A SURVEY OF PROPIONIC ACID BACTERIA AND THE OPTIMISATION OF VITAMIN B12 IN MOTOHO, AN AFRICAN BEVERAGE

by

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DECLARATION

I, Letlotlo Nyenye, declare that the contents of this dissertation represent my own unaided work, and that the thesis/dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date: 13 December 2023**

ABSTRACT

Cereal-based fermentation is an extensive, common and popular practice across the African continent. The variety of fermented food products and beverages produced by means of sorghum fermenting is testament to the practice. Motoho is a sour, porridge-like snack beverage made from brown or red sorghum. Motoho is produced by means of spontaneous fermentation by back slopping with tomoso (starter culture). Lactic acid bacteria (LAB) are reported to be the most dominant microorganisms in spontaneous cereal fermentation, followed by yeasts. Vitamin B12 (vit B12) deficiency is reported to have negative health effects, and therefore, fortification by means of cereal fermentation with Generally Recognised as Safe Organisms (GRAS) has been proposed as a method for addressing vitamin deficiencies. The vitamin content of motoho, especially vit B12 is unknown. *Propionibacterium freudenreichii*. *P. freudenreichii* biosynthesises vit B12 naturally and has GRAS status. The aim of this study was to isolate *Propionibacterium* spp. present in motoho, and optimise the production of vit B12 by means of co-inoculating tomoso with *P. freudenreichii* subsp *shermanii* (PAB-J17) at low and high concentrations. The traditional method (back-slopping with tomoso) being utilised as the control.

Motoho was prepared using two methods: (i) the traditional method (TFM) (back-slopping with tomoso) and (ii) by co-inoculating tomoso with *P. freudenreichii* (PABJ-17) in a low (LPAB-J17) and high (HPAB-J17) cell concentrations, respectively. Fermentation was facilitated at 32 °C for 12 h with sampling performed every 3 h. The pH was monitored and the following organisms were enumerated: Total Bacterial Counts, coliforms, lactic acid bacteria (LAB), yeasts and moulds. LAB were the most dominant organisms during the whole fermentation process, with cell counts obtained for TFM being significantly higher ($p \leq 0.05$) than LPAB-J17 and HPAB-J17. For total coliform counts, there was no significant difference ($p \geq 0.05$) between LPAB-J17 and HPAB-J17. After 12 h of fermentation, there was no yeast growth for TFM. HPAB-J17 obtained the lowest final pH (3.6) after fermentation, with TFM and LPAB-J17 obtaining the same final pH of 4.75. Isolate MFS1 was isolated from MFS by pre-enrichment in Yel-broth (30 °C for 5 d) and sub-culturing on Yel-agar (30 °C for 7 d anaerobically). Isolate MFS1 was Gram-positive with an endospore. The NCBI BLAST resulted in 99.5 % similarity with seven strains. It had 99.5% similarity to the type species of *Bacillus rugosus sp. nov*. *B. rugosus* is an emerging organism of the *Bacillus* genus. MFS1 formed a relatively stable phylogeny at 68 %; the cluster was located in a clade that was 99 % stable.

The optimisation of vit B12 was accomplished by co-inoculating tomoso, which was back-slopped with PAB-J17 in a low cell inoculum of 1×10^4 cfu.g^{-1} (LPAB-J17) and a high cell inoculum of 1×10^8 cfu.g^{-1} (HPAB-J17). The traditionally fermented motoho (TFM), which was the control obtained a significantly higher ($p \leq 0.05$) vit B12 concentration than LPAB-J17;

LPAB-J17 vit B12 concentration was significantly ($p \leq 0.05$) lower than TFM and HPAB-J17. Inoculum optimisation was successful for HPAB-J17, obtaining vit B12 which was 2-fold higher than the control (TFM) and 5-fold higher than LPAB-J17.

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DEDICATION

I dedicate my dissertation work to God, my family and friends for their support, encouragement and prayers throughout this journey. I would especially like to express my deepest gratitude to my gradmothers, Machere Makuebu and Josephina Nyenye, Ma'letlotlo Nyenye (mother), Phole Nyenye (brother) and Banyane Makwebu (aunt) for your constant and overwhelming love, prayers and support throughout this journey.

Lastly, I dedicate this dissertation to myself; for seeing this work through and for not giving up whenever it got hard and it seemed impossible.

Psalm 46:10- "*Be still and know that I am God. I will be exalted among the nations; I will be exalted in the earth."*

TABLE OF CONTENTS

BIBLIOGRAPHY

LIST OF FIGURES

PAGES

LIST OF TABLES **PAGES**

ABBREVIATIONS AND ACRONYMS

CVD	Cardiovascular disease
DMB	Dimethylbenizmidazole
FAO-UN	Food and Agriculture Organization of the United Nations
FF	Functional food
FUFOSE	Functional Food Science in Europe
ILSI Europe	International Life Science Institute
LAB	Lactic acid bacteria
PAB	Probionic acid bacteria
SSA	Sub-Saharan Africa
Vit	Vitamin

GLOSSARY

Deoxyribonucleic acid (DNA):	A nucleic acid containing the genetic instructions used in the development and functioning of all known living organisms (with the exception of RNA viruses). The DNA segments carrying this genetic information are called genes. DNA is one of the three major macromolecules that are essential for all known forms of life.
Fermentation:	The desired action of converting carbohydrates to alcohol or organic acids by means microbial action of either yeasts or bacteria under anaerobic conditions.
Fortification:	The practice of deliberately increasing the content of an essential micronutrient, i.e. vitamins and minerals (including trace elements) in a food, so as to improve the nutritional quality of the food supply and provide a public health benefit with minimal risk to health.
Functional Foods:	Are similar in appearance to conventional foods, are consumed as part of a usual diet, and are known to improve health status beyond basic nutritional function expected of conventional foods.
Polymerase chain reaction (PCR):	A scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.
Vitamin B12 (cobalamin):	The largest and most structurally complex vitamin and can be produced industrially only through bacterial fermentation. It is a water-soluble vitamin with a key role in the normal functioning of

the brain, nervous system and for the formation of blood. It is normally involved in the metabolism of every cell of the human body, especially affecting DNA synthesis.

CHAPTER 1
INTRODUCTION

1.1. Background to the research problem

There is increasing consumer awareness concerning the relationship between food and health. This is coupled with an increasing global population, malnutrition/vitamin deficiencies, climate change and food security concerns, especially in underdeveloped and developing countries (Siro *et al.*, 2008:457; Rathore *et al.*, 2012:239). There is also mounting pressure regarding the production of food products that meet the daily nutritional requirements. This is only possible by means of employing technological methods that are economic, coupled with the utilization of indigenous cereal crops with favourable agronomical traits (Garrity *et al.*, 2010:197-198; Ringler *et al.*, 2011:1; Thierfelder *et al.*, 2014:328).

Cereals and their constituents are accepted as functional food (FF) deemed necessary for human health. This is due to their content of vitamins, minerals, antioxidants, proteins and dietary fibre. Cereals also contain various phenolic acids, antioxidants, folate, selenium, phytoestrogens, linoleic acid and vitamin E. (Achi & Ukwuru, 2015:71-72; Hadebe *et al.*, 2016:179,184). Sorghum [*Sorghum bicolor (L.) Moench*] is a cereal crop that is ranked second in Africa with regards to its acreage and production and is estimated to feed approximately 500 million people (Taylor *et al.*, 2014b:257; Proietti *et al.*, 2015:174). This cereal grain has gained great attention due to its various potential uses and applications, namely to address food security, and the nutritional quality and content of foods (Achi & Ukwuru, 2015:72-73; Taylor *et al.*, 2014b:257).

Fermentation of cereal grains is exceptionally popular in Africa, because it has been a simple, common and home-based practice that has been utilized for decades to produce various foods and beverages (Guyot, 2012:1109; Kumari *et al.*, 2015:134-135). Cereal grains like sorghum have been utilized for centuries to produce fermented food products, which constitute one of the main dietary staples for consumers (Guyot, 2012:1109-1110; Achi & Ukwuru, 2015:72; Mokoena *et al.*, 2016:2). Motoho is a snack-beverage produced by means of spontaneous fermentation or back-slopping (Bajpai & Tiwari, 2013:2387). Lactic acid bacteria (LAB) and yeasts are the most studied microorganisms in spontaneous fermentation, therefore the knowledge on other microflora and their probiotic potential in fermented indigenous African foodstuffs is limited (Franz *et al.*, 2014:87; Mokoena *et al.*, 2016:1-2).

Inhabitants of Sub-Saharan Africa (SSA) are reported to have the highest vitamin deficiency, and fortification of staple foods by means of fermentation has been demonstrated

to enhance the vitamin B12 (vit B12; cobalamin) content (Micronutrient Forum, 2015:2; Che *et al.*, 2016:11040; Walther & Schmid, 2017:132). Vitamin B12 is an essential vitamin that participates in the synthesis of nucleotides, and its deficiency can have adverse health implications. Vit B12 is unique; it can only be synthesized by a few microorganisms and archaea. *Propionibacterium freudenreichii* is an effectual producer of vit B12 and has Generally Recognised as Safe (GRAS) status (Deptula *et al*., 2015:2; Chamlagain *et al*., 2017b:67). The effectual production of vit B12 has been explored in dairy-based products, but utilisation of *P. freudenreichii* in cereal fermentation has yet to be thoroughly exploited (Chamlagain, 2016a:12).

1.2. Statement of the research problem

Food security has become a global priority due to increasing global populations and depleting water resources. This is more evident in areas such as SSA, which is reported to have the highest food insecurity globally (Achi & Ukwuru, 2015:72; Hadebe *et al.*, 2016:177). It is accompanied by vitamin deficiencies in populations in SSA, which is one of the highest in the world and can negatively affect the development, working ability and the overall quality of life and human health (Micronutrient Forum, 2015:2; Che *et al.*, 2016:11040).

Cereal-based fermented foods form part of the daily diet of many communities in Africa and the diverse variety of fermented foodstuffs is due to the availability of a wide range of indigenous raw materials (Schoustra *et al.*, 2013:1; Mokoena *et al*., 2016:1). Fermented foodstuffs are regarded as functional foods due to their probiotic and/or prebiotic content, which is beneficial for the maintenance of good health. Fermentation is an inexpensive practice and improve the nutritional, technological and functional qualities of raw materials (Van Hylckama-Vlieg *et al.*, 2011:211; Marsh *et al.*, 2014:113-114). Sorghum is Africa's second most cultivated cereal grain and is known to contain vitamins, minerals and fermentable sugars which can sustain microbial fermentation (Kumari *et al.*, 2015:136). The microbial starter cultures used during the preparation of the most indigenous African fermented foods and beverages are unknown or poorly characterized. Vitamin fortification by means of fermentation has been suggested to be employed in order to produce fermented food products which contain specific naturally synthesized vitamins and minerals by food-grade microbial starter cultures (Burgess *et al.*, 2009:2; Marsh *et al.*, 2014:113; Hadebe *et al.*, 2016:178).

There is a need to evaluate the *Propionibacterium* strains that may be present in naturally fermented sorghum beverages. Their contribution to the production of vit B12 during fermentation needs to be defined and methods to further increase the vit B12 content must be evaluated. This could contribute to the development of a suitable *Propionibacterium* sp. starter

culture, which can be employed for vitamin fortification during cereal-based fermentation, and the production of FF products.

1.3. Aim of the study

The aims of this study were to, (a) evaluate the microbial content of traditionally fermented motoho (control) and the control co-inoculated with Propionic Acid Bacteria (PAB) as well as to isolate and identify PAB species in the control sample.

(b) to detect and quantify the vitamin B12 content in the traditionally fermented motoho and optimise vitamin B12 production.

1.4. Specific objectives of the study

The specific objectives of this study were:

1. To evaluate the nutritional composition (proximate analysis) of the control and optimised samples.
2. To evaluate the microbial content (total viable count, coliforms, *E. coli*, lactic acid bacteria, yeasts and moulds) during traditional fermentation of motoho (control).
3. To isolate, characterise and identify *Propionibacterium* spp. in the control.
4. To detect and quantify vitamin B12 in the traditionally fermented motoho.
5. To optimise the production of vitamin B12 by co-inoculation of the back-slopped starter culture (tomoso) with a low and high inoculum concentrations of *Propionibacterium freudenreichii* subsp. *Shermanii.*
6. To collate, integrate and interpret the data collected

1.5. Hypotheses

It was hypothesised that the isolates from the traditionally fermented motoho would include *Propionibacterium* spp.

Considering the concentration of vitamin B12 synthesised by *P. freudenreichii* (Chamlagain, 2016), it was hypothesised that the co-inoculation with *P. freudenreichii* with higher inoculum concentrations will increase the vitamin B12 concentration in motoho.

1.6. Delineation of the study

For the study, Yel-media was employed for the isolation for Propionic Acid Bacteria isolates. For the optimisation of vitamin B12, *P. freudenreichii* subsp. *shermanii* (ATCC13637) strain J17 was employed in low and high inoculum concentrations. The back-slopped starter culture (tomoso) inoculum strength for the three models was the same, and fermentation temperature and time at 32 °C for 12 h. Sampling was performed every 3 h throughout the 12 h of

fermentation. For the vit B12 analysis, High-Performance Liquid Chromatography (HPLC) was employed. The data was statistically analysed using SPSS® 22.0.

1.7. Significance of the study

The significance of this research study is the possible isolation of a *Propionibacterium* sp. strain from motoho, which could contribute to the probiotic potential of motoho and its classification as a functional food. This includes the evaluation of the vit B12 content and the optimisation of the vit B12 content by employing *P. freudenreichii* to an inoculum cell concentration that meets or exceed the daily recommended intake of vit B12 (2.4 µg/day). This will therefore allow for the promotion of motoho as FF, and the continuous utilisation of *P. freudenreichii* for vitamin fortification during cereal fermentation.

1.8. Expected outcomes, results and contributions of the research

The discovery of a novel *Propionibacterium* sp. strain in motoho. This also included the detection and quantification of vit B12 in motoho and successfully optimisation of the vit B12 content. The consumption of motoho is mostly isolated within the Basotho community in Lesotho and parts of the Free State province. It is a food product that they are familiar with. The increased vit B12 will allow for the promotion and commercialisation of the product, as well as exploring fortification with other indigenous raw materials.

