated by *Lactobacillus* spp. of which *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Pediococcus acidilactici* and *Weisella cibaria* were the most frequently identified from all the central regions. *Weisella confusa* and *Lactobacillus graminis* were only isolated from Otjozondjupa and Omaheke region respectively. On the other hand, two non-beneficial LAB species were identified as *Enterococcus hirae* (10.5%) and

Enterococcus feacalis (2.6%). The numbers of LAB isolates identified in beef biltong samples from each region are presented on Figure 14.

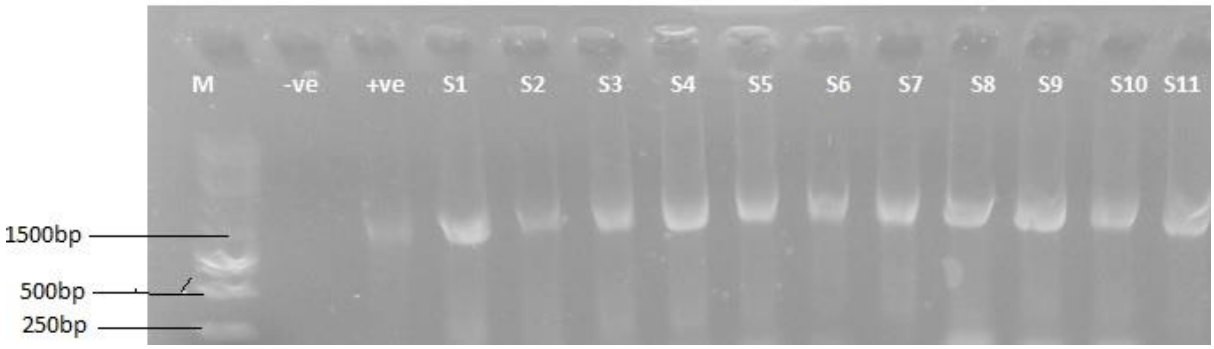

Figure 13: Example of amplified 16S rRNA fragments from LAB isolates in 1% agarose gel. Lane M: Molecular weight marker (1kb), -ve: negative control, +ve: positive control, S1-S11: LAB samples 1-11.

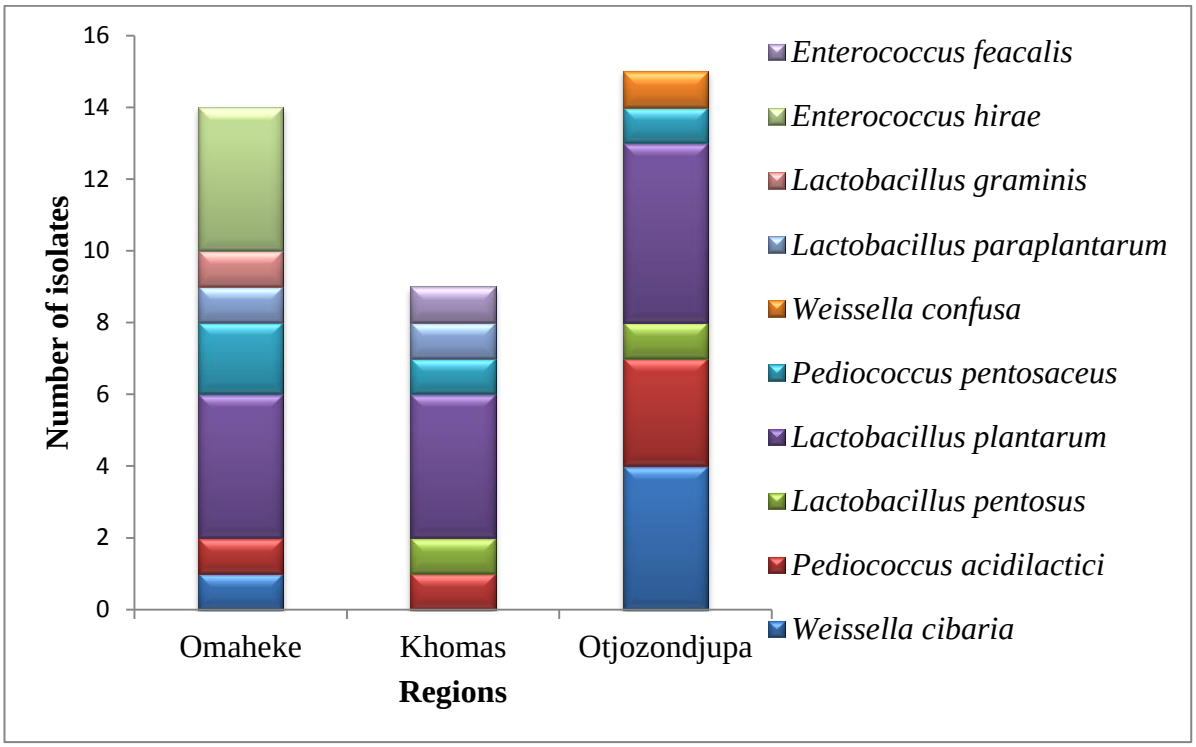

Figure 14: LAB identified by sequencing the 16S rRNA genes isolated from beef biltong samples.

Table 5: BLAST results of the LAB identification based on (1500bp) 16S rRNA gene sequence comparison.

Sequence origin	Most related reference	Scores	E value	Identities	Accession number
1A	*Weissella cibaria* II-I-59	1330	0.0	97%	NR036924.1
2A	*Pediococcus acidilactici* DSM20284	1600	0.0	99%	NR042057.1
3A	*Pediococcus acidilactici* DSM 20284	1600	0.0	99%	NR042057.1
4A	*Lactobacillus pentosus* 124-2	1561	0.0	99%	NR029133.1
5A	*Lactobacillus plantarum* NBRC	1598	0.0	99%	NR1138338.1
6A	*Lactobacillus plantarum* JCM 1149	1541	0.0	99%	NR115605.1
8A	*Pediococcus pentosaceus* DSM 20336	1502	0.0	98%	NR042058.1
10A	*Weissella confusa* JCM 1093	634	0.0	93%	NR113258.1
11A	*Lactobacillus plantarum* NBRC 15891	1600	0.0	99%	NR113338.1
12A	*Pediococcus acidilactici* DSM 20284	1605	0.0	99%	NR042057.1
14A	*Lactobacillus plantarum* NBRC 15891	1592	0.0	99%	NR113338.1
17A	*Weissella cibaria* II-I-59	1225	0.0	99%	NR036924.1
18A	*Pediococcus acidilactici* DSM 20284	1550	0.0	99%	NR042057.1
19A	*Weissella cibaria* II-I-59	1613	0.0	100%	NR036924
21A	*Lactobacillus plantarum* NBRC 15891	1602	0.0	99%	NR113338.1
22A	*Pediococcus pentosaceus* DSM 20336	1428	0.0	99%	NR042058.1
23A	*Pediococcus acidilactici* DSM 20284	1604	0.0	99%	NR042057.1
26A	*Lactobacillus plantarum* NBRC 15891	1604	0.0	99%	NR113338.1
28A	*Pediococcus pentosaceus*	1360	0.0	97%	NR042058.1
34A	*Pediococcus pentosaceus* DSM 20336	1223	0.0	98%	NR042058.1
35A	*Lactobacillus paraplantarum*	1147	0.0	98%	NR025447.1
44A	*Pediococcus acidilactici* DSM 20284	1356	0.0	99%	NR042057.1
46A	*Lactobacillus graminis* G90	1007	0.0	97%	NR042438.1