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CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

There is an increasing consumer awareness and demand for natural food products with health promoting benefits. This increasing movement is fuelled by evolving eating habits, religious and lifestyle choices promoted by urbanization (Siro *et al.*, 2008:457; Rathore *et al.*, 2012:239). Consumers have become aware that the purpose of food goes beyond the intake of nutrients, but goes to the point of promoting and ensuring physical and mental health, as well as the prevention of diseases related to nutrient deficiencies (Siro *et al.*, 2008:457; Rathore *et al.*, 2012:239; Salmeron *et al.*, 2015:106). This in return has placed food manufacturers under pressure to produce new food products with functional properties which have beneficial health properties for the consumers (Hugenholtz, 2013b:155). Indigenous and/or traditional foods and beverages could be the vehicle to addressing health and nutrition in developed and developing countries (FAO, 2008a:1; 2017:48-49). Motoho, a Basotho traditional sorghum snack-beverage produced by means of spontaneous fermentation holds potential to be utilized to address vitamin deficiencies in Sub-Saharan Africa (SSA).

Consumer interest in traditional food products associated with specific cultural traditions and origins is increasing. This is evident in both developed and developing countries. This interest and demand are coupled with the willingness to pay more for traditional foods (Ackermann, 2010:1; FAO, 2017b:110). The various factors such as changing local and global food laws and regulations, increasing health-care costs and great advances in science and technology have also contributed to the growing demand for health promoting food products (Stanton *et al.*, 2005:198; Danilova *et al.*, 2012:48; Rathore *et al.*, 2012:239; Hugenholtz, 2013b:155).

2.2. Functional foods

The FF market and market share is expected to increase as more resources are invested in the research, development and promotion of FF (Doyon & Labrecque, 2008:1134; Vicentini *et al.*, 2016:343). The promotion and growth of the FF market will also depend on the development of a globally accepted and defined definition which clearly stipulates what FF are, and which foods and beverages are regarded as FFs (Verbeke, 2005:45; Doyon & Labrecque, 2008:1135). This will in turn reduce the confusion experienced by consumers (Frewer *et al.*, 2003:727).

2.2.1. Defining functional foods

In 1991 the Japanese government established the Foods for Specific Health Use (FOSHU), which is responsible for the systematic analysis and development of food functions. This entails

the evaluation of the physiological regulation of FF, the molecular design and analysis of FF (ILSI, 1999:1; Ashwell, 2006:4).

All health promoting food products have been termed as FF, even though a universal unitary accepted definition was yet to be established (Krystallis *et al.*, 2008:525; Siro *et al.*, 2008:457; Crowe *et al.*, 2013:1097). In 1999, the European Commission's Concerted Action on Functional Food Science in Europe (FUFOSE) was facilitated by the International Life Science Institute (ILSI Europe) and a proposed working definition for FFs was constructed (Siro *et al.*, 2008:457; Pravst, 2012:165). It stated that food can be regarded as "functional" if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease (ILSI, 1999:6; Ashwell, 2006:5; Pravst, 2012:165). The quantity and form of intake of the FFs should be in its normal expected form for dietary purposes, therefore, the FF is to be in its normal form and not in capsule or pill form (Verbeke, 2005:45; Crowe *et al.*, 2013:1097,1099).

Various studies have stated that the motivation behind FFs is to assist in the alleviation of all or most of the associated risks of some chronic diseases such as diabetes, obesity, some cancers, some cardiovascular diseases and atherosclerosis (Shahidi, 2004:146; American Dietetic Association, 2009:735,738). Therefore, the objective of FFs is optimizing the consumers health by strengthening the immune response, increase energy, as well as health preservation and longevity (Shahidi, 2004:146; Topolska *et al.,* 2021:1).

2.2.2. Identification and classification of functional foods

The science behind FFs is founded in the relationship of certain nutrients and their interaction with specific food components, which in turn affects the targeted biological responses of the consumer (Henry, 2010:64; Topolska *et al.*, 2021:1). Functional foods have earned their reputation based on their high content of various bioactive compounds and phytochemicals which are of great interest and highly valued for nutritional wellness (ILSI, 1999:5; Ashwell, 2006:5; Crowe *et al*., 2013:1099). Functional foods have been categorized into four groups based on their form and use:

1. **Conventional/whole foods:** unmodified/whole/organic foods such as whole grains, nuts, fruits (citrus fruits, raspberries, and kiwi) and vegetables (kale, broccoli and cauliflower), which respectively contain high contents of bioactive components such as polyphenols, phytosterols, carotenoids, vitamins and minerals (American Dietetic Association, 2009:736-376; Liu, 2013b:3845-3846).

2. **Modified foods:** subdivided into three categories: (i) micronutrient fortified foods with the potential to significantly reduce deficiencies and increase serum micronutrient concentrations in staple foods. Mandatory fortification and biofortification with iron and vitamin A of wheat and maize flour has been employed in South Africa to help combat various micronutrient deficiencies. Food fortification initiatives have increased globally, with 86 countries having mandatory cereal fortification programs. Figure 2.1 illustrates the global map of mandatory legislated cereal fortification initiatives of maize, wheat, and rice. South Africa, Zimbabwe, Burundi and Kenya are some of the African countries amongst 14 other global countries (shaded green) that have legislation for the fortification of their maize and wheat flours. Argentina, Cameroon, Australia and Ghana are amongst the 67 countries (shaded red) that have legislation for wheat flour. The United States of America (USA) is one of two countries that has legislation for the fortification of wheat, rice and maize flours (shaded blue); (ii) enhanced foods (e.g. yoghurt, snack bars and energy beverages) have their nutrient content enhanced with particular bioactive components such as specific amino acids, saw palmetto, fish oils and ginkgo biloba, and (iii) enriched foods such as folate-enriched breads which can be consumed by expecting mothers for fetal development. Modified foods are intentionally produced and promoted to meet the daily nutritional dietary needs of consumers especially those who are not able to obtain all the vital micronutrients required to alleviate health deficiencies and maintain a healthy lifestyle (Ashwell, 2006:29-31; American Dietetic Association, 2009:736-737; Micronutrient Forum, 2015:5-7).
3. **Medical foods**: the Orphan Drug Act has defined medical food as food which is specifically formulated to be administered/ consumed entirely under the supervision of a medical practitioner. The intended use is for the distinctive nutritional requirements for the specific dietary management of a disease or condition. It is established by a medical evaluation based on recognized scientific principles. An example of such foods is Banatrol Plus which is used for the treatment of diarrhea (American Dietetic Association, 2009:736; Crowe *et al.*, 2013:17-18).
4. **Foods for special dietary needs:** foods that are formulated, processed or prepared to meet the special dietary needs of consumers with special dietary needs. Foods which fall in this group are hypoallergenic food (gluten-free and lactose-free foods), infant foods and weight-loss foods (Ashwell, 2006:6-14; American Dietetic Association, 2009:736; Codex, 1997).

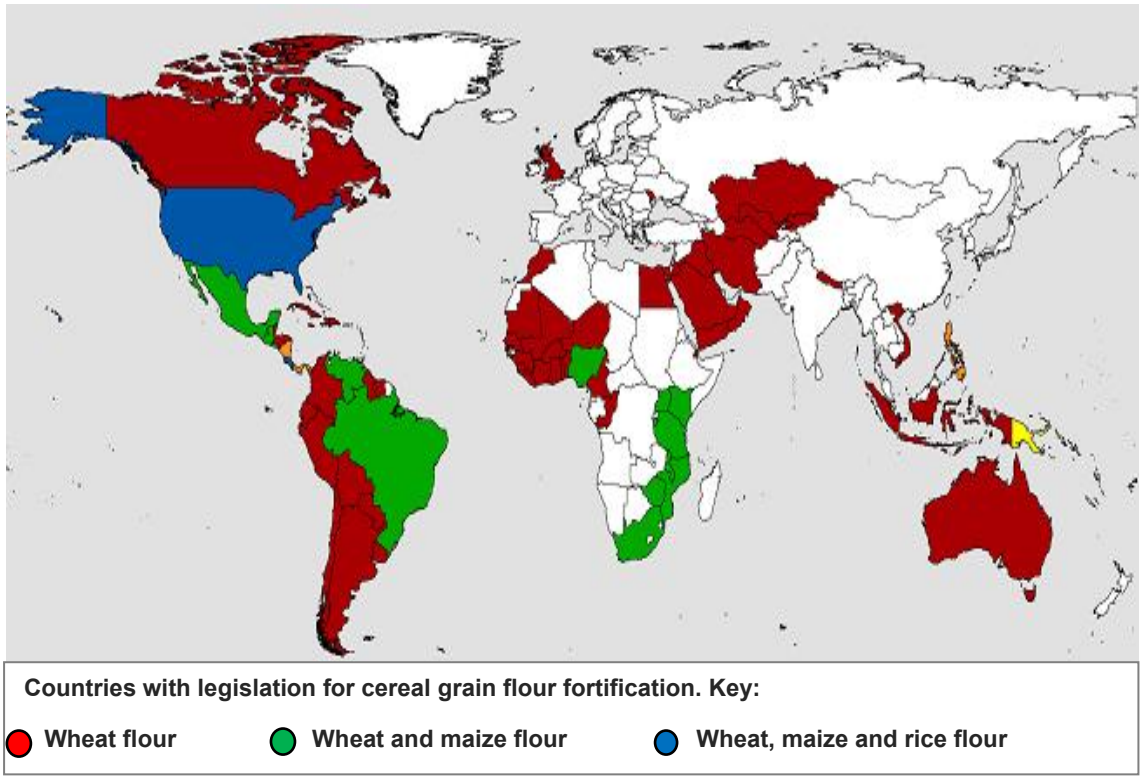

Figure 2.1: Countries with legislated mandatory cereal grain fortification.

(Source: Food Fortification Initiative http://www.ffinetwork.org/global_progress.php).

2.2.3. Health benefits associated with functional foods

Functional foods possess similar characteristics (appearance) as conventional foods consumed daily, whereas nutraceuticals are produced from animals, plants and microbial sources. Therefore, nutraceuticals are in the form of pharmaceutical products such as powders, tablets/capsules, potions and solutions (Shahidi, 2004:146; Krystallis *et al.*, 2008:525; Nasri *et al.,* 2014:1487). Health benefits associated with nutraceuticals and FFs are continuously being observed and evaluated (Bigliardi & Galati, 2013:121).

Consumers have shown to be motivated to include nutraceuticals and FFs in their diets in order to prevent and/or assist in the management of chronic diseases such as osteoporosis, cardiovascular disease (CVD), diabetes and Alzheimer's disease (Shahidi, 2004:146,149; Khan *et al.*, 2013a:27). Nutraceuticals and FFs are also consumed to support and boost immune system function, increase energy and overall health as well as vitality (Khan *et al.*, 2013a:27). Nutraceuticals and FFs are not intended to cure diseases, but rather assist, decrease and/or prevent the risk of nutrition-related illnesses, such as chronic and lifestyle diseases, therefore improve and support overall health (Menrad, 2003:181; Bigliardi & Galati, 2013:119; Nasri *et al.*, 2014:1487-1488).

2.2.4. Potential market and financial impact of functional foods

The food industry has a huge responsibility with regards to the type and quality of food products that they produce. Products therefore need to meet the expectations of consumers, especially the demand for a healthy lifestyle where FFs play a specific role (Menrad, 2003:181; Szakály *et al.*, 2012:406-407). The category of FFs has increasingly become one of the most popular and interesting areas of research and innovation (Annunziata *et al*., 2011:223; Falguera *et al.*, 2012:276). The lack of an accepted global definition for FFs has made it challenging to fully estimate the precise overall financial market turnover and value (Doyon & Labrecque., 2008:1134; Henry, 2010:657).

The development and promotion of FFs has shown to be continuously growing with a long term trend with great market potential (Khan *et al.*, 2013a:27). There have been various estimations of the global market for FFs (Vicentini *et al.*, 2016:343-344). Based on the perspective of the food industry, the development of FFs can provide an increase in the global competition for quality, reliability and variety of products against a guarantee of an already saturated consumer demand (Doyon & Labrecque, 2008:1141,1146; Falguera *et al.*, 2012:276,279). Even though exact data is not yet available regarding the exact value and market turnover of FFs, the rise of the FFs market is increasing due to consumers becoming more aware of the strong relationship between food, health and wellness (Siro *et al.*, 2008:458; Bigliardi *et al.*, 2013:119).

The FFs market growth is increasing annually. There are three concentrated dominating markets, i.e. Europe, Japan (most developed) and the USA, which contribute over 90% of the FFs sales globally with each country having its own definition of FFs (Vicentini *et al.*, 2016339-340; Balogh *et al,* 2020:162). It was reported by Siro *et al*. (2008:458) that the USA was the leading market of FFs with a market share of 50%. Five years later Bigliardi & Galati. (2013:119) reported that between 1988 and 1998 circa 1700, new FF products were produced, launched and marketed in Japan, therefore making it the largest market. This was not unexpected due to the fact that Japan is regarded the birthplace of most FFs. Szakály *et al.* (2012:407) reported that in 2005 the Japanese market had approximately 500 products which were labelled as FOSHU and that the market was estimated to be valued at US$ 5.73 billion in 2006. According to Basu *et al.* (2007:637-638) and Doyon & Labrecque (2008:1134), they predicted FFs market growth was estimated at 7-10% per year with an estimated market size of US$ 11-115 billion, which was dependent on the definition used. The estimation corresponds with other estimations made by other researchers. Euromonitor estimated the FFs global market value to be US$ 168 billion, which is about 2.5 times higher than the dietary supplement and vitamin market (Euromonitor, 2018). These significant rapid growths in the food industry led to a compounded 8.6% growth rate estimation in 10 years to 2012. There is

an observed evident growth in the global market value of FFs, with 7.2% being the predicted continuous growth by 2017 (Khan *et al.*, 2013a:27; Vicentini *et al.*, 2016:346).

There are lucrative opportunities in emerging markets of FFs. The observation was specifically made in developed and some developing countries and this has been reported as an ideal opportunity to develop the local market (Frewer *et al.*, 2003:715; Vicentini *et al.*, 2016:346). Asia-Pacific (Malaysia, South Korea and China) is one of the most developed and fastest growing markets of FFs, contributing about 34% of the total global revenue (Vicentini *et al.*, 2016:347). Countries such as Italy, Germany, France, The Netherlands and the United Kingdom have seen substantial growth in FFs, with Germany reporting an estimated US$ 600 million market value of FFs in 2002/2003 (Menrad, 2003:181-182; Annunziata & Vecchio, 2011:223; Bigliardi & Galati, 2013:119; Vicentini *et al.*, 2016:346). The specific needs, demand, and acceptability of FFs in the various markets are heterogeneous. The cultural traditions and heritage of the various populations will contribute significantly in the variety of FF-products manufactured and therefore consumed (Annunziata *et al.*, 2011:223; Vicentini *et al.*, 2016:345-346).

2.3. Sorghum: potential novel food

Food security has become a global priority due to the global increase of populations, political-economic crises and climate change (Garrity *et al*., 2010:197-198; Giller, 2020:2). This is more evident in SSA countries, as reported by the Food and Agriculture Organization of the United Nations (FAO), in terms of food insecurity globally (Lewandowski, 2013:70; Hadebe *et al.*, 2016:177). A report published in 2019 by the FAO, revealed that 239 million people in SSA were undernourished, which is higher than the 17 million from the Northern African population (FAO, 2019c:3). Communities in rural areas are reported to make up approximately 83% of the entire extreme poverty group, with approximately 85% of the poor population relying on agriculture for sustaining their livelihoods in SSA. Subsistence farming at household-level is vital and deemed necessary for improving food security, especially in developing and underdeveloped countries where food is consumed locally in low-income households (Garrity *et al.*, 2010:198; Lewandowski, 2013:74,76; Hadebe *et al*., 2016:177).

Cereal grain farming forms part of a major source of income and food security, whereby it meets the dietary nutritional needs for a great percentage of the African population (Macauley, 2015:1; Hadebe *et al.*, 2016:177). Cereal grains and their constituents are accepted as FFs and deemed necessary for human health, whereby their use in the production of FFs has garnered great attention and investment (Charalampopoulos *et al.*, 2002:132; Oboh Amusan, 2009:18; Das *et al.*, 2016:1). This is due to their content of vitamins, minerals, antioxidants, proteins and dietary fibre (Achi & Ukwuru, 2015:72; Macauley, 2015:1).

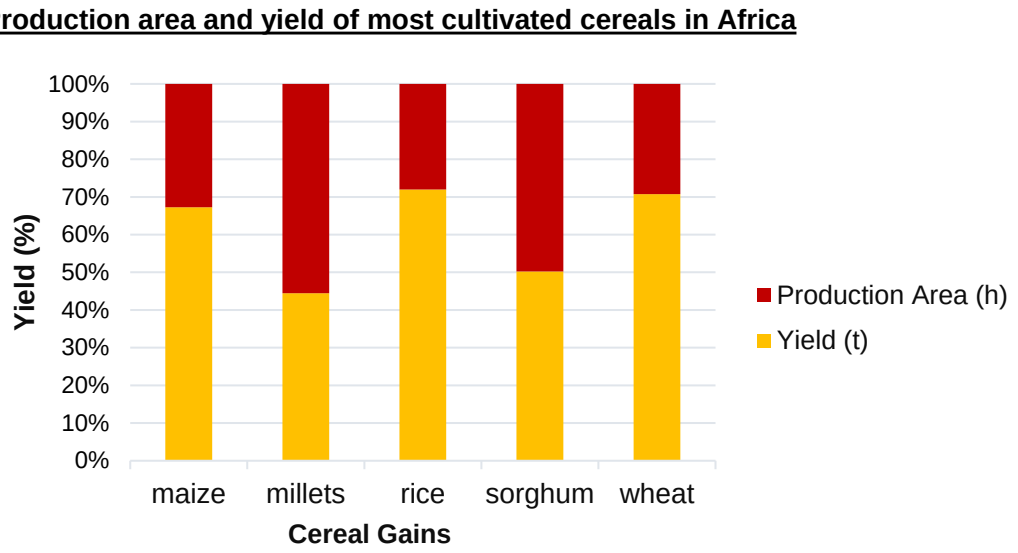

Figure 2.2: Production area and yield of the most cultivated cereal grains in Africa. (Source: FAOSTAT http://Faostat.fao.org)

Cereals contain various folic acids, antioxidants, folate, selenium, phytoestrogens, linoleic acid and vitamin E, a group of fat-soluble compounds with distinctive antioxidant activities (Charalampopoulos *et al.*, 2002:136; Sidhu *et al.*, 2007:232; Oboh & Ukwuru, 2009:18; Hadebe *et al.*, 2016:179). The most cultivated cereal crops in SSA (Figure 2.2) are maize, wheat, sorghum, millet and rice, with sorghum being regarded as the second most valued on the continent (Taylor, 2003a:1; Lindsay, 2010:15; Macauley, 2015:1). In Africa, sorghum occupies the second highest cultivation area (22%), with an estimated area of 23 million ha and production yield of 20 million tonnes (Macauley, 2015:6).

2.3.1. Sorghum: an ancient grain

Sorghum [*Sorghum bicolor (L.)* Moench] is one of the oldest known indigenous African cereal crops which was previously regarded as a minor crop (Taylor *et al.*, 2014c:257; Ratnavathi, 2016:312). The cereal crop is ranked fifth globally amongst the major cereal grains and second in Africa with regards to its acreage and production. It is estimated to feed approximately 300 to 500 million people in developing countries (Proietti *et al.*, 2015:174; Che *et al.*, 2016:11040; Tian *et al.*, 2016:1). The cereal crop primarily provides food in Africa, Latin America and Asia, but in countries such as America and Australia the crop is primarily used for animal feed, fibre and fuel (Taylor *et al.*, 2014c:257; Waniska *et al.*, 2016:116; Wu *et al.*, 2017b:199). In recent years the crop has gained great attention due to its various potential uses and applications, namely to address food security, cultivation challenges, nutritional content and FFs potential and production (Dicko *et al.*, 2006:387-388; Queiroz *et al.*, 2015:104; Anunciacao *et al.*, 2017:984-985). The increasing global population, decreasing water supplies and consumer

awareness of dietary needs have shifted the focus on sorghum and has resulted with the grain being tagged as the crop of the future (Ringler *et al.*, 2011:1; Hadebe *et al.*, 2016:180).

Figure 2.3: Sorghum [*Sorghum bicolor (L.) Moench*]

(Source: https://www.pakhousebrands.com/sorghum-in-south-africa/)

2.3.2. Classification of sorghum

The genus *Sorghum* (Figure 2.3) belongs to the grass family *Graminea* and the tribes of *Andropogoneae*, *Eragrostideae* and *Paniceae* are the most domesticated species of this genus. It is also related to the *Triticeae* cereal tribe which consists of barley, wheat and rye (Ritter *et al.*, 2007:161-162; Taylor *et al.*, 2014c:257-258). Sorghum is classified into five races based on the phenotypic traits of the spikelet and panicle. The five races are (i) *bicolor,* the primitive progenitor from which all the other races are derived; (ii) *caudatum*, used in sorghum breeding. It has excellent seed quality and is known to have its origins in East Africa; (iii) *durra,* which has adapted to arid environments and has a compact inflorescence; (iv) *guinea*, originally from West Africa, has a more lose and open inflorescence architecture compared to *durra* and is more adapted to moist environments; and (v) *Kafir* has its origins in Southern Africa and has participated greatly in sorghum grain breeding (Ritter et al., 2007:161-162; Department of Agriculture, 2010:1; Brown et al., 2011:224).

2.3.3. Cultivation of sorghum

The agronomic characteristics of sorghum make it an ideal cereal crop which can be cultivated in tropical, arid and semi-arid environments (Hadebe *et al.*, 2016:180). It is an ideal crop to be exploited and promoted in SSA, because it is drought and heat tolerant (Lindsay, 2010:3;

Hadebe *et al.,* 2016:180). Sorghum can grow in various temperature ranges, but for optimum growth and germination the temperature needs to be in the higher range (Lindsay, 2010:4). The average temperature range for seed germination is 21-35°C, for vegetative growth and crop development 26-34°C, and for reproductive growth 25-28°C. It must be noted that high-temperature stress and low water availability affect crop yield significantly (Prasad *et al*., 2008:1911; Department of Agriculture, 2010:6; Hadebe *et al*., 2016:183). Hadebe *et al*. (2016:180) stated that sorghum is capable of growing in temporal water-logging areas, which makes the crop ideal in regions that experience erratic and unexpected rainfall seasons. The extreme heat and erratic rainfall climate in SSA has greatly affected cereal crop farming in general, due to the high water requirements of most cereals (Department of Agriculture, 2010:6-7; Queiroz *et al*., 2015:104; Tian *et al*., 2016:2). Hadebe *et al*. (2016:180) also reported that cereal crops that have agronomic advantages, especially desirable water usage traits such as sorghum are not effectively promoted and/or given the attention they deserve. This has resulted in the exploitation of other cereals based on their consumption rates, production areas, quantities and research investment (Taylor, 2003a:231; Macauley, 2015:3-4). Climate change has had a profound impact on the local and global agricultural markets (Ringler *et al.*, 2011:1). This has resulted in the search for agricultural methods and crops that utilize water effectively and efficiently. The output of rain-fed and irrigated agricultural crops have been highly affected and SSA has had great constraints with regards to its annual rainfall, development and management of renewable water resources (Boutraa, 2010:1-2; Lewandowski, 2013:218).

2.3.4. Sorghum production yield

Léder (2004:7) reported that sorghum consumption in developing countries was estimated to increase by 4 million tonnes to 30 million tonnes, while Belton *et al.* (2004:94) reported that approximately between 49-55% of sorghum production land was situated in Africa. A third of the sorghum produced globally was produced in Africa (Taylor, 2003a:2). Global farmlands where sorghum is cultivated are estimated to be approximately 8 million hectares, with Nigeria (4.8 million metric tonnes) being the highest sorghum producer in Africa (Tian *et al.*, 2016:2). Between 2008 and 2018, Africa (Figure 2.4) maintained the highest production share (41%) which was significantly higher than Asia and the Americas (Lewandowski, 2013:184-186; Mundia *et al.*, 2019:5).

Approximately 65% of the global sorghum production yield is intended for industrial applications, including alcohol and animal feed production, while only 35% is for human consumption (Dicko *et al.*, 2006:385; Mundia *et al.*, 2019:5). Waniska *et al*. (2016:116) reported that Botswana, Nigeria, and South Africa were the major sorghum cultivators in Africa. There is an increasing trend for sorghum consumption, especially in developed countries. This is due

to sorghum's antioxidant potential, polyphenolic phytochemical content and its nutritional benefits (Wu *et al.*, 2017b:347-348).

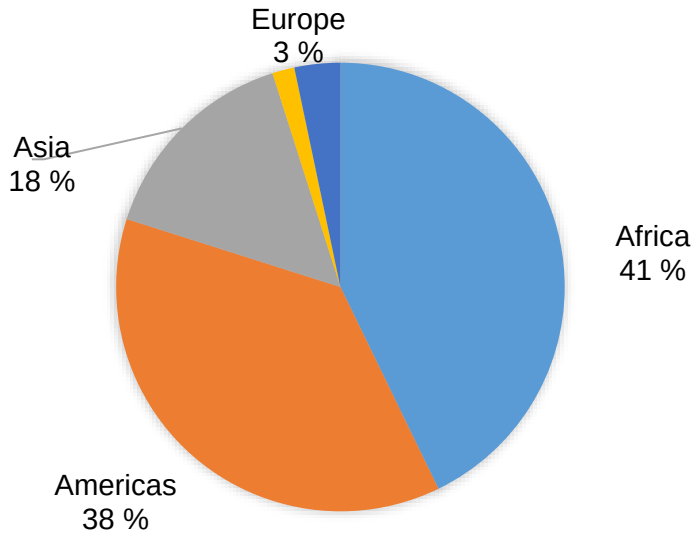

Figure 2.4: Global sorghum production yield (2008-2018).

(Source: FAOSTST http://www.fao.org/faostat/en/#data/QC/visualize)

Climate change and the variability of rainfall is said to have a significant impact on the agricultural sector, with a predicted cereal crop production decline in SSA (Ringler *et al.*, 2011:1-2; Arora, 2019:1). The estimated decline is set at net 3.2% by the year 2050. Production yields are expected to decrease for maize (5%), wheat (22%) and rice (2%) crop, and is coupled with an estimated 4.7-15% price increase. The price increase will also affect cassava (20%), millets (5%), sorghum (4%), potatoes and yams (26%). The price increase will greatly affect consumers in developing and underdeveloped countries who are already struggling to afford high food prices (Ringler *et al.*, 2011:1-2). Even though the price of cereal crops in general will increase, the estimated production yields of sorghum are said to increase together with those of millet due to their drought resistant properties. This further motivates for capital investment and research into drought resistant indigenous cereal crop cultivars. It will also be of great interest to invest in research, production and promotion of foodstuffs produced from sorghum (Prasad *et al.*, 2008:1912; Ringler *et al.*, 2011:2; Hadebe *et al.*, 2016:181).