4.9. Determination of bacteriocin production by lactic acid bacteria

The CFS and NCFS of the LAB strains isolated from beef biltong samples were screened for their antimicrobial activity against 6 food borne pathogens; *E. coli*, *S. aureus*, *B. subtilis*, *C. perfringens*, *A. niger* and *C. albicans* using agar well diffusion method. The antimicrobial activity exhibited by LAB CFS was due to organic acid while those exhibited by the NCFS were due to bacteriocins or bacteriocin-like substances. Examples of the illustration of the antimicrobial activity against indicator strains are shown in Figure 15.

The diameters of zones of inhibition due to antimicrobial activities are presented on Table 6. The results showed that, the CFS from the LAB strains had antimicrobial activity against the indicator strains with exception of *W. confusa* against *C. perfringens*, *L. paraplantarum* against *C. albicans* and *L. brevis* against *A. niger*. In addition, *S. aureus* was the most resistant with only the CFS from *L. plantarum*, *L. lactis*, *L. paraplantarum*, *W. confusa* and *L. pentosus* showing a small zone of inhibition. The biggest inhibition zone (18mm) was observed from *L. plantarum* extracts against *A. niger*.

Antimicrobial activity of most LAB CFS decreased after neutralization with NaOH (as shown on table 6), however; the zone of inhibition from *L. brevis* against *B. subtilis*, *L. lactis* ssp. *lactis* against *E. coli*, *L. plantarum* against *C. albicans* and *W. cibaria* against *A. niger* remained the same. The biggest zone of inhibition after neutralization (16mm) was observed in *L. plantarum* against *A. niger* while the lowest was 1mm observed in most of the LAB strains against 2 or more indicator strains (shown on Table 6). *S. aureus* was the least sensitive indicator strain while *A. niger* was the most sensitive. Their average antimicrobial activities are presented on Figure 16.

Table 6: Showing the inhibition zones (mm) of LAB CFS and NCFS against *E. coli*, *B. subtilis*, *S. aureus*, *C. albicans* and *A. niger* using agar well diffusion method.

Indicator strains	*E. coli*		*B. subtilis*		*S. aureus*		*C. perfringens*		*C. albicans*		*A. niger*	
LAB extracts	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS
L. plantarum	4	5	4	8	1	1	2	8	8	8	16	18
L. brevis	2	3	4	4	0	0	1	2	1	1	1	4
L. mesenteroides ssp. *mesenteroides*	2	4	2	5	0	0	2	4	4	6	4	7
L. lactis ssp. *Lactis*	4	4	2	7	2	2	4	5	4	4	5	7
L. graminis	0	1	2	2	0	0	0	2	2	5	3	9
W. cibaria	3	4	5	6	0	0	2	3	5	7	4	4
L. paraplantarum	4	5	4	5	1	2	1	1	0	0	2	5
W. confuse	1	1	2	3	0	1	0	0	1	2	1	5
L. pentosus	3	4	3	5	1	1	1	3	0	4	2	3
P. pentosaceus	1	12	1	1	0	0	2	3	3	3	4	6
P. acidilactici	2	2	4	6	0	0	2	6	4	4	3	5

NCFS: Neutralized cell free supernatant CFS: Cell free supernatant

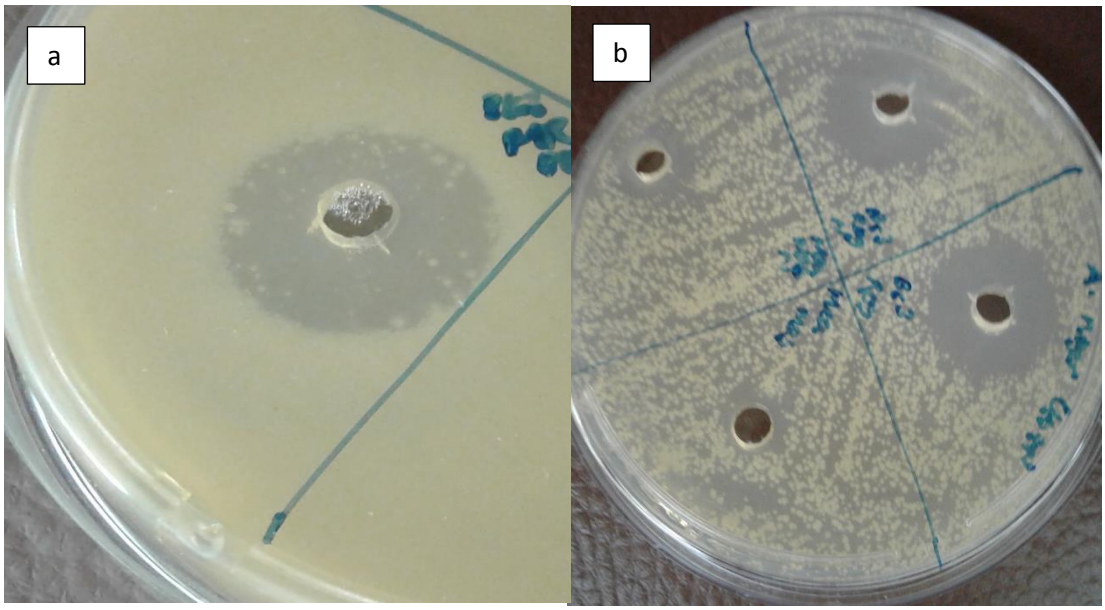

Figure 15: Example of antimicrobial assay a) antimicrobial activity against *E.coli* by *P. pentosaceus* CFS and b) Bacteriocin production against *A. niger* by *L. plantarum* isolated from beef biltong.