2.3.5. Consumption levels of sorghum

Sorghum is regarded as a staple cereal crop in many African countries such as Ethiopia, Lesotho, Nigeria, Sudan, and South Africa. Other countries include India, Japan and Brazil. In these countries, sorghum is cultivated primarily for human consumption and also used as animal feed (Dicko *et al.*, 2006:384-385; Taylor *et al.*, 2006b:253; Achi & Ukwuru, 2015:71-72). Sorghum is referred to in different names in various countries, regions and languages: (Dicko *et al.*, 2006:385; Department of Agriculture, 2010:1):

1. East Africa : mtatam, shallu or feterita
2. South Africa : mabele, kaffir corn, amabele or amazimba
3. USA : milo, sorgo, Sudan-grass, or sorghum
4. United Kingdom : chicken corn, guinea corn or sorghum
5. India : durra, jola, cholam, jawa/jawar, bisinga

Waniska *et al*. (2016:116) stated that approximately 30-40% of the 62 million tonnes of sorghum harvested annually is utilized in various traditional foods and beverages in Africa, Latin America, and Asia where sorghum is used as a staple food. Dicko *et al*. (2006:387) estimated that approximately 300 million people in developing countries rely on sorghum as their staple food. Sorghum is used daily to produce various alcoholic and non-alcoholic beverages, porridges, baked goods, couscous, rice-like products and many other foodstuffs (Taylor, 2003a:10; Ratnavathi, 2016a:312). Sorghum is generally consumed as:

1. **Baked products**: sorghum grains are milled into a fine flour which is used in the production of unleavened flat bread such as roti (India), tortilla (Latin America) and injera (Ethiopia and Eritrea). In East and West Africa, kisra is made from sorghum flour and dark-rye bread in Central and Eastern Europe, which can be made from fermented or unfermented dough (Taylor *et al.*, 2006b:254-256; Ratnavathi, 2016a:318; Waniska *et al.*, 2016:120).
2. **Porridges and Beverages**: fine and/or course sorghum flour (Figure 2.5a) is used in the production of various fermented and unfermented thick or thin porridges. The porridge is usually prepared as a weaning food and breakfast porridges (Dlamini *et al.*, 2007:1413; Moodley, 2015a:20-21). Fermented beverages can either be alcoholic or non-alcoholic and they play a significant role in cultural and traditional events (funerals, wedding celebration and other indigenous cultural practices). Various beverages such as mahewu (Southern Africa), motoho (Lesotho), bushera (East Africa), ogi (West Africa), umqomboti (Figure 2.5b) and chibuku (Southern Africa), are produced from fermented sorghum and are consumed across Africa (Taylor, 2003a:14; Achi & Ukwuru, 2015:75-77; Mokoena *et al.*, 2016:2; Ratnavathi, 2016b:87-88). Sorghum is also used in the production of opaque beer (low alcohol African beer, which has suspended particulates) and mass produced clear beer (lager, draft and ale) (Taylor *et al.*, 2006b:259-260; Ratnavathi *et al.*, 2016b:77-78).
3. **Other forms of consumption**: in West Africa, sorghum is used in the preparation of couscous, while sorghum noodles are commonly prepared in China (Dicko *et al.*, 2006:387-388; Taylor *et al.*, 2006b:259). In India, sorghum flakes and pops are popular. The flakes are produced by soaking the grains in water for up to 18 hours, then drained and air dried. Once dried, the grains are roasted, rolled into flakes and packaged

(Figure 2.5c). To produce the sorghum pops, the cereal grains are preconditioned by soaking for up to 6 hours followed by air drying in a cool dry area. After preconditioning the grains are popped (Figure 2.5d) (Kulamarva *et al.*, 2009:55-56; Ratnavathi, 2016a:320-321).

Figure 2.5: (a) sorghum flour, (b) umqomboti, (c) sorghum flakes, and (d) sorghum pops

Sorghum is a gluten-free cereal, which further increases its application potential for the production of gluten free foodstuffs for consumers who are sensitive to gluten or suffer from celiac disease (Taylor *et al.*, 2006b:253, 2014c:258). Sorghum can be used in conjunction with other gluten-free grain flours for the production of baked goods such as breads, biscuits and cakes (Taylor *et al.*, 2006b:255; Kulamarva *et al.*, 2009:65). It has also been established that sorghum can be used in the production of various extrusion products such as pasta, puffed snacks and ready-to-cook/eat breakfast cereals (Waniska *et al.*, 2016:120). The digestibility of sorghum can be increased by popping, extrusion, flaking and fermentation, which also increases the organoleptic characteristics, quality, price and shelf-life of the various foodstuffs that can be produced from sorghum (Dicko *et al.*, 2006:387-388; Taylor *et al.*, 2014c:269-271; Ratnavathi, 2016a:312; b:64-65).

2.3.6. Nutritional composition of sorghum

Various cereal grains play an important role in providing sufficient energy, protein, carbohydrates, fibre, micro and macro-nutrients (Adebiyi, 2005:1089; Rooney, 2016:201; Waniska *et al.*, 2016:116). Sorghum is primarily harvested for the production of fuel and animal feed in western countries (Léder, 2004:7; Rooney, 2016:201). This is, however, slowly changing mainly due to the nutritional content of sorghum. The potential of sorghum as a staple crop is attracting the attention of many consumers and food manufacturers (Taylor *et al.*, 2014c:271).

The promotion and inclusion of sorghum as a staple crop is vital, especially in SSA countries and other regions known to struggle with water insecurity (Hadebe *et al.*, 2016:179). This is because of its resistance to droughts and its nutritional content, which can be compared with those of other better known or major cereals such as maize and wheat (Dicko *et al.*, 2006:388; Hager *et al.,* 2012:239; Taylor *et al.*, 2014c:258). In western countries sorghum has gained noticeable attention for its functional properties and potential due to its content of dietary fibre, vitamin E, carotenoids, various minerals, and bioactive compounds. Many sorghum genotypes are known to contain tannins which are of interest in the production and promotion of FFs (Anunciacao *et al.*, 2017:984-985).

2.3.6.1. The proximate composition of sorghum

The proximate nutritional value and composition of sorghum is similar to other commonly consumed staple grains. The average energetic value of sorghum and other flours is illustrated in Table 2.1.

Table 2.1: The average energetic value of common cereal grain flours (Dicko *et al.*, 2006; Hager *et al.*, 2012; Hadebe *et a*l., 2016)

Cereal flours	Average energetic value (kcal/100g)
Sorghum	356-375
Wheat	361
Maize	362
Whole wheat	366
Rice	359

The average energetic value of sorghum flour is 356-375 kcal/100g, which is comparable with other cereal flours such as wheat (361 kcal/100g) and maize (362 kcal/100g) (Dicko *et al.*, 2006:388; Hager *et al.*, 2012:239; Hadebe *et al.*, 2016:184). The proximal nutrient content of the various sorghum genotypes differs significantly and is greatly affected by the availability of water; protein (7.3-16.8%), fat (1.5-6.3%), fibre (0.1-6.5%), ash (1.2-7.0%), moisture (8.0-11.2%) and carbohydrates (65-90.3%) (Belton & Taylor, 2004:95; Dicko *et al.*,

2006:388-389; Ragaee *et al*., 2006:34; Oboh & Amusan, 2009:21; Kayitesi *et al.*, 2012:839; Queiroz *et al.*, 2015:106; Singh *et al.*, 2015:97; Waniska *et al.*, 2016:117,119).

2.3.6.2. Sorghum starch, protein, and lipid contents

The main component of sorghum is starch. It constitutes approximately 70% of dry grain weight (Sang *et al.*, 2008:6680). The starch is regarded as resistant starch, which is deemed desirable for the production of foodstuffs that are targeted for diabetic and obese consumers (Khan *et al.*, 2013b:585). Grains and food products produced from slowly digestible starch have a low glycaemic index with the added value of reaching desirable satiety without overeating (Dicko *et al.*, 2006:391; Sang *et al.*, 2008:6680).

In Africa, Asia, Central America and Europe cereal-based food products provide more protein than meat products, with cereal-grain protein content ranging between 7-18 % dry matter (Poutanen *et al.*, 2022:1650). Kayitesi *et al*. (2012:839) reported that the protein content of sorghum is low (9.9 g/100 g), like other cereals. Proietti *et al.* (2015:174) stated that the protein content of sorghum is not significantly different to that of wheat but is higher than that of rice and wheat. The composition of the essential amino acids of wheat, sorghum and maize are comparable due to the limited content of arginine, threonine, and lysine. Even with the low protein content, Belton *et al*. (2004:94, 96-97) and Kayitesi *et al*. (2012:837) suggested that sorghum can be considered as a source of protein in Africa when used in combination with other protein rich legumes and/or cereals. A study conducted by Adebiyi (2005:1089) supported the suggestion made by Belton *et al.* (2004:94) that sorghum had a lower fat content and higher protein content than maize. Resistant starch is a low caloric component of FFs, which is ideal since it is resistant to enzymatic hydrolysis during digestion (Dicko *et al.*, 2006:391; Debabandya *et al.*, 2017:180,181). The starch undergoes partial or complete fermentation in the colon, and this leads to the beneficial production of short-chain fatty acids. This also leads to the production of beneficial gut microflora (Khan *et al.*, 2013b:578).

The fats and lipids present in the germ of cereal grains are rich in polyunsaturated fatty acids (Hadebe *et al.*, 2016:184). The composition of the fatty acids comprising of linoleic (49%), lenolenic (2.7%), stearic (2.1%), oleic (31%) and palmitic acids 14% are higher than the fat in rice and wheat (Léder, 2004:5; Dicko *et al.*, 2006:390). Sorghum wax contains unique health promoting benefits and is concentrated in the pericarp surface of the cereal grain. The pericarp surface is suggested to contain 0.7% hydrocarbons, 1% triacylglycerols, 1.4% sterol esters and wax, 7.5% fatty acids, 41% fatty alcohols and 46% fatty aldehydes (Taylor *et al.*, 2006b:253). Wang *et al.*, (2005:1883) reported that sorghum was gaining interest due to its content of unsaturated fatty acids, sterols and tocopherols, which could have positive health benefits.

2.3.6.3. Phytochemical composition

Phytochemicals are regarded as bioactive compounds in plants, which have gained attention due to their potential health benefiting attributes and disease prevention in humans (Awika & Rooney, 2004:1199; Saxena *et al.*, 2012:130; Taylor *et al.*, 2014c:258). Sorghum has been reported as a diverse and abundant source of phenolic compounds (Althwab *et al.*, 2015:352). One of the most contributing factors to the health benefits associated with sorghum is the phenolic content which ranges from 3 to 43 mg/100 g (Ragaee *et al*., 2006:36; Taylor *et al.*, 2014c:258). There are different classes of phenolic compounds (flavonoids, phenolic acids, phenols, lignans, quinines, xanthones, etc.), which together form a foundational group of compounds, which contain phenol functional groups. Their classification can be based on tannins, increase in molecular weight into phenolic acids and the flavonoid-type compounds (Dykes *et al*., 2006b:238; Ragaee *et al.*, 2006:36; Taylor *et al*., 2014c:258).

2.3.6.4. Phenolic acid and flavonoids

The phenols in sorghum are classified into two groups; (i) phenolic acids which are situated in the endosperm, testa, pericarp and aleurone layer of the kernel (Awika *et al*., 2004:1199; Dykes *et al.,* 2006b:238), and (ii) flavonoids which are grouped into flavanones, flavones, flavanols, anthocyanins, isoflavones, flavon-3-ols and anthocyanidnes, which are situated in the bran layers of the grain (Dykes et al., 2005a:6813; Ghasemzadeh *et al.*, 2011:6698; Del Rio *et al.*, 2013:1820; Althwab *et al.*, 2015:352). Phenolic acids and flavonoids are termed bioactive compounds which are present in plants and are natural antioxidants and contain other biological properties such as anti-diabetic, antiviral, antiulcer, anti-inflammatory, antitumor and anti-cytotoxicity functions (Shahidi, 2004:147; Saxena *et al*., 2012:130-131; Wu *et al.*, 2012a:199).

The chemical structures of flavonoids are distinguished by means of two phenyl rings and a heterocylic ring, which are differentiated by the position and form of the functional groups (Althwab *et al.*, 2015:352). Del Rio *et al*. (2013:1820) stated that the majority of flavonoids occurs naturally as glycosides rather than aglycones. Flavonoids contain 15 carbon atoms comprising of two aromatic rings linked through a heterocyclic pyrane ring. They share the same C6-C3-C6 skeletal structure, having two aromatic C6 rings and a heterocyclic ring with one oxygen atom (Ghasemzadeh *et al.*, 2011:6698-6699; Saxena *et al.*, 2012:131; Del Rio *et al*., 213:1820). The vast health promoting attributes associated with flavonoids are known to be crucial components for medicinal, pharmaceutical, cosmetic and nutraceutical applications and products (Panche *et al.*, 216:1)

2.3.6.5. Tannin content

Tannins and/or condensed tannins (proanthocyanidins) are present in most sorghum varieties, being previously referred to as “brown sorghum” (Waniska *et al.*, 2016:119). The kernels which

contained the condensed tannins were characterized as having a testa that was distinctively thick and highly pigmented, but now “brown sorghum” is classified as tannin sorghum (Awika & Rooney, 2004:1201; Waniska *et al.*, 2016:119). Tannin sorghum has had both positive and negative effects upon consumption, but tannin sorghums are excellent and potent sources of antioxidants (Awika & Rooney, 2004:1201; Dykes *et al.*, 2005a:6813). The antioxidant potency of sorghum bran fractions and extracts has been determined. It is reported that the oxygen radical absorbance capacity (ORAC) levels are remarkably high (tannin sorghum bran 2400 µmol TE.g^{-1}, Sumac sorghum bran 11200 µmol TE.g^{-1}) when compared with commonly consumed tannin-rich fruits such as grapes, cherries, blueberries and cranberries (Awika & Rooney, 2004:1204; Waniska *et al.*, 2016:120).

Waniska *et al*. (2016:120) reported that foodstuffs such as bakery products that contain sorghum bran have a higher antioxidant potential, fibre content and an appealing natural brown to chocolate colour. Anunciacao *et al*. (2017:988) reported that breakfast cereals produced from whole-grain sorghum or tannin sorghum have the potential to be promoted into the market as an alternative for cereals produced from corn and wheat.

2.3.6.6. Vitamin and mineral content

Malnutrition in Africa is a huge problem and coupled with micro and macronutrient deficiencies makes it more serious. Malnutrition and micronutrient deficiencies do not only have an effect on human health, but also have a direct impact on the economy (Micronutrient Forum, 2015:2; Che *et al.*, 2016:11040; FAO, 2019:2). The utilization of indigenous cereal crops such as sorghum can assist in combating food insecurity, malnutrition and nutrient deficiencies (Lindsay, 2010:9).

Sorghum is known to contain various vitamins and minerals which are required for the function of various biological processes and the maintenance of good health (Dicko *et al.*, 2006:390; Achi & Ukwuru, 2015:72-73). Vitamins and minerals are regarded as organic compounds which are deemed as nutritionally essential macro and micronutrients required for biochemical functions and reactions. Humans are unable to synthesise the required amount and therefore need to obtain them through their daily diet (Flynn *et al.*, 2003:119; Walther & Schmid, 2017:131). Macronutrients such as potassium, calcium, magnesium, phosphorus, sulphur, chlorine, and sodium are classified as essential and are therefore required in large quantities. This also includes the essential micronutrients such as iron, silicon, iodine and boron (Ng’uni *et al.*, 2011:436). The bioavailability of various minerals in sorghum is known to be high, with potassium and phosphorus being reported to be abundant (Léder, 2004:5; Ng’uni *et al.*, 2011:347).

Sorghum contains the following fat-soluble vitamins (Dicko *et al.*, 2006:390; Hadebe *et al.*, 2016:184):

1. Vitamin A - a group of unsaturated organic compounds such as retinal, retinol, retinoic acid, and other various pro-vitamin-A carotenoids. Only certain yellow sorghum endosperms contain small concentrations of β-carotene which is a precursor of vitamin A (Che *et al.*, 2016:10042).
2. Vitamin D - secosteroids are responsible for increasing the intestinal absorption of calcium, magnesium, phosphate and zinc.
3. Vitamin E - is regarded as an excellent antioxidant.
4. Vitamin K - a group of structurally similar vitamins required for complete synthesis of certain proteins. Daily recommended intake of the vitamin is 50-120 µg per day (Walther & Schmid, 2017:138).

Sorghum has a good concentration of B-complex vitamins, excluding vitamin B12, which can be produced through microbial fermentation (Hadebe *et al.*, 2016:184; Walther & Schmidt, 2017:150). The B-complex vitamins are in the germ and aleurone and participate in energy metabolism. This cereal grain also contains a high concentration of pantothenate, thiamin, niacin, vitamin B6 and riboflavin (Kulamarva *et al.*, 2009:60; Lindsay, 2010:5).

2.4. Cereal fermentation

In today's society and market, fermentation has become a means of producing products with unique signature organoleptic traits, which can even be more beneficial to consumers by employing further natural processes (Hugenholtz, 2013:155; Marsh *et al.*, 2014:113). Food fermentation is defined as the production of foods and beverages through the controlled growth of desired microorganisms and the conversion of various food constituents by enzymes (Sahlin, 1999:9; Kumari *et al.*, 2015:134; Marco *et al.*, 2017:94). Fermentation is one of the oldest forms and most cost-effective means of preservation known to man and is practiced world-wide for the production and preservation of various food products (Blandino *et al.*, 2003:528; Marsh *et al.*, 2014:113). Food is fermented in order to extend its initial shelf life, improve quality, preserve and create old or new organoleptic characteristics (Van Hylckama-Vlieg *et al.*, 2011:211; Marsh *et al.*, 2014:113). Fermentation has shown to develop and enhance flavour compounds, produce vital enzymes and fatty acids, produce active substances (antimicrobial substances), reduce or eliminate raw material toxicity by removing undesired compounds, increase digestibility and increase nutrient availability as well as the quality (Schoustra *et al.*, 2013:1; Walther & Schmid, 2017:113).

In various underdeveloped and developing African countries, fermented foods and beverages are known to play an important role in the traditional, cultural, and socio-economic

environment of the communities. The fermented foodstuffs are traditionally prepared by women for various cultural rituals and celebrations (e.g., weddings and funerals) or being sold locally at markets (Schoustra *et al*., 2013:1; Kumari *et al*., 2015:134).

2.4.1. The history of fermentation

Fermentation was previously a home-based artisanal practice, but has been up-scaled over the years to semi or full industrial scale to accommodate consumer demands (Blandino *et al.*, 2003:528; Van Hylckama-Vlieg *et al.*, 2011:211; Schoustra *et al.*, 2013:1). The variety of indigenous fermented food products and beverages found in Africa and the rest of the world is primarily based on the availability and affordability of local and/or indigenous raw materials (Guyot, 2012:1109; Mahlomaholo, 2017:7). Many of these methods, ingredients and recipes were passed down from generation to generation (Blandino *et al.*, 2003:528; Schoustra *et al.*, 2013:1).

Cereal-based fermentation is a common practice in many tropical regions. It is exceptionally extensive in Africa, because it has been a simple, common and home-based practice to rely on for producing food for adults, children and weaning foods for infants for centuries (Guyot, 2012:1109; Kumari *et al.*, 2015:134-135). Cereal grains such as sorghum have been utilized for centuries to produce fermented food products which constitute one of the main fundamental dietary components for the various consumers (Guyot, 2012:1109-1110; Achi & Ukwuru, 2015:72; Mokoena *et al.*, 2016:2). The characteristic properties (colour, taste, aroma and texture) of fermented indigenous foodstuffs have shown to be desired by the local consumers (Mokoena *et al.*, 2016:1). The fermentation process is uncontrolled and therefore the sensory attributes of the products is due to the local environmental conditions (Chaves-López *et al.*, 2014:1031).

Sorghum grain possesses ideal substrates for fermentation, including sterols, vitamins, and minerals (Charalampopoulos *et al.*, 2002:132; Kumari *et al.*, 2015:136). Traditional methods of fermentation are either by means of spontaneous fermentation or by back-slopping. In Lesotho, one of the popular snack-beverages produced from fermented sorghum is motoho (Bajpai & Tiwari, 2013:2387).

2.4.2. Motoho: a non-alcoholic fermented beverage

Motoho is consumed daily by the Basotho people of Lesotho. Motoho is a non-alcoholic, sour product and is produced by means of spontaneous fermentation. This is done by back-slopping with a starter culture (tomoso) obtained from a previous batch (Bajpai & Tiwari, 2013:2387; Mahlomaholo, 2017:28). Motoho is a porridge-like slurry produced from brown or red sorghum grains, giving the product a pale brown or brown-red colour (Figure 2.6). The type of sorghum used is based on the region. People in the northern districts of Lesotho prefer using red or

brown/ tannin sorghum whereas yellow or white is preferred in the central and southern districts. Motoho is traditionally produced by women, and has become available in retail stores in recent years (Moodley, 2015a:24-25; Mahlomaholo, 2017:28). It is a convenient refreshment that is offered to visitors (Bajpai & Tiwari, 2013:2387-2388; Moodley, 2015a:24; Mahlomaholo, 2017:28-29).

Figure 2.6: Commercially available Motoho: a) motoho produced from yellow sorghum, and (b) motoho produced from brown sorghum

2.4.3. Production of motoho

In Lesotho, motoho is produced (Figure 2.7) from fine sorghum flour which is mixed with lukewarm water to form a thin slurry, thereafter tomoso is added to the slurry. In the summer, the mixture is allowed to ferment for 10-12 hours, at an average temperature of 30-35 ˚C. In the winter, the mixture is covered with a blanket and allowed to ferment for 24-48 hours. In the highlands of Lesotho, temperatures range from -5-10 ˚C and 5-15 ˚C in the lowlands. When tomoso is not available, stainless-steel utensils (spoon or fork) or a raw potato wedge is added to the flour-slurry in order to facilitate the fermentation process. After fermentation, the liquid at the top of the mixture will be boiled with water, while a small quantity of the remaining liquid and paste will be set aside as tomoso; starter-culture for the next batch. Once the water mixture has reached boiling point, the remaining fermented paste is added to the boiling water and allowed to cook for 15 to 30 minutes. The final product, motoho, is allowed to cool down before consumption. Motoho has a sour taste and in order to combat that, sugar is added to it before consumption (Bajpai & Tiwari, 2013:2388; Moodley, 2015a:24; Mahlomaholo, 2017:28-29).

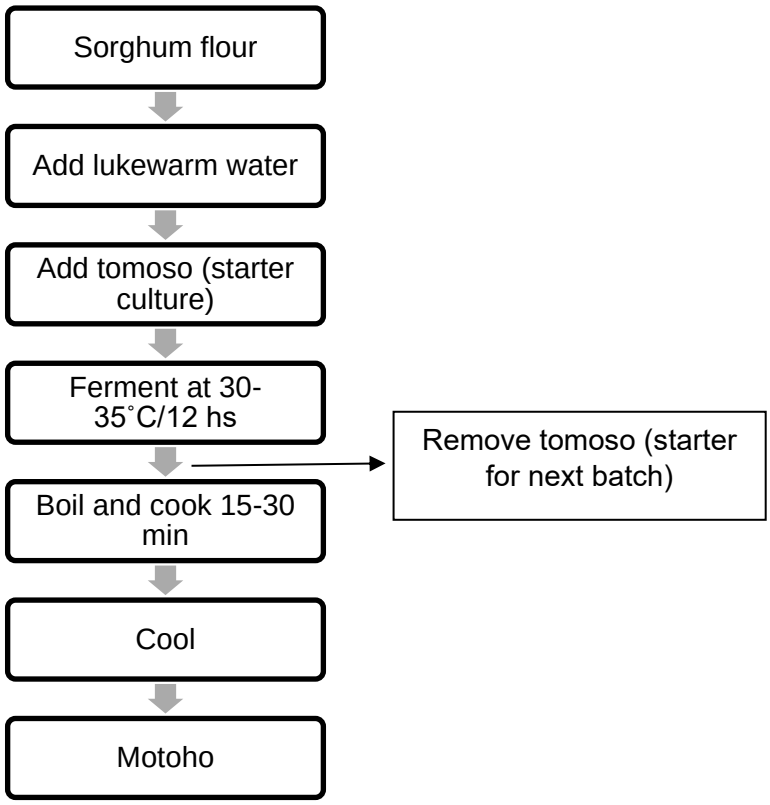

Figure 2.7: The traditional Motoho production process

2.4.4. Health benefits associated with fermented foodstuffs

The human diet consists approximately of one third of fermented foods and beverages (Van Hylckama-Vlieg *et al.*, 2011:211). Rural communities are the major consumers of fermented products and are aware of the health benefits associated with their consumption (Schoustra *et al.*, 2013:1; Mokoena *et al.*, 2016:1). In most African countries, 80% of total caloric consumption including adequate dietary protein is obtained from foodstuffs prepared by spontaneous cereal fermentation (Franz *et al.*, 2014:86).

Several health benefits related to the urogenital tract, cardiovascular, endocrine, gastrointestinal tract, respiratory, nervous and immune systems are associated with the consumption of fermented foodstuffs (Van Hylckama-Vlieg *et al.*, 2011:212-213; Marsh *et al.*, 2014:116-117; Marco *et al.*, 2017:96). This is due to the bioactive peptides which are released during fermentation by proteolytic microorganisms (Marco *et al.*, 2017:96). Health conditions that can be addressed from the consumption of probiotics produced by fermented foods include gastroenteritis, inflammatory bowel disease, genitourinary tract infections and diarrhea (Van Hylckama-Vlieg *et al.*, 2011:157; Hugenholtz, 2013b:156; Marco *et al.*, 2017:96). Fermentation is known to participate in calorie reduction by converting sugars into ethanol and various organic acids which can be beneficial in the production of FFs that address the needs

of obese consumers and those with special dietary needs (Hugenholtz, 2013b:157; Marsh *et al.*, 2014:117).

2.4.5. Fermentation and probiotics

Fermented food products are known to contain probiotics and/or prebiotics (Van Hylckama-Vlieg *et al.*, 2011:211). Probiotics are viable microbial food constituents which stimulate the growth of the desired microflora, and prebiotics are non-digestible food constituents such as starch which positively affect the activity of some selected bacteria in the colon. They are both beneficial to the health of the consumer as their core purpose is to maintain intestinal balance and health (Charalampopoulos *et al.*, 2002:132; Siro *et al.*, 2008:459; Shori, 2016:1). Maintenance of intestinal balance results in the reduction of blood pressure and serum cholesterol level, improved immune system and anti-carcinogenic activity as well as the optimum utilization of nutrients (Tripathi & Giri, 2014:226).

Food products containing probiotics and/or prebiotics are estimated to account for 60-70% of the total FFs market globally (Schrezenmeir & de Vrese, 2001:361; Salmeron *et al.*, 2015:106). Certain microbial strains of lactic acid bacteria (LAB) and propionic acid bacteria (PAB) possess probiotic potential, and synthesize vitamin B12 (Hugenschmidt *et al.*, 2010a:852). They are classified as food-grade microorganisms and have been utilized in the production of various food products, with the concentration of the synthesized vitamins being dependent on the incubation environment, microbial species and strain (Hugenholtz *et al.*, 2002a:104; Hugenschmidt *et al.*, 2010a:852, 2011b:1063; LeBlanc *et al.*, 2011:1297). For this reason fermented food products have been recommended by numerous health practitioners and researchers as part of the daily dietary intake (Marsh *et al.*, 2014:121-122; Marco *et al.*, 2017:99).

2.4.6. Vitamin fortification by means of fermentation

Vitamin deficiency among the low socio-economic populations in Southern Africa is reported to be one of the highest in the world, and also had a significant impact in other parts of the world (Micronutrient Forum, 2015:2; Che *et al.*, 2016:11040). Vitamin deficiencies have negative effects on fetus development, cognitive development and function as well as the overall human working ability, general health and quality of life (Brinch-Pedersen *et al.*, 2007:308-309; Soh *et al.*, 2020:1).

In order to sustain human life, there are approximately 49 various vital nutritional components, including carbohydrates, water, 13 vitamins, 10 essential amino acids, linoleic and linolenic acids, seven mineral macro-elements and 16 minerals which are required through diet (Brinch-Pedersen *et al.*, 2007:308; Walther & Schmid, 2017:131). Various studies have illustrated that vitamin deficiencies such as vitamin B12 can lead to negative health

consequences (Burgess *et al.*, 2009:6; Hugenschmidt *et al.*, 2011b:1036). Inadequate dietary intake of vitamin B12 (vit B12) is regarded to be prevalent in middle to low-income countries, especially in expecting mothers, young children and the elderly (Venkatramanan *et al.*, 2016:879; Soh *et al*., 2020:1). It is therefore of great importance to find ways to address such vitamin deficiencies (Chamlagain, 2016a:17). One of the proposed approaches is by mandatory fortification or the development and promotion of fortified fermented food products and employing microbial cultures which specifically synthesize the vitamin in the required concentrations (LeBlanc *et al.*, 2011:1305; Deptula *et al.*, 2017b:2).

2.5. Cobalamin (vitamin B12)

The ability to synthesize cobalamin is reported to be restricted to a few bacterial and archaea species (Burgess *et al.*, 2009:4; Hugenschmidt *et al.*, 2010a:852). Humans are unable to synthesize cobalamin (Walther & Schmid, 2017:150). Cobalamin is commonly referred to as vit B12, which is a stable, water soluble, sensitive to light, reducing and oxidizing agent (Walther & Schmid, 2017:150). Vit B12 is also the general name used for corrinoids that contain the biological activity of cyanocobalamin (Burgess *et al.*, 2009:4; Fang *et al.*, 2017:1). Vit B12 is synthesized by means of microbial fermentation, in a fed or batch process on an industrial scale (Piao *et al.*, 2004:167). Vit B12 can also be found in animal-based food products due to the presence of microorganisms in animal tissue, which are able to synthesize the vitamin (Chamlagain, 2016a:11; Walther & Schmid, 2017:150).