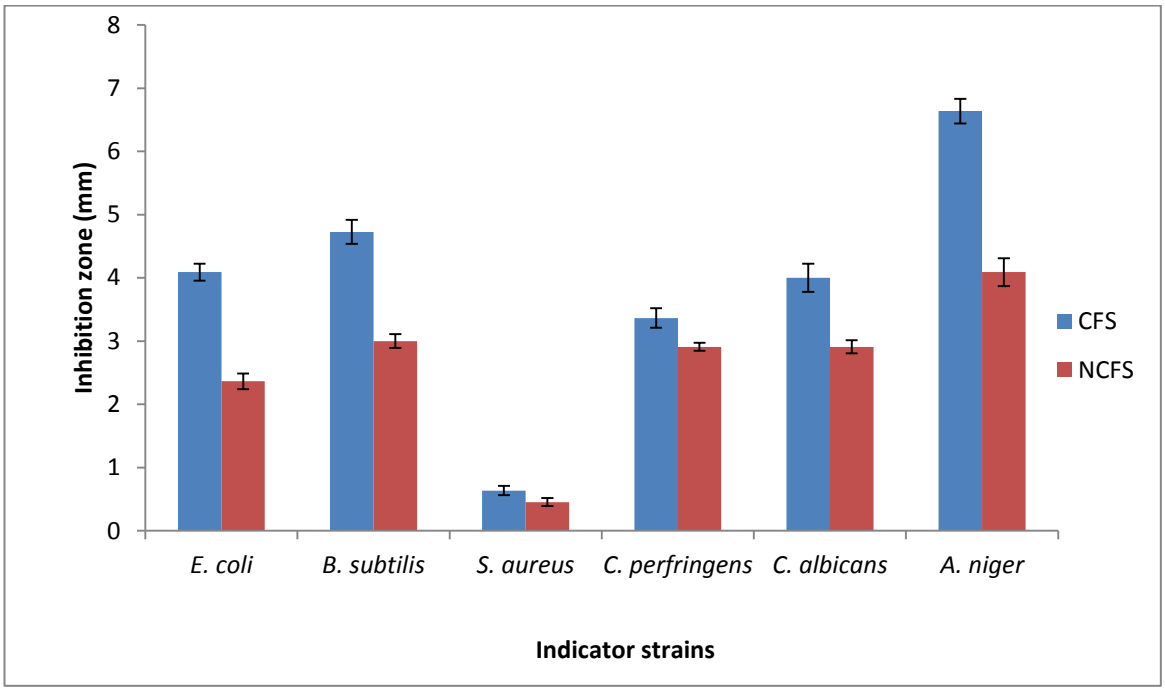

Figure 16: Average antimicrobial activity against indicator strains, *P*> 0.005

4. 10. Enzymatic profile of LAB strains

Enzymatic profiles of LAB strains were assayed using API ZYM (bioMerieux, France) galleries. The results are presented on Table 7. The results showed that, all LAB strains were characterized by strong to good esterase and leucine arylamidase activity. However, esterase-lipase, α-galactosidase, valine arylamidase, cysteine arylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase, lipase, trypsin, alkaline phosphatase and α-chymotrypsin generally showed either good, very weak or no enzymatic activities (as shown on Table 7). Moreover, α-fucosidase and α-mannosidase enzymatic activities were not exhibited by any of the LAB strains tested. Strong enzymatic activities were detected for β-galactosidase (in *L. plantarum*, *L. brevis*, *L. paraplantarum*, and *L. mesenteroides*) and leucine arlylamidase (in *L. mesenteroides and P. pentosaceus*). The activity of β-glucuronidase was only present in *L. brevis* strain.

Table 7: Enzymatic activity (expressed as numbers of scores due to their colour intensity) of predominant LAB strains isolated from beef biltong.

Strains/ Enzymes	1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20
L. plantarum	0	2	3	2	0	4	3	0	0	0	3	2	2	5	0	5	4	4	0	0
L. graminis	0	0	1	0	0	4	3	0	0	0	0	0	0	3	0	4	0	0	0	0
L. paraplantarum	0	0	2	2	0	4	2	2	0	0	2	2	0	5	0	2	2	5	0	0
L. lactis ssp. Lactis	0	1	3	3	0	3	0	1	0	1	4	3	0	0	0	0	0	0	0	0
L. mesenteroides ssp. mesenteroides	0	1	1	0	0	5	3	1	0	0	3	3	0	4	0	2	3	4	0	0
L. brevis	0	0	2	0	0	4	4	3	0	0	5	3	3	5	4	5	4	0	0	0
W. cibaria	0	1	4	3	0	3	1	2	0	0	5	1	0	0	0	0	0	0	0	0
W. confusa	0	1	1	0	0	3	0	0	1	4	0	0	0	0	0	0	0	0	0	0
L. pentosus	0	0	2	2	0	4	3	0	0	0	4	4	0	2	0	3	3	2	0	0
P. pentosaceus	0	0	2	2	0	5	4	4	4	0	0	3	3	0	0	4	4	4	0	0
P. acidilactici	0	0	1	0	4	3	3	0	0	5	3	0	0	0	0	0	0	0	0	0

Color intensity: 0 (no activity) to 5 (strong activity). The numbers in the top rows; 1 is a control and 2-20 correspond to the following enzyme: E2, alkaline phosphatase, E3; esterase, E4;esterase-lipase, E5; lipase, E6; leucine aminopeptidase, E7; valine aminopeptidase, E8; cysteine aminopeptidase, E9; trypsin, E10; chymotrypsin, E11; acid phosphatase, E12; phosphohydrolase, E13; α-galactosidase, E14; β-galactosidase, E15; β-glucuronidase, E16; α-glucosidase, E17; β-glucosidase, E18; α-glucosamidase, E19; α-mannosidase, E20; α-fucosidase

CHAPTER 5: DISCUSSION

5.1. Beef biltong processing methods

The dry curing process of beef biltong production is often passed on from generation to generation (Dzimba et al., 2007) and therefore several basic steps exist for biltong production. The methods of beef biltong production from the central regions is in agreement with the production methods outlined in the study of Naidoo (2010) as well as the methods used in manufacturing biltong in the study of Engez et al. (2012). Beef biltong production steps in Omaheke, Otjozondjupa and Khomas regions are in agreement with each other, however variation exist on the spices used during marinating and the drying periods. The slight differences that exist in the drying periods of beef biltong have an impact on the quality of the final product. This is because, longer drying periods may be associated with dry beef biltong products, which have less water content and low a_w. On the other hand, a shorter drying period will be associated with wet beef biltong products which have higher water content and higher a_w. Therefore these products will possess different microflora and their quality may differ.