2.5.1. The chemical structure and function of vitamin B12

The structure of vit B12 (Figure 2.8) consists of a corrin ring with a central cobalt ion (Chamlagain, 2016a:13), a dimethylbenizmidazole (DMB) moiety at the base, and various upper axial ligands (“R”), i.e., adenosylcobalamin, methylcobalamin, glutathionyclcobalamin, hydroxocobalamin, and cyanocobalamin (Chamlagain, 2016a:13; Froese *et al.*, 2019:674). Vit B12 is an essential vitamin for humans and is required as a co-factor for methionine synthase and methylmalonyl-CoA mutase (Walther & Schmid, 2017:150; Froese *et al*., 2019:674). Vit B12 therefore plays a vital role in the synthesis of DNA, hemoglobin as well as the metabolism of fatty acids and amino acids (Deptula *et al*., 2015a:2; Walther & Schmid, 2017:150; Froese *et al.*, 2019:674).

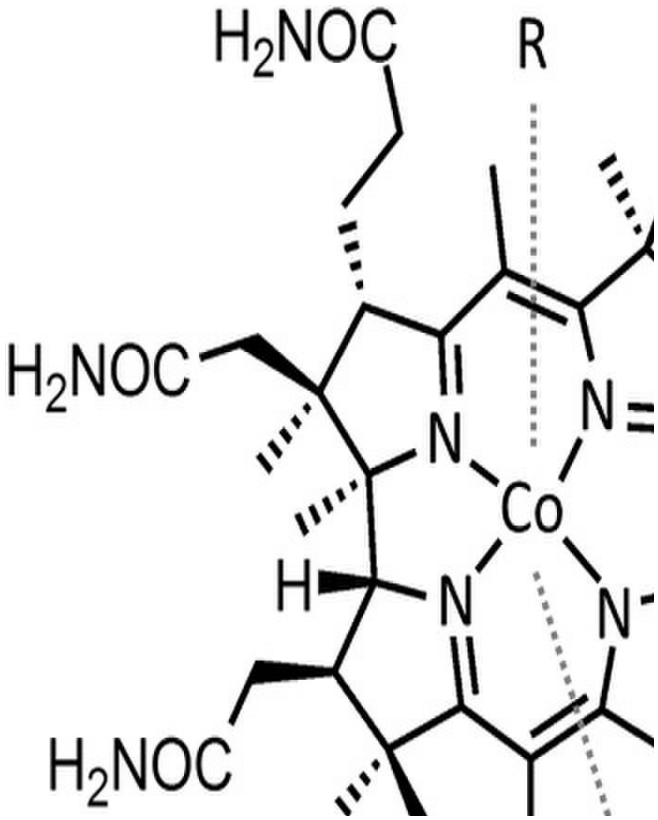

Figure 2.8: The chemical structure of vitamin B12
DBM: dimethylbenizmidazole
("R"): axial legands i.e., adenosylcobalamin, methylcobalamin, glutathionyclcobalamin, hydroxocobalamin, and cyanocobalamin

(*Froese *et al., 2018).

2.5.2. Health benefits associated with vitamin B12

The consumption of foodstuffs containing vit B12 is vital for the development and maintenance of good health (Deptula *et al.*, 2015a:2; 2017b:2). The recommended daily intake of vit B12 is 2.4 mg/day for adults (Van Wyk *et al.*, 2011b:69; Chamlagain, 2016a:16). In a normal diet, food products of animal origin are almost the only source of vit B12, which increases the probability of vit B12 deficiency in consumers who are adhering to strict vegan or vegetarian diets (Burgess *et al.*, 2009:4; Capozzi *et al.*, 2012:1390; Venkatramanan *et al.*, 2016:879). It was suggested that shellfish is an ideal source of the vitamin, for they scavenge microbes in their surroundings, which result in a high content of the vitamin (Burgess *et al.*, 2009:4; Chamlagain, 2016a:16). A deficiency in vit B12 can have negative health effects, with symptoms not being immediately evident upon initial observations (Green *et al*, 2017:1-2). Pernicious anemia is one of the well documented results of vit B12 deficiency. It is known to manifest with neuropathy and megaloblastic anemia (caused by failed or delayed synthesis of DNA and RNA in the intestinal mucosa and bone marrow, where there is a high cell production rate) as the primary symptoms (Burgess *et al.*, 2009:4; Chamlagain, 2016a:14; Green *et al.*, 2017:2). Other

deficiencies associated with vit B12 are psychiatric disorders and neurological damage (Hugenschmidt *et al.*, 2011b:852; Chamlagain, 2016a:14; Green *et al.*, 2017:1).

2.5.3. Vitamin B12 fortification and optimization

Walther & Schmid (2017:150) reported that the concentration of vit B12 in fermented milk products such as yoghurt decreased during fermentation, because certain LABs required vit B12 for growth. This promotes the notion of cereal-based fermentation as a means of vit B12 fortification and optimization (Chamlagain, 2016a:63-34). Cereal grains possess adequate levels of vitamins, minerals and fermentable sugars, which can be utilized by microorganisms during fermentation (Achi & Ukwuru, 2015:72-73). For cereal fermentation in Africa, LAB are known to be the most common and domninant bacteria involved in the process (Mokoena *et al.*, 2016:1-2). For traditional fermentation, the process occurs by pure chance (spontaneous fermentation) (Aka *et al.*, 2014:104). With spontaneous fermentation, mixed bacterial cultures and/or yeasts are present, with *Bacillus*, *Leuconostoc, Pediococcus, Streptococcus, Lactobacillus* and *Micrococcus spp.* being the most commonly identified microorganisms (Blandino *et al.*, 2003:529; Achi & Ukwuru, 2015:74). *Lactobacillus* is known to possess the ability to synthesize vit B12 (Achi & Ukwuru, 2015:74,78; Deptula *et al.*, 2017b:2). With regards to vit B12 fortification of fermented food products, it has not been exploited significantly (Chamlagain, 2016a:63).

2.5.4. Selection of microorganisms for fermentation

The development, production and promotion of FFs containing vital and/or beneficial nutrients by means of cereal fermentation can assist in attending to the growing need for healthy foodstuffs (Stanton *et al.*, 2005:199; Achi & Ukwuru, 2015:73). In order to produce the desired food, it is vital to ensure the selection of suitable raw materials, fermentation parameters and microbial starter cultures (Van Hylckama-Vlieg *et al.*, 2011:216). The selection of food-grade microbial cultures assists in providing and improving the nutritional content and quality of the food product. It is of great importance to identify, study, and promote microbial strains which show potential in elevating the nutritive quality of natural vitamins when employed during fermentation (Sahlin, 1999:56; LeBlanc *et al.*, 2011:1297-1298; Walther & Schmid, 2017:152).

2.5.5. Isolation and identification of microorganisms from motoho

Studies which have focused on traditionally fermented African food products and beverages have established that yeasts and LAB are the most populous microorganisms present (Achi & Ukwuru, 2015:74; Moodley *et al.*, 2019b:1). Mahlomaholo (2017:58-59) stated that *Firmicutes* and *Proteobacteria* were the dominating phyla, with a notable decrease in *Proteobacteria* as fermentation progressed. The decrease in the *Proteobacteria* and increase in *Firmicutes* is preferable, as it indicates reduction or elimination of harmful components, which can compromise the safety of the product (Moodley, 2015a:5; Mahlomaholo, 2017:58). Moodley *et*

al. (2019b:5) conducted a study whereby genotypic and phenotypic traits were employed to isolate and identify 24 yeast and LAB strains to species level. *Lactobacillus fermentum* was the most dominant species during fermentation and suggested as a possible starter culture.

2.6. Propionibacterium

The genus *Propionibacterium* belongs to the order *Actinomycetales* and include approximately 12 species (Thierry *et al.*, 2011:20). Propionibacteria are characterized as being Gram-positive, hetero-fermentative, mesophilic, pleomorphic rods, aerotolerant organisms with a high GC-content. Propionic acid bacteria are also characterized by their anaerobic metabolism, which involves various carbon rearrangement reactions (Vorobjeva *et al.*, 2008:110; Liu *et al.*, 2012a:375; Turgay *et al.*, 2020:2). Propionic acid bacteria are categorized into two functional categories (Table 2.2), i.e. dairy PAB and cutaneous PAB. The dairy PAB are characterized as non-pathogenic, anaerobic to aerotolerant and non-motile and with most strains being catalase positive (Faye *et al.,* 2011:549; Thierry *et al.,* 2011:20; Turgay *et al.,* 2020:2). Propionic acid bacteria have a unique metabolism which allows them to ferment various sugars and lactate in order to produce propionic acid as their main product (Danilova *et al.*, 2012:49; Liu *et al*., 2012a:375).

Table 2.2*:* Categorization of propionic acid bacteria (Cousin *et al*., 2011:3)

Dairy propionibacteria	**Cutaneous propionibacteria**
Propionibacterium freudenreichii subsp. freudenreichii	*P. acnes*
P. freudenreichii subsp. shermanii	*P. avidum*
P. acidipropionici	*P. acidifaciens*
P. jensenii	*P. australiense*
P. microaerophilum	*P. granulosum*
P. thoenii	*P. propionicum*
P. cyclohexanicum	

Propionic acid bacteria are considered GRAS (Generally Recognized as Safe) by the United States Food and Drug Administration (US FDA). GRAS microorganisms do not produce endotoxins and/or exotoxins. They are therefore authorized to be utilized as natural food preservatives, due to their bacteriocidic effect on microorganisms which are closely related to them (Danilova *et al.*, 2012:49; Deptula *et al.,* 2015a:2). It has also been established that certain species of *Propionibacterium* exhibit great potential in synthesizing vit B12 intracellularly by means of fermentation and have therefore been regarded as probiotics (Piao *et al*., 2004:168; Zhang *et al.*, 2010:139; Thierry *et al.*, 2011:20; Danilova *et al.*, 2012:49).

2.6.1. The use of propionic acid bacteria in food manufacturing

Propionibacterium spp. have attracted great attention due to the economic advantages of producing vit B12 (Zhang *et al.*, 2010:139; Chamlagain *et al.*, 2016b:118). One of the well documented PAB strains is *Propionibacterium freudenreichii (P. freudenreichii).* It is popularly used in the manufacturing of fermented food products due to its GRAS status. It is well known for its use in the production of Swiss Emmental cheese (Cousin *et al.*, 2011a:4; Turgay *et al.,* 2020:7-8). *P. freudenreichii* is reported to produce vit B12 concentrations of approximately 15 $\mu g.mL^{-1}$ and trace concentrations of pseudocobalamin (Deptula *et al.*, 2015a:2; Wang *et al.*, 2015:123; Chamlagain, 2016b:118). *P. freudenreichii* is one of four dairy species isolated from milk and the recorded genome population ranges around 2.6 Mb with a G+C content of 67% (Thierry *et al.*, 2011:20; Turgay *et al.*, 2020:4). With regards to microbial fermentation, it has been established that *P. freudenreichii* is capable of fermenting various substrates such as polyols and carbohydrates (legumes, cereals, fruits and vegetables) (Vorobjeva *et al.*, 2008:110; Thierry *et al.*, 2011:21; Chamlagain, 2016a:22). By-products produced during fermentation include carbon dioxide, and succinic, propionic, and acetic acids (Faye *et al.*, 2011:550; Turgay *et al.,* 2020:4).

Vitamin fortification by means of fermentation with food-grade microbial cultures such as *P. freudenreichii* has been suggested and is gaining momentum due to its bifidogenic and probiotic potential and application (Cousin *et al.*, 2011a:19; Van Hylckama-Vlieg *et al.*, 2011:216; Chamlagain, 2016:117, 122-123). The method will allow to increase the nutritive value of food products economically and without the preparation of synthetic vitamin compounds (Piao *et al.*, 2004:167; Wang *et al.*, 2015:123; Chamlagain *et al.*, 2016b:117, 122-123). Other advantages of using propionibacteria include (i) they do not have complex nutritional requirements for growth, and (ii) they are able to grow in a wide range of temperature and pH environments (growth temperature range, 15-40°C; pH range, 5.1-8.5; optimum growth environment, 30°C and neutral pH) (Thierry *et al.*, 2011:22; Turgay *et al.*, 2020:3,7).

Vit B12 concentration in fermented milk products is reported to be low, due to the utilization of vit B12 by LAB (Walther & Schmid, 2017:150). *P. freudenreichii* growth and survival in milk fermentation is strain dependent, due to the inability to utilize lactose (Chamlagain *et al.,* 2016b:119; Turgay *et al.,* 2020:8-9). Some dairy propionibacteria have been reported to grow poorly in milk, with *P. freudenreichii* adapting poorly in milk. This is because of *P. freudenreichii* being unable to utilise the nitrogen in milk (Cousin *et al.*, 2012b:136). Deptula *et al.* (2017b:6, 8-9) noted that the growth of *P. freudenreichii* in milk and whey-based mediums can be overcome by means of supplementing the growth medium with yeast extracts. Cousin *et al*. (2012b:135-136, 145) produced a fermented dairy product from an exclusive dairy *P. freudenreichii* strain. The development of a dairy *Propionibacterium*

culture further increases the potential for further exploitation of *P. freudenreichii* and other food-grade *Propionibacterium* species. Van Wyk *et al*. (2011b:73) reported that vit B12 concentrations in fermented kefir increased by 9-folds from 2.2 mg.100 mL^{-1} to 9.2 mg.100 mL^{-1} following fermentation with *P. freudenreichii*. Cereal fermentation by *Propionibacterium* has not yet been exploited and with the characteristics of *P. freudenreichii* illustrating its advantages, it still remains unexploited (Chamlagain, 2016a:58,64).

2.6.2. The prospects of indigenous fermented functional foods

Traditional foodstuffs are defined as a representation of the culture and lifestyle of a population as factors such as the local climatic, agricultural and economic conditions determine the production, processing and consumption practices. It represents the expression of the communities which consume them (FAO, 2008a:2; Bhaskarachary *et al.*, 2016:1565-1566). Fermented foods and beverages have been regarded as FFs due to their probiotic and/or prebiotic properties that have been proven to be beneficial to the health of consumers (Stanton *et al.*, 2005:199; Marco *et al.*, 2017:96). Various fermented foodstuffs have been developed on household level and form part of the staple foods typically consumed in communities (Hugenholtz, 2013b:155). Several of these products are presently considered artisanal and are produced on industrial scale (Van Hylckama-Vlieg *et al.*, 2011:211; Walther & Schmid, 2017:131). These include products developed from meats, fish, fruits, vegetables, legumes and cereals.