During the marinating step, coriander, black pepper, brown sugar, nitrite, vinegar and salt are used. These ingredients may be important in the inhibition of microbial growth due to their antimicrobial properties. The use of vinegar is important in reducing the pH of the product which inhibits microbial growth (Burfoot et al., 2010). The spices are also important in the contribution of flavour and antimicrobial activity against spoilage and pathogenic microorganisms as they have been shown to possess antimicrobial properties (Tajkarimi, Ibrahim and Cliver, 2010).

Nitrite used during marinating could contribute to colour, cured flavour and aroma as well as antioxidant and antimicrobial activity on beef biltong as described in

literature on the importance of nitrite to dried cured meat products (Sullivan, 2011). The use of salt in the processing of beef biltong is important as it aids in the drying process, and contributes to the reduction of a_w and moisture content of the product (Attwell, 2003), which in turn leads to the inhibition of microbial growth on the product. Therefore the spices together with salt and the drying process provide a good hurdle for inhibiting pathogenic and spoilage microorganisms on beef biltong, leading to the stability and safety of the products.

5.2. Physicochemical parameters of beef biltong

Physicochemical parameters such as pH, NaCl content, MC and a_w have an influence on microbial growth. NaCl is commonly used in meat products to inhibit microbial growth by reducing a_w and extend shelf-life of products. The NaCl content of the 36 beef biltong samples tested ranged from 1.7g/ 100g to 4.89g/ 100g which did not differ significantly ($P>0.005$). These data were close to the 3% - 13% range found in other published data (Burfoot et al., 2010) and can be compared to salt content of biltong in the study of Engez et al., (2012) which was among 2.68g/100g - 3.30g/100g.

pH is one of the factors that affect microbial growth in meat products and can enhance the shelf-life of the products. Biltong is reported to be generally microbially stable at the pH values ranging from 4.8-5.8 (Dzimba at al., 2007). In this study, the pH values of beef biltong samples ranged from 4.94-6.15. These data were in accordance with published data where pH was found to range from 4.81-6.26 (Petit et al., 2014; Dzimba et al., 2007; Attwell, 2003; Osterhoff and Leistner, 1984). The low pH values associated with the beef biltong samples may be attributed to the production of lactic acid by the LAB isolated from the products.

The MC and a_w are important elements in food influencing microbial growth. However, a_w may be the best parameter to predict the quality and shelf life of the products (Wolter et al., 2000). The a_w is the amount of water in the product that support microbial growth, with the value ranging from 0 and 1. Biltong can be classified as moist biltong or dry biltong with reference to a higher moisture content and a corresponding higher water activity ranging from 0.85- 0.93 (Petit et al., 2014; Osterhoff and Leistner, 1984).

In the current study, a total of 11 out of 36 beef biltong samples investigated were considered as moist biltong samples due to a higher MC and higher a_w even though there was no statistical difference in a_w values as well as moisture content of beef biltong samples (P> 0.005). Six moist biltong samples were from Omaheke region with MC ranging from 20.04% to 29.87% and a_w in a range of 0.85 to 0.9, two samples were from Otjozondjupa region with the MC ranged from 25.47% to 28.79% and a_w of 0.83 and other three samples were from Khomas region with MC ranged from 30.84% to 33.74% and a_w of 0.82 to 0.83. These data were close to those reported in moist biltong from literature (Petit et al., 2014).

Furthermore, 25 out of 36 beef biltong samples tested were considered as dry biltong samples. This was based on the low a_w ranging from 0.68 to 0.84 with a corresponding MC ranging from 5.88% to 24.85%. The data were closer to those reported by Petit et al. (2014) who found the MC of dry biltong to range from 21.5 to 25.3g/100g and a_w between 0.65 to 0.68 as well as closer to those reported by Van den Heever, 1970 (average MC of 25.2g/100g and average a_w of 0.74). The low MC and reduced a_w associated with beef biltong may be attributed to longer drying periods and presence of salt on the product.

The shelf- stable intermediate moisture foods like biltong are usually defined as having a_w between 0.65 and 0.90, although this classification seems not to be absolute (Dzimba et al., 2007; Nortje et al., 2005; Attwell, 2003, Wolter et al., 2000). The a_w of all the beef biltong samples from Omaheke, Otjozondjupa and Khomas regions fell in that range. The a_w values observed in the study were lower than those required by the growth of most pathogens for example *Salmonellae* require a minimum a_w of 0.93, *E. coli* require a_w of 0.95 and *Listeria monocytogenes* require a_w of 0.92 (USDA, 2005). However, two samples from Omaheke region had a a_w value of 0.9 which is the minimum a_w for the growth of *S. aureus* and this means that the biltong processors should increase their drying process to ensure a reduction of a_w in their final products to prevent the risk of microbial hazards in their products.

5.3. The relationship between microbiological characteristics and physicochemical parameters of beef biltong

Beef biltong from Omaheke, Otjozondjupa and Khomas regions carried a TPC load ranging from 5.24 log CFU/g to 7.02 log CFU/g. These fell in range of microbial loads found in associated with biltong in previous studies (Lindsay and Naidoo, 2010; Wolter et al., 2000). The less TPC of 5.24 log CFU/g associated with biltong may be attributed to the combination of NaCl content, pH, MC and a_w of the commodity. Pearson Correlation Coefficient showed that there was a correlation between physicochemical properties and TPC, meaning that there was a relationship between them even though they were not statistically significant. The physicochemical parameters are reported to control microbial growth (Burfoot et al., 2010; Dave and Ghaly, 2011). Removal of water by the drying process lead to a reduction of a_w within the commodity, low pH in products may disrupt microbial growth, application of NaCl on meat may bind water molecules and reduce a_w, then;

microbial growth on food products may be reduced. It is therefore important that, biltong processors should reduce a_w and maintain low pH of their products to prevent microbial hazards.

The total LAB in the 36 beef biltong samples ranged from 5.35 to 7.39 log CFU/g which indicated high counts of fermentative bacteria associated with biltong and this may also be responsible for the low pH observed in the samples. The yeast and mould counts ranged from 4.03- 6.4 log CFU/g. These microbial ranges were also similar to those reported in the study of Burfoot et al. (2010). The LAB, yeast and moulds in beef biltong samples were found to correlate with the physicochemical parameters by Pearson Correlation and this means that there was a relationship but not statistically significant except the relationship between pH and total LAB which was found significant (P<0.005). Therefore, the presence of LAB, yeast and moulds on beef biltong samples may be due to that the microorganisms were able to tolerate the low pH, reduced MC, presence of NaCl and low a_w that was associated with the biltong samples.

According to literature, most microbial growth ceases at elevated salt content, low pH values and reduced water activity; however, microbes such as LAB, yeast and moulds are able to thrive in such conditions, for example yeast was found to grow at a minimum a_w of 0.6 (Dave and Ghaly, 2011; Wolter et al., 2000). Therefore this may explain the dominance of the microbiota isolated in the present study. Furthermore, the existence of yeast with a high count of LAB observed in the study may also indicate the synergistic relationship between the microorganisms.

5.4. Dominant microbiota associated with beef biltong

The dominant LAB that were identified in beef biltong samples belonged to *L. plantarum*, *P. acidilactici*, *P. pentosaceus*, *L. pentosus*, *L. paraplantarum*,

Leuconostoc mesenteroides ssp. *mesenteroides*, *L. lactis* ssp. *lactis*, *L. brevis*, *L. graminis*, *W. confusa* and *W. cibaria*. These LAB have been isolated in meat and vegetable products in previous studies and some such as *P. acidilactici*, *P. pentosaceus*, *Lactococcus lactis* and *W. cibaria* are reported to be used in food fermentation as starter cultures or as indigenous microflora in natural or controlled fermentations. They may contribute to flavour, texture and nutritional attributes to products (Crowley et al., 2013; Taheri et al., 2011). Some strains are also known as potential producers of antimicrobial metabolites such as organic acids and bacteriocins (Reis et al., 2012) therefore they can be used as alternatives for improving food safety and biopreservation.

LAB such as *L. plantarum*, *P. pentoaceus*, *L. pentosus*, *Leuconostoc mesenteroides*, *L. lactis*, *L. brevis*, *W. confusa* and *W. cibaria* are considered as probiotic source of food production. Therefore, they can be applied in production of foods with health benefits and in shelf life extension of foods. According to literature LAB such as *W. confusa*, *Leuconostoc* and *W. cibaria* are able to produce exopolysaccharides with anticancer and immune modulating functions (Park et al., 2013). It was demonstrated that, *W. cibaria* and *L. plantarum* in Kimchi possess anticancer activities (Kwak et al., 2014). This means that, these LAB isolated from beef biltong may also be applied in the development of functional foods.

Yeast and moulds are reported as part of natural microflora of meat (Wolter et al., 2000). The presence of yeast on beef biltong may be attributed to their ability to tolerate low pH, high salt content and low a_w. Biochemical and genetic identification of yeast isolates from beef biltong samples showed that, the commodities were dominated by *Y. lipolytica*, *C. guilliermondii*, *C. zeylanoides*, *M. guilliermondii*, *C. krusei*, *C. tropicalis* and *C. famata*. These findings are in agreement with Matsheka

et al. (2014) who isolated *Candida famata*, *C. zeylanoides* and *Y. lipolytica* from biltong. These results can be compared to other findings on dry cured meat products by Ozturk (2015) who isolated *C. zeylanoides* and *Y. lipolytica* from Pastirma; a Turkish dry cured meat product. The dominance of genus *Candida* in the present study is also in accordance with the published data (Matsheka et al., 2014).

Although the genus *Candida* is reported to consist of opportunistic pathogens, some strains may have applications in food industries. *C. krusei* is reported to contribute to fermentations of food. Yeasts may produce antimicrobial metabolites or bind bacteria on their surface therefore inhibiting spoilage or potentially pathogenic microorganisms (Fleet, 2006). Furthermore, *C. guilliermondii* is reported to be permitted by Food and Drug Administration (FDA) to be used in food for human consumption as Secondary Direct Food additives in the production of citric acid while *C. tropicalis* has applications in food industry as it can produce xylitol, a product of great economic interest due to its anticariogenicity and sweetening properties which have been exploited in food production (Lima, Filipe and Torres, 2003). However, similar yeasts *(C. krusei* and *C. tropicalis*) have been isolated in cancer patients (Darko et al., 2001) even though causal connection was not established and such possibilities were not investigated in the current study. Therefore further study needs to be done on the properties of the isolated strains. *M. guilliermondii* is known for the production of flavour compounds in fermented food products. In a study with soybean paste fermentation, *M. guilliermondii* was found to efficiently produce isoflavone aglycone which is a widely known bioactive compound for its various health promoting functions (Romi et al., 2014).

Further, yeasts are reported to contribute to the formation of characteristic flavours and aroma of products through the enzymatic activities of lipases and proteinases

(Concconcelli and Fontana, 2015; Ozturk, 2015). For example, *C. zeylanoides* and *Y. lipolytica* were investigated in the study of Ozturk (2015) and were found to exhibit lipolytic activity as well as proteolytic activity. The use of *C. zeylanoides* is also reported in production of lacon, a dried cured meat product whereby its effects on aroma of lacon were investigated (Purrinos, Carballo and Lorenzo, 2013). *Y. lipolytica* is also reported for aroma formation in munster and parmesan cheeses (Bourdichon et al., 2012). Therefore the presence of yeast in beef biltong may be important in the contribution to aroma and flavour of the products and the strains may also have an application in the production of other dried cured meat products and fermented food as starter cultures.

However, some of microorganisms identified in beef biltong may not play a beneficial role in it. They were identified as *Enterococcus feacalis*, *Enterococcus hirae*, *Stephanoascus ciferii*, *Trichosporon* mucoides, *Cryptococcus laurentii*, *Rhizopus stolonifer*, *Candida parapsilosis* and *Filobasidium unigutulattum*. Their presence in beef biltong samples may be due to contamination or unhygienic equipment used during slaughtering or manufacturing process. This means that biltong manufacturers should consider hygienic practices to prevent microbial hazards associated with their products.