Cereal fermentation is commonly practiced in Africa (Blandino *et al.*, 2003:529). Sorghum is one of the most cultivated cereals in Africa and is commonly fermented to produce various foods and beverages (Hadebe *et al*., 2016:178; Debabandya *et al.*, 2017:180), but the positive agricultural, economic and nutritional traits are far overshadowed by maize (Hadebe *et al.*, 2016:181). These traits, as well as the potential applications in SSA are not sufficiently exploited. More effort is needed for promoting the cultivation and use of sorghum (Taylor, 2003a:18-19; Belton & Taylor, 2004:94; Debabandya *et al.*, 2017:182). Motoho is a traditional food product and conforms to The European Food Information Resource (EuroFIR) Consortium's definition of traditional food. Motoho has specific features which distinguish it from similar characteristics in the same category with regards to orthodox composition, utilization of raw materials and processing or production (Weichselbaum *et al.*, 2009:4-6; Bhaskarachary *et al.*, 2016:1566).

Various microbial species involved during the fermentation of cereals in Africa are documented, but LAB are commonly known and well-studied (Schoustra *et al.*, 2013:1; Mokoena *et al.*, 2016:1). Although food-grade *Propionibacterium* synthesize vit B12, sorghum fermentation with this bacterium has not yet been adequately explored (Chamlagain,

2016a:63-34; Turgay *et al.*, 2020:6). The development of motoho as a FF produced from sorghum with the application of *Propionibacterium* as the starter culture can give rise to new research and product development scope (Lindsay, 2010:23; Chamlagain, 2016a:64; Debabandya *et al.*, 2017:182). This would lead to the production of a range of food products that meet the nutritional requirements for consumers especially in SSA (Van Wyk, 2011a:858; Achi & Ukwuru, 2015:79; Mahlomaholo, 2017:8). The further development of sorghum foodstuffs can increase the research, development and promotion of indigenous African foods produced by means of fermentation.

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CHAPTER 3:
MICROBIAL SUCCESSION, ISOLATION AND IDENTIFICATION OF *PROPIONIBACTERIUM* IN MOTOHO

3.1. Abstract

Motoho is a fermented sorghum beverage, produced by means of spontaneous fermentation and back-slopping with a small quantity of previously fermented product called tomoso. The aim of the study was to observe microbial succession, isolate and identify propionic acid bacteria (PAB) and other dominant bacterial strains from motoho. Motoho was prepared using two methods; the traditional method (back-slopping with tomoso) and by co-inoculation with tomoso and *P. freudenreichii* (PABJ-17) culture (broth and freeze-dried) at low and high inoculum cell concentrations. Fermentation was facilitated at 30 °C for 12 h. Sampling, pH, and microbial counts were performed every 3 h. The following organisms were enumerated: total viable counts (TVCs), coliforms, lactic acid bacteria (LAB), Yeasts and moulds (Y&M) using PCA, VRBA, MRSA and RBA culture media, respectively. For isolation and identification of Propionibaterium in motoho, the fermented slurry (MFS) (control) was inoculated into yeast extract sodium lactate-broth, incubated at 30 °C for 5 d and streaked onto Yel-agar, which was incubated anaerobically for 7 d. The dominant isolate obtained was identified by applying Gram-staining, DNA extraction, PCR, Vitek analysis and phylogenetic analyses.

After 12 h of fermentation, the TVCs for MFS (9.30 ± 0.58 log cfu.g^{-1}) were significantly higher ($p \leq 0.05$) then LPAB-J17 (8.02 ± 0.06 log cfu.g^{-1}), with no significant difference between MFS and HPAB-J17 (9.24 ± 0.02 log cfu.g^{-1}). and The MFS (3.85 ± 3.26 log cfu.g^{-1}) coliforms were ($p \leq 0.05$) lower than HPAB-J17 4.41 ± 0.95 log cfu.g^{-1}. The LAB was the most dominant organisms after 12h, with MFS (9.02 ± 0.6 log cfu.g^{-1}) being significantly ($p \leq 0.05$) higher than LPAB-J17 (8.32 ± 0.83 log cfu.g^{-1}) and HPAB-J17 (7.78 ± 1.52 log cfu.g^{-1}). MFS (3.52 ± 0.87 log cfu.g^{-1}) yeast count were significantly lower ($p \leq 0.05$) than LPAB-J17 (5.21 ± 0.33 log cfu.g^{-1}) and HPAB-J17 (4.78 ± 0.56 log cfu.g^{-1}). The colonies isolated on Yel-agar were small, light cream in colour, smooth surfaces with entire edges. The isolate obtained stained Gram-positive, short rods with a central endospore. Vitek analysis gave a low discrimination result, which was inconsistent with PAB. Furthermore, the results for the PCR identification for PAB were inconclusive, which disqualified the isolate as PAB. The 16S rRNA sequence analysis of the isolate gave a 99.50 % similarity to *Bacillus rugosus* SPB7, which is an emerging organism that has been isolated from sea sponges.

KEYWORDS

Motoho, tomoso, *Propionibacterium freudenreichii,* Vitek, 16S rRNA sequencing, phylogenetic analysis.

3.2. Introduction

Sorghum [*Sorghum bicolor (L.) Moench*] (Tian *et al.*, 2016) is drought and heat tolerant as well as being able to handle temporal waterlogging, which makes it ideal for cultivation in the Sub-Sahara African (SSA) climate (Lindsay, 2010:4; Hadebe *et al.*, 2016:178). There is an increasing trend in sorghum consumption especially in developed countries. This is due to the antioxidant potential, polyphenolic phytochemicals and nutritional benefits offered by the cereal grain (Althwab *et al.*, 2015:351; Wu *et al.*, 2017:347). In Lesotho, sorghum is used to prepare motoho, which is a non-alcoholic, sour porridge produced by means of fermentation. Motoho is produced by means of spontaneous fermentation or by back-slopping with small amount of previously fermented motoho, called tomoso (Mahlomaholo, 2017:28). Motoho is consumed at household level, prepared as weaning food, snack-beverage as well as sold in the local market (Bajpai & Tiwari, 2013:2388; Mahlomaholo, 2017:28).

Fermentation enhances the nutrient content and availability of various vitamins such as vitamin B12 (cobalamin; vit B12) (Hugenholtz, 2013:156; Walther & Schmid, 2017:131). This is accomplished by employing specific microorganisms such as yeasts, LABs and PABs (Sahlin, 1999:9; Giraffa, 2004:252). Fermented products are regarded as functional foods due to their probiotic and/or prebiotic content (Stanton *et al.*, 2005:198-199; Marco *et al.*, 2017:98). Microbial strains of LAB and PAB have been reported to exhibit probiotic and/or prebiotic potential (Charalampopoulos *et al.*, 2002:133; Leblanc *et al.*, 2007:1297). In most African communities, cereal fermentation is achieved by spontaneous fermentation or back-slopping (Achi & Ukwuru, 2015:74). In African cereal fermentation, LAB have shown to be the most dominant microorganisms followed by yeasts (Guyot, 2012:1111; Mokoena *et al.*, 2016:1). Though interest has increased in fermented foodstuffs, data pertaining to the microflora and probiotic potential of African cereal fermented foodstuff is still limited (Franz *et al.*, 2014:87; Mokoena *et al.*, 2016:1-2).

There is very little information known regarding the microflora involved during the fermentation process of motoho and in the finished product (Bajpai *et al*., 2013:2388; Moodley, 2015:24). PAB have a unique metabolism which allows them to ferment various sugars and lactate in order to produce propionic acid as their main product (Turgay *et al.,* 2020:5-6). Food-grade propionibacteria synthesize vit B12 significantly in fermented food products. Cereal

fermentation by *Propionibacterium* has not been exploited and with the advantageous characteristics exhibited by *P. freudenreichii*, it still remains unexploited (Chamlagain, 2016:12,22; Turgay *et al*., 2020:7-8). This study aimed to (1) evaluate microbial populations during fermentation in three models: traditional method (control), co-inoculation of starter culture with PAB-J17 at two different inoculum cell concentrations); and (2) isolate, identify and characterise the dominant strain present in motoho.

3.3. Materials and methods

3.3.1. Chemical reagents and other chemicals

All the chemicals employed in the study were of Analytical-grade and therefore prepared according to their standard analytical methods. Milli-Q water (18.2 MΩ.cm-1), purified using the Milli-Q water purification system (Millipore, Microsep, South Africa) was employed for preparing solutions and dilutions. All prepared reagents were stored under conditions that will prevent deterioration or contamination.

3.3.2. Preparation of growth media

3.3.2.1. Yeast extract-peptone lactate media (Yel-media (broth/agar)

Yel-media was prepared according to Van Wyk & Britz (2010). To 1 L of Milli-Q water 1.0 g Tween 80 (Saarchem), 2.0 g peptone (Biolab), 5.0 g yeast extract (Biolab,Merk), 10.0 g KH_2PO_4 (Merck), 20.0 g sodium lactate syrup (16.6 mL of 60 % solution) (United Scientific). To prepare solid media, 15.0 g bacteriological agar (Merck) was added. The Yel-media pH was adjusted to pH 7 using 2 M NaOH (Sigma Aldrich). Media was used for the isolation of presumptive positive PAB isolates.

3.3.2.2. Trypticase soy broth (TSB)

Trypticase soy agar/broth was prepared by adding 10.0 g yeast extract (Biolab, Merck), 10.0 g trypticase soy (Merck), 20.0 g sodium lactate syrup (16.6 mL of 60 % solution) (United Scientific) and 1.0 g Tween 80 (Saarchem) were added to 1 L of Milli-Q water to prepare the broth. To prepare the solid media, 15 g bacteriological agar (Merck) was added. The pH for both the TSA and TSB was adjusted to pH 7 using 2 M NaOH (Sigma Aldrich). The Yel-broth and TSB were respectively decanted into 250 mL bottles. The media was used to grow PAB freeze-dried cultures which were used as positive controls; inoculate was incubated at 30 °C for 7 days.

3.3.2.3. Vitamin B12 media (VBM)

Vit B12 media was prepared according to Van Wyk & Britz (2010). To 1 L of Milli-Q water, the following were added: 0.0003 g (+) Biotin (Fluka), 0.004 g Ca-pantothenate (Fluka), 0.01 g

$FeSO_4.7H_2O$ (Merck), 0.018 g $CoCl_2.6H_2O$ (Merck), 0.07 g 5,6-dimethyl-benzimidazole (DMBI) (Merck), 1.0 g Tween 80 (Saarchem), 1.76 g K_3PO_4 (Merck), 2.25 g $NaH_2PO_4.2H_2O$ (Merck), 2.5 g yeast extract (Difco), 5.0 g polypeptone (BBL), 11.0 g casamino acid (Difco), 40.0 g glucose (BDH chemicals) and 40.0 g $MgCl_2.6H_2O$ (Merck). Media was used to grow the PAB-J17 freeze-dried culture, which was used for co-inoculation with tomoso (Morkel, 2016:59-60).

After preparation, the media (Yel, TSA/TSB and VBM) were autoclaved at 121°C for 15 min., cooled, inoculated and incubated at 32 °C for 7 days.

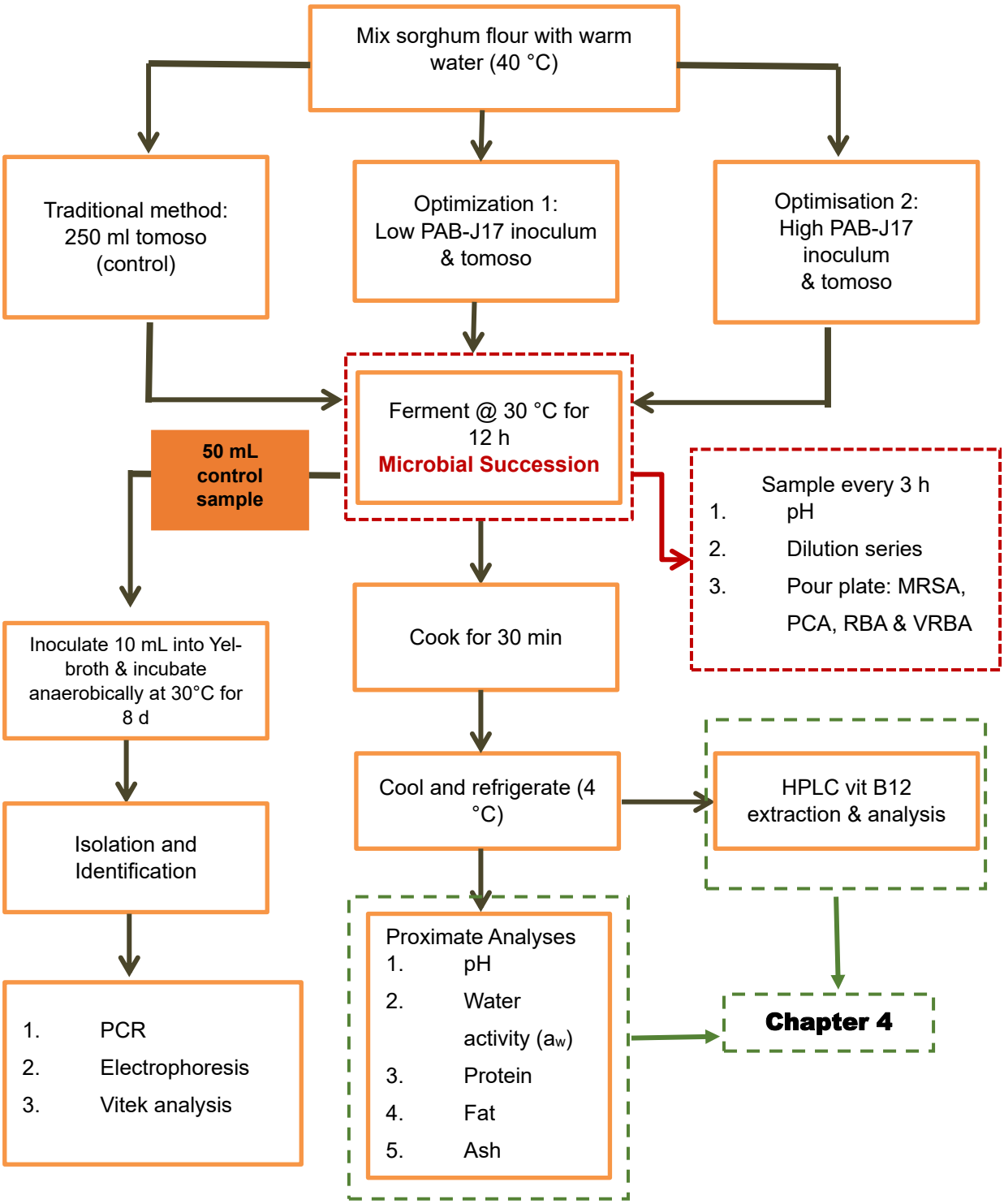


Figure 3.1: An outline of the research methodology

3.3.3. Preparation of Motoho

Motoho was prepared by employing two methods: (i) the traditional method (back-slopping with tomoso) and (ii) modified method by co-inoculating tomoso with PAB-J17 at low and high inoculum cell concentrations. PAB-J17 was a reconstituted freeze-dried culture of *P. freudenreichii* subsp. *shermanii* ATCC13637, which was renamed to strain J17 in the culture collection of the Department of Food Science and Technology, Cape Peninsula University of Technology.