5.5. Bacteriocin production

LAB are known to produce antimicrobial substances that exert antagonistic activity against gram positive and gram negative bacteria as well as fungi (Reis et al., 2012). The antimicrobial substances include metabolites such as organic acids, hydrogen peroxide and bacteriocins. These may explain the varying degrees of inhibitory activity (Table 6) exhibited by the LAB strains in the present study against *E. coli*, *B. subtilis*, *C. perfringens*, *A. nige*r, *C. albicans* and *S. aureus* (most resistant). Some

strains showed a higher activity than others. These findings can be compared to those of Heita (2014) who observed antimicrobial activity of LAB CFS against *E. coli*, *Candida famata*, *Geotrichum klebahnii* and *Bacillus cereus*.

After neutralization of CFS, antimicrobial activity continued to be observed in all the LAB strains against two or more indicator strains with a varying degree (Table 6). The production of antimicrobial activity after neutralization of CFS means that, LAB strains may indicate potential production of bacteriocins or bacteriocin like substances. The exhibition of antimicrobial activity by NCFS of LAB strains were also observed in the study of Georgieva et al. (2015) who found antimicrobial activity against *E. coli*, *S. aureus* and *B. cereus* as well as Taheri et al. (2011) who reported on the antimicrobial activity of *Lactococcus lactis* and *Enterococcus* ssp. against *S. aureus*. The antimicrobial activity exhibited by NCFS in the present study showed that, the activity was not just the result of organic acids but may also be due to bacteriocin or bacteriocin-like substances.

Furthermore; there was no change in antimicrobial activity in *L. brevis* against *B. subtilis*, *L. lactis* ssp. *lactis* against *E. coli*, *L. plantarum* against *C. albicans* and *W. cibaria* against *A. niger*. These may suggest that activity was due to bacteriocins or bacteriocin-like substances. The LAB species isolated in the present study like *L. lactis* ssp. *lactis*, *Pediococcus* ssp. and *Lactobacillus* ssp. (example *L. plantarum*) have been reported as potential producers of bacteriocin (nisin, pediocin and plantaricin) respectively (Gyawali and Ibrahim, 2014; Suskovic et al., 2010). Therefore the antimicrobial activity exhibited by LAB NCFS in the study may be due to bacteriocins or bacteriocin-like substances which are a beneficial characteristic as the strains can be used in food biopreservation or as probiotic.

5.6. Enzymatic profiling

The LAB isolates were screened for enzymatic activities. The enzymatic activities detected include esterase-lipase, valine arylamidase, cysteine arylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase, esterase, leucine arylamidase, β galactosidase and α-galactosidase. These results were in agreement with those in literature (Mechai et al., 2014; Pisano et al., 2014; Bassyouni et al., 2012). The absence of α-mannosidase and α-fucosidase activity in the LAB strains is consistent with the findings of Arias et al. (2014). The absence of β- glucuronidase in LAB strains means that those strains could be used as probiotic flora for humans; however the *L. brevis* strain which was found to possess β-glucuronidase activity cannot be used as probiotic due to the carcinogenic property of the enzyme (Mechai et al., 2014). The exhibition of phosphatase activity by LAB strains indicated that the strains could have a possible role in phytic acid degradation in fermented products (Abdelbasset et al., 2014).

L. plantarum, L. brevis, L. paraplantarum and *L. mesenteroides* strains were found to exhibit β-galactosidase activities and this could suggest that they can be used as probiotic because they may be suitable for lactose intolerant patients. Good and strong enzymatic activities were also detected in leucine arylamidase, valine and cysteine arylamidase among LAB strains screened. These are important technological characteristic of LAB strains that may be intended for use as starter cultures for the manufacture of fermented products as they can contribute to the catabolism of proteins and peptides generating free amino acids which are precursors of flavor compounds in the final product (Mora et al., 2015; Ammor et al., 2005). Moreover, the low proteinase, low esterase-lipase activities and high peptidase activities that were observed in *L. plantarum, L. lactis, W. cibaria, L. paraplantarum,*

L. pentosus and *P. pentosaceus* means that the strains may be useful in reducing bitterness, enhance flavor and texture of food products (Arias et al., 2014; Georgieva et al., 2009).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Beef biltong is a commonly consumed meat product in Namibia and it is produced by dry curing process. The beef biltong production methods differs from each other in terms of spices used during marinating and drying periods, therefore beef biltong products differs among the regions. The physicochemical characteristics; pH, NaCl, a_w and MC were found to have a relationship with microbial characteristics, therefore they may have an effect on microbial growth of beef biltong. Some of the beef biltong samples that were found to have a higher TPC and higher a_w did not comply with standard hygienic quality, therefore; biltong producers need to take the hygienic practices into consideration to prevent microbial hazards and improve the safety of the products.

Based on the findings of the current study, beef biltong may contain beneficial yeasts and LAB with important functions. The LAB were represented by *Lactobacillus brevis, L. graminis, L. plantarum, L. pentosus, L. paraplantarum, Lactococcus lactis* ssp. *lactis, Leuconostoc mesenteroides* ssp. *mesenteroides, Pediococcus acidilactici, P. pentosaceus, Weisella cibaria,* and *W. confusa* of which *Lactobacillus* ssp. was predominating in all the regions. Beneficial yeasts were represented by *Candida zeylanoides, C. guilliermondii, C. famata, C. krusei, C. tropicalis, Meyerozyma guilliermondii* and *Yarrowia lipolytica* of which *Y. lipolytica* was the predominant one in all the regions. LAB strains were found to exhibit important functional properties with industrial potentials such as enzymatic activities and antimicrobial properties which was due to organic acids and after pH neutralization of the CFS, the activity was due to bacteriocins or bacteriocins-like substances. The use of bacteriocins or LAB bacteriocin producing strains in food industries may help to

replace the use of chemical preservatives, resulting in natural preserved food with improved organoleptic properties produced. Therefore, LAB strains with important functions may be used as starter cultures in fermented foods in order to improve their quality and safety.

6.2. Recommendations

It is recommended that, further studies are needed to be done on the isolation, identification and characterization of the bacteriocins producing LAB strains. The use of bacteriocins or bacteriocins producing LAB associated with food may provide a novel or innovative approach of food biopreservation against spoilage or pathogenic microorganisms. Furthermore, technological properties of beneficial yeasts such as proteolytic activity, lipolytic activity and growth under different conditions (NaCl, temperature and pH) are needed to be studied to evaluate if the strains can be used as starter cultures in food fermentation.

On biltong producers, the presence of beneficial microorganisms in the studied beef biltong samples is an indicator to their safety and quality in terms of organoleptic properties. This is because LAB is well known to contribute to the flavour and aroma of meat products. Therefore, a feedback to these producers is recommended.

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Appendices

Appendix 1

1. Procedures for DNA extraction using ZYMO Bacterial or Fungal DNA Extraction Kit

1. Fungal/ bacterial cells were suspended in 200µl of phosphate buffered saline (PBS) and transferred into a ZR bashingBead lysis tube.
2. The lysis solution (750 µl) was added into the tube followed by voltexing at high speed for 15 minutes.
3. The ZR bashingBead lysis tube was centrifuged in a microfuge (Eppendorff, USA) at 10,000×g for 1minute.
4. The supernatant (400 µl) was transferred to a Zymo-spin IV spin filter in a collection tube and centrifuged at 7,000 rpm for 1 minute.
5. Then, 1200 µl of fungal/ bacterial DNA binding buffer was added to the filtrate in the collection tube from step 4.
6. The mixture from step 5 (800 µl) was transferred to a Zymo-spin^{TM} 11C colum in a new collection tube and centrifuged at 10000g for 1minute.
7. The flow through from the collection tube was discarded and step 6 was repeated.
8. Then 200 µl of DNA pre-Wash Buffer was added to the Zymo-spin TM 11C column in a new collection tube and centrifuged at 10000g for 1 minute.
9. Funga/Bacterial DNA Wash Buffer (500 µl) was then added to Zymo-spinTM 11C column and centrifuged at 10000g for 1 minute.
10. The Zymo-Spin 11C column was transferred to a clean 1.5 ml microcentrifuge tube followed by the addition of 100 µl DNA Elution Buffer

directly to the column matrix and centrifuged at 10000g for 30 seconds to elute the DNA.

11. The resulting DNA was quantified at wavelength range 260nm to 280nm in the Thermo nanodrop 2000 (Thermo Scientific, Wash., D.C., USA) and stored at -20^{o}C for PCR assays.

2. Gram staining procedures

1. A loopful of a freshly prepared bacterial colony was transferred to a clean microscope slide followed by the addition of a drop of double distilled water and spread in a circle on a slide.
2. The slide was then heat fixed by passing through a flame 3 times.
3. The slide was then flooded with a primary stain (crystal violet) for 1 minute and rinsed with water.
4. Gram's iodine solution was then used to flood the slide for 1 minute and rinsed with distilled water.
5. The slide was then decolourized using ethanol for 3 seconds and rinsed with distilled water.
6. They were then flooded with secondary stain (Safranin) for 1 minute and rinsed with distilled water.
7. Lastly, the slide was air dried and viewed under light microscope (Olympus BX%!, Japan) using oil immersion.

3. Catalase test

A drop of 3% solution of hydrogen peroxibe (H_2O_2) was placed on a clean microscope slide and a 24hour old culture colony from MRS, Rogosa, and M17 plate

were picked using a sterile inoculating loop. The production of bubbles indicated the catalase positive test.

Appendix 2

1. Average microbial counts and physicochemical parameters in beef biltong from Otjozondjupa region

Sample ID	LAB (log cfu/g	TPC (log cfu/g)	Yeast and moulds (log cfu/g)	NaCl (g/100g)	pH	a_w	MC (%)
S25	7.23	6.4	6.29	1.7	6.15	0.83	25.47
S26	7.24	6.31	6.11	2.1	6.06	0.83	28.79
S27	7.23	6.24	6.14	1.4	6.2	0.84	18.63
S28	6.99	6.4	6.1	3.8	5.82	0.81	16.13
S29	6.97	6.39	6.13	3.2	5.83	0.82	20.5
S30	7.02	6.37	6.12	3.9	5.84	0.8	17.95
S31	7.39	6.95	6.35	3	6.09	0.79	24.85
S32	7.37	7.02	6.29	3.3	6.12	0.8	23.7
S33	7.31	6.95	6.27	3	6.14	0.81	14.27
S34	6.45	6.04	5.93	4.2	5.84	0.73	7.85
S35	6.54	6.27	5.97	4.3	5.83	0.71	7.82
S36	6.46	6.13	5.95	4	5.85	0.68	5.88

2. Average microbial counts and physicochemical parameters in beef biltong from Omaheke region

Sample ID	TPC (log cfu/g)	LAB (log cfu/g)	Yeast and moulds (log cfu/g)	NaCl (g/100g)	pH	a_w	MC (%)
S1	5.4	5.5	5.6	2.7	5.31	0.9	25.17
S2	6.1	6.4	6.3	2.6	5.33	0.89	25.07
S3	6.2	6.3	6.4	2.6	5.24	0.9	14.83
S4	5.5	5.5	5.6	4.6	5.22	0.75	9.41
S5	6.2	6.3	6.2	4.6	5.35	0.74	8.81
S6	6.1	6.2	6.2	4.5	5.16	0.76	11.76
S7	5.4	5.4	5.5	4.8	5.08	0.84	6.32
S8	6.1	6.3	6.1	4.7	5.02	0.84	7.55
S9	6.1	6.4	6	4.6	5.06	0.85	12.63
S10	5.5	5.4	5.5	4.05	4.94	0.89	26.09
S11	6.3	6.2	6.1	4.1	5	0.88	29.87
S12	6.2	6.2	6.4	3.9	4.96	0.89	20.04

3. Average microbial counts and physicochemical parameters in beef biltong from Khomas region

Sample ID	LAB(log cfu/g)	TPC (log cfu/g)	Yeast and moulds (log cfu/g)	NaCl (g/100g)	pH	a_w	MC (%)
S13	6.45	5.41	4.03	3.5	5.36	0.83	33.74
S14	6.24	5.4	4.23	3.4	5.37	0.82	30.84
S15	6.3	5.34	4.25	3.2	5.34	0.83	31.68
S16	5.44	5.25	5.21	2	5.26	0.81	8.54
S17	5.37	5.32	5.22	2.3	5.24	0.81	8.62
S18	5.47	5.25	5.26	2.2	5.25	0.8	11.2
S19	5.35	5.32	6.16	3.8	5.95	0.77	18.28
S20	5.5	5.28	6.28	3.5	5.92	0.78	14.86
S21	5.46	5.28	6.38	3.7	5.94	0.76	10.7
S22	5.45	5.27	5.33	3.5	5.54	0.81	18.58
S23	5.4	5.24	5.32	3.4	5.55	0.82	19.6
S24	5.48	5.27	5.37	2.8	5.55	0.82	19.63

4. Statistical data generated using SPSS software

One way ANOVA

Bacteria count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.990	2	4.995	28.835	.000
Within Groups	5.717	33	.173		
Total	15.707	35			

Multiple Comparisons for total bacteria count in post hoc

Dependent Variable: Bacteria count

LSD

(I) Region	(J) Region	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Omaheke	Otjozondjupa	-.9117*	.1699	.000	-1.257	-.566
	Khomas	.3350	.1699	.057	-.011	.681
Otjozondjupa	Omaheke	.9117*	.1699	.000	.566	1.257
	Khomas	1.2467*	.1699	.000	.901	1.592
Khomas	Omaheke	-.3350	.1699	.057	-.681	.011
	Otjozondjupa	-1.2467*	.1699	.000	-1.592	-.901

*. The mean difference is significant at the 0.05 level.