3.3.3.1. Preparation of tomoso

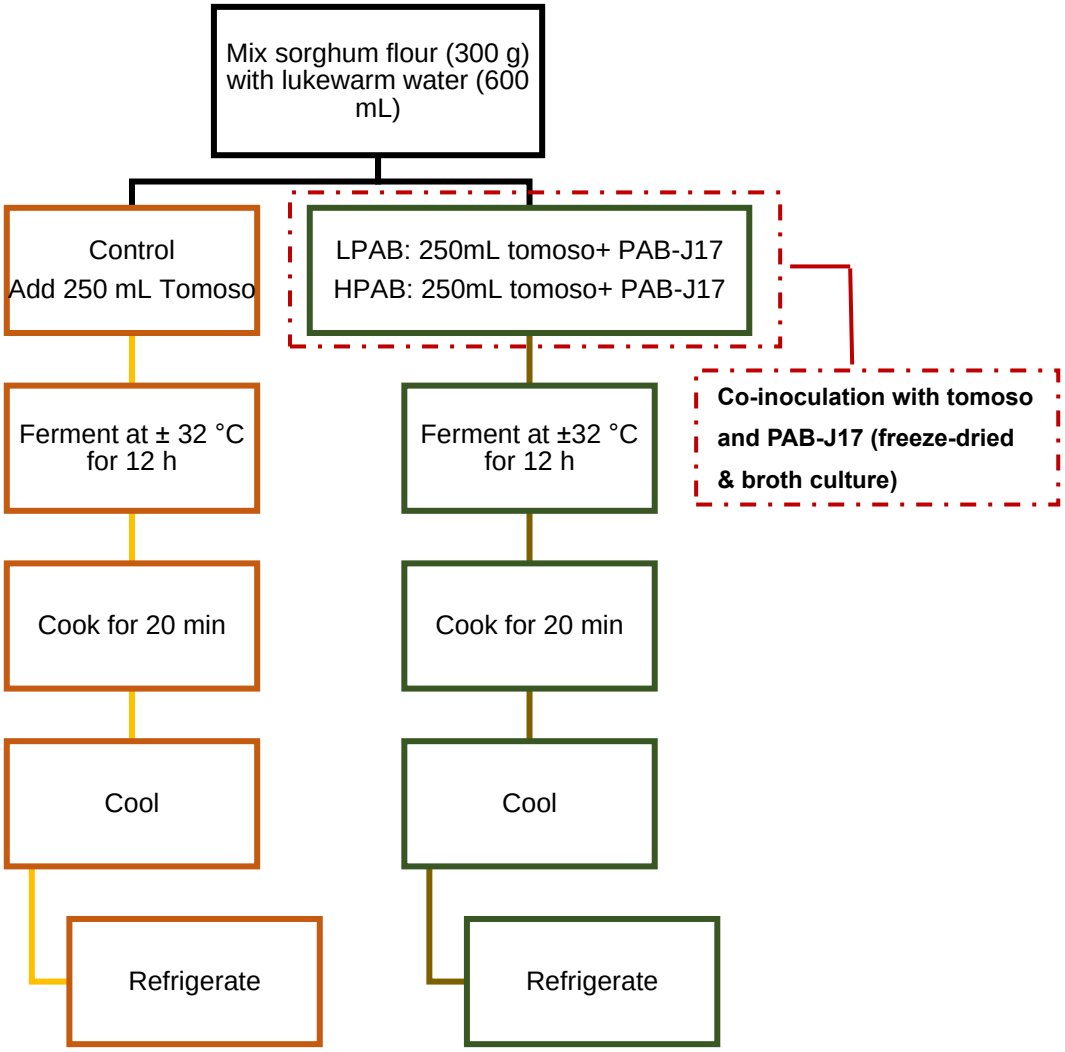
Tomoso (starter culture) was prepared by mixing 100 g fine sorghum flour purchased from a market in Maseru, Lesotho, with 500 mL lukewarm water. The slurry was homogenized by shaking the bottle vigorously for a few seconds. The slurry was incubated at 37 °C for 48 h. The fermented slurry was regarded as tomoso, and was used as the back-slopping starter culture.

3.3.3.2. Traditional method of fermentation

Motoho was prepared according to the traditional method (Figure 3a) by first preparing tomoso. Motoho was prepared by mixing 300 g of sorghum flour with 600 mL of lukewarm water to form a slurry, and then 250 mL of tomoso was back-slopped into the slurry. The mixture was fermented at 30 ˚C for 12 h. After fermentation, 700 mL water was boiled in a pot, and the fermented liquid (supernatant) was added to the pot and allowed to boil. Once the mixture was boiling, the fermented paste (sediment) was added to the pot while stirring continuously to prevent the formation of lumps. The mixture (motho) was cooked for 20 min over low heat. The final product, motoho, was then poured into sterile bottles and allowed to cool to room temperature before being refrigerated.

3.3.3.3. Optimisation of inoculum strength: co-inoculation with tomoso and PAB-J17 at low and high inoculum cell concentrations

Inoculum preparation: a freeze-dried PAB-J17 culture was inoculated into VBM media and incubated at 30 °C for 7 days for pre-enrichment. After incubation, the active culture was centrifuged at 12 × 1 000 g for 5 min (Morkel, 2016:59-60). The supernatant was discarded and the sediment (pellet) re-suspended in 5 mL VBM, and together with the tomoso, used as inoculum. The PAB-J17 inoculum was standardised to $1x10^8$ cfu/mL using serial dilutions in VBM. Two PAB-J17 inoculum strengths were prepared, namely LPAB-J17 (low inoculum) and HPAB-J17 (high inoculum). Three batches of motoho were prepared: 300 g of sorghum flour was mixed with 600 mL of lukewarm water to form a slurry. The batches were each inoculated with (i) 250 mL of tomoso (control), (ii) 250 mL of tomoso and LPAB-J17, and (iii) 250 mL tomoso and HPAB-J17, respectively.



3.3.4. Microbial succession: microbial isolation and enumeration

Motoho was fermented at 32 ˚C for 12 h, during which a 50 mL sample was extracted every 3 h. For each sample, the pH was analysed, serial dilutions (10^{-1} to 10^{-8}) were prepared and pour plated in triplicates. The media used was prepared according to manufacturer's instructions. The media used was Plate Count Agar (PCA), Violet Red Bile agar (VRBA), Rose Bengal Chloramphenicol Agar (RBCA) and De Man, Rogosa and Sharpe Agar (MRSA). The PCA plates together with the MRSA and VRBA plates were incubated at 37 ˚C for 48 h, whereas the RBCA-plates were incubated at 25 ˚C for 5 days. Once the incubation periods had elapsed, the colonies were enumerated with the aid of a colony counter.

3.3.5. Isolate identification and characterisation

For isolate identification and characterisation, 10 mL of the fermented motoho slurry (MFS) (control), was inoculated into 90 mL Yel-broth for pre-enrichment at 30 °C for 5 days. After incubation, the broth culture was streaked onto Yel-agar, incubated anaerobically at 30 °C for 7 days. The colonies obtained were subjected to Gram-staining, Vitek 2 system analysis, PCR identification as well as 16S rRNA sequencing.

3.3.6. DNA extraction and Polymerase Chain Reaction

DNA-extraction and purification was performed with an ABIOpure (version 2.0), Total DNA/ Blood/ Cell/ Tissue Extraction kit (cat no: M501DP100).

Isolated colonies from motoho fermented slurry (MFS) obtained from Yel agar plates were utilised for DNA extraction and PCR, with PAB-J17 used as the positive control. The isolated colonies were suspended into 1.5 mL micro-centrifuge tubes containing 200 µL of CL-buffer. The suspension was vortexed for 30 sec and then centrifuged at 13 000 × g for 1 min. The supernatant was discarded. The cells were re-suspended in 200 µL CL-buffer and 20 µL Proteinase K, the mixture was vortexed vigorously for 1 min. The samples were incubated in a water bath at 56 ˚C until the lysis was complete (mixture was clear after 25 min). After cell lysis, samples were spun-down briefly and 200 µL of BL-buffer added to each sample. The samples were vortexed for 30 sec and incubated at 70 ˚C for 10 min in a water bath. After incubation, 200 µL of absolute ethanol was added to sample, pulse- vortexed and briefly spun-down. The sample was then cautiously transferred to mini columns, centrifuged for 1 min at 6000 × 10 g, after which the collection tube was replaced. Caution was taken to ensure that all the solution had passed through the membrane. Six hundred µL of BW-buffer was added, the sample centrifuged for 1 min at 6000 × 10 g, and the collection tube replaced. Then 700 µL of TW-buffer was added to sample, which was centrifuged 1 min at 6000 × 10 g, the pass-through was discarded and the mini column was reinserted into the collection tube. The sample was centrifuged at 13000 ×10 g for 1 min to remove the residual wash buffer and the mini column inserted into a clean 1.5 mL Eppendorf tube. Two hundred µL of AE-buffer was added and the sample was incubated at room temperature for 1 min before centrifuging for 1 min at 13000 ×10 g. The DNA-extract was then regarded as the pass-through liquid which was collected after centrifugation. The DNA-extracts were then placed on ice.

3.3.7. Optimization of the polymerase chain reaction (PCR)

Polymerase chain reaction amplifications were prepared in duplicate. The amplification within the 16 rRNA-gene was obtained with the aid of primers; Prop1 (5'GATACGGGTGACTTGAGG-3') and Prop2 (5'GCGTTGCTGATCTGCGATTAC-3') (Whitehead Scientific). The Taq DNA Polymerase master-mix is obtained as a ready-to-use system, which contains 1.5 mM Ampliqon Taq DNA Polymerase, the NH_4^+ buffer system, dNTPs and magnesium chloride. The PCR-reaction was prepared to a final volume of 50 µL, which consisted of 25 µL Ampliqon Master-mix (Lasec), 17 µL PCR-water, 1 µL of each primer (Prop1 and Prop2) and lastly 6 µL DNA sample. The mixture was vortexed for 1 min and then placed back on ice before annealing. The negative blank-reaction was prepared by substituting the DNA template with PCR-grade water and used for each PCR run. The PCR reaction mix was amplified in 0.2 mL thin-walled tubes using the Eppendorf Master cycler (Merck, Germany).

The PCR amplification was carried under the following conditions; initial denaturation at 95˚C for 5 min, and then followed by 98 ˚C for 20 sec; annealing at 52 ˚C for 15 sec and chain elongation at 72 ˚C for 1 min to complete the cycle, with chain elongation at 72 ˚C for 5 min. The cycle was completed by final elongation at 72 ˚C for 5 min and cooling of the reaction products to 4 ˚C.

3.3.7.1. Electrophoresis gel preparation

A 0.5 × concentration TBE buffer (Sigma Aldrich) which was utilised to prepare the agarose gel was prepared by mixing 950 mL of Milli Q cold water with 50 mL concentrated 10 × TBE buffer. One hundred mL of 1% agarose gel was prepared by adding 1 g agarose powder to 100 mL 0.5 × TBE buffer, which was heated up in the microwave on high power until all the granules were dissolved. The agar was incubated in a water-bath set at 55 ˚C to cool down. Once cooled, 5 µL of ethidium bromide was added to the agarose before casting it into a gel-mould containing a comb. The gel was left to solidify at room temperature prior to removing the comb from the gel and extracting the gel from the mould.

3.3.7.2. Inoculation of agarose-gel wells and Electrophoresis

The solidified agarose-gel was carefully submerged into the electrophoresis bed which contained 0.5 × TBE buffer and 5 µL of ethidium bromide. In the first well, the DNA-marker [4 µL 1 kB DNA ladder (Promega, Anatech) and 1 µL bromophenol loading dye (Promega, Anatech)] was loaded with the second well left empty. From the third well onwards, 3 µL of amplified sample DNA products and controls were loaded into their respective wells. The PAB bands are known to fluoresce in the 750 bp regions when compared to the fluorescent bands of the DNA-ladder.

The sample PCR-products were electrophoresed in 1% agarose gel (w/v) in 0.5 × TBE buffer with 5 µL ethidium bromide to obtain sufficient separation of the respective DNA fragments. Electrophoresis was conducted at 90 V for 90 min, until the samples migrated approximately 10 cm down the agarose gel. After electrophoresis was complete, the gel was removed and placed in a Uvidoc gel imaging system (Whitehead Scientific) and visualised under UV light.

3.3.8. Statistical analyses

All analyses done in this study were performed in triplicate and Analyses of variance (ANOVA) were performed with SPSS® 21.0 (SPSS® Inc., Chicago, Illinois, USA) to test and compare the microbial succession during the 12 h of fermentation. Microbial growth means and standard deviations were compared using the Duncan multiple comparison Post-hoc test, where significant differences were found in terms of the main effects. Multivarient analyses of variance was performed with SPSS® 21.0 to calculate the corresponding p-values for microbial growth in 3 models (MFS, LPAB-J17 and HPAB-J17).

3.4. Results and discussion

3.4.1. Microbial succession and pH analysis

The descriptive statistics for the microbial succession of motoho during fermentation at 30 °C for 12 h, with sampling performed every 3 h, are summarized in Table 3.1 and each model in Figures 3.3-3.5 below. During fermentation, the microbial counts were analysed by pour plating the samples on PCA, VRBA, MRSA and RBA. Motoho fermented slurry (MFS