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
yeast and molds	Between Groups	5.393	2	2.696	10.759	.000
	Within Groups	8.270	33	.251		
	Total	13.663	35			
Salt content	Between Groups	5.738	2	2.869	4.148	.025
	Within Groups	22.826	33	.692		
	Total	28.564	35			
PH content	Between Groups	4.262	2	2.131	52.759	.000
	Within Groups	1.333	33	.040		
	Total	5.594	35			
water activity	Between Groups	.020	2	.010	4.374	.021
	Within Groups	.076	33	.002		
	Total	.096	35			
Moisture content	Between Groups	45.824	2	22.912	.299	.744
	Within Groups	2532.153	33	76.732		
	Total	2577.977	35			
lactic acid B	Between Groups	9.438	2	4.719	37.851	.000
	Within Groups	4.114	33	.125		
	Total	13.552	35			

Multiple comparisons by LSD test in post hoc

Dependent Variable	(I) Region	(J) Region	Mean Difference (I-J)	Sig.
yeast and molds	Omaheke	Otjozondjupa	-.1458	.481
		Khomas	.7383*	.001
	Otjozondjupa	Omaheke	.1458	.481
		Khomas	.8842*	.000
	Khomas	Omaheke	-.7383*	.001
		Otjozondjupa	-.8842*	.000
Salt content	Omaheke	Otjozondjupa	.82083*	.021
		Khomas	.87083*	.015
	Otjozondjupa	Omaheke	-.82083*	.021
		Khomas	.05000	.884
	Khomas	Omaheke	-.87083*	.015
		Otjozondjupa	-.05000	.884
PH content	Omaheke	Otjozondjupa	-.84167*	.000
		Khomas	-.38333*	.000
	Otjozondjupa	Omaheke	.84167*	.000
		Khomas	.45833*	.000
	Khomas	Omaheke	.38333*	.000
		Otjozondjupa	-.45833*	.000
water activity	Omaheke	Otjozondjupa	.05667*	.007
		Khomas	.03917	.054
	Otjozondjupa	Omaheke	-.05667*	.007
		Khomas	-.01750	.379
	Khomas	Omaheke	-.03917	.054
		Otjozondjupa	.01750	.379
Moisture content	Omaheke	Otjozondjupa	-2.39333	.508
		Khomas	-2.39333	.508
	Otjozondjupa	Omaheke	2.39333	.508
		Khomas	.00000	1.000
	Khomas	Omaheke	2.39333	.508
		Otjozondjupa	.00000	1.000
lactic acid B	Omaheke	Otjozondjupa	-.6275*	.000
		Khomas	.6267*	.000
	Otjozondjupa	Omaheke	.6275*	.000
		Khomas	1.2542*	.000
	Khomas	Omaheke	-.6267*	.000
		Otjozondjupa	-1.2542*	.000

Pearson correlation statistical analysis results

Correlations

		Salt content	PH content	water activity	Moisture content
Bacteria count	Pearson Correlation	.009	.415*	-.030	.060
	Sig. (2-tailed)	.959	.012	.860	.727
	N	36	36	36	36
lactic acid B	Pearson Correlation	-.188	.559**	-.033	.272
	Sig. (2-tailed)	.273	.000	.849	.108
	N	36	36	36	36
yeast and molds	Pearson Correlation	.075	.350*	-.075	-.322
	Sig. (2-tailed)	.664	.036	.662	.055
	N	36	36	36	36

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).p

Appendix 3

INFORMED CONSENT FOR SAMPLING AND KNOWLEDGE ON BEEF BILTONG PROCESSING METHODS

Researcher's Name: Maria Angula,University of Namibia Biological Sciences Department, Email: mariaangula48@gmail.com

Supervisor's Name: Dr. Ahmad Cheikhyoussef,University of Namibia, Multidisciplinary Research Centre, tell:, Email: acheikhyoussef@unam.na

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Dear participant

We would appreciate your assistance with this research project on identification and characterization of beneficial microbiota in beef biltong from Namibian central regions.

The purpose of this study is: 1. To determine the main physico-chemical parameters which affect microbial growth such as pH, water activity, moisture content and sodium chloride concentration of beef biltong samples. 2. To identify the LAB, yeast and molds associated with beef biltong samples using culture dependent and independent methods. 3. To profile the enzymatic activities of LAB isolates and screen them for bacteriocin production. 4. To document the indigenous knowledge on the traditional methods of making beef biltong from Namibian central regions.

Therefore we would like to purchase the samples from your butchery for analysis and to obtain an explanation on the steps that are involved in the production of beef biltong.

The results will be used for educational purposes only and not for any sort of commercialization. Confidentiality will be maintained. All the butcheries will be encoded in numbers in all research notes. The results will be provided to the production points upon request.

CONSENT

I have read and I understand the provided information and have had the opportunity to ask questions. I understand that I will be given a copy of this consent form. I voluntarily agree to take part in this study.

Participant's signature _____ Date _____

Researche's signature _____ Date _____

Supervisor's signature _____ Date _____

Appendix 4

QUESTIONNAIRE ON THE PROCESSING STEPS FOR BEEF BILTONG PRODUCTION

Researcher's Name: Maria Angula,University of Namibia Biological Sciences Department, Email: mariaangula48@gmail.com

Supervisor's Name: Dr. Ahmad Cheikhyoussef,University of Namibia, Multidisciplinary Research Centre, tell:, Email: acheikhyoussef@unam.na

1. Production point number: .....
2. Region.....
3. Name of the selling point.....
4. Steps involved in beef biltong production.....
5. Ingredients used.....
6. How long does it take for biltong to be ready for consumption?.....

Preservation

technique.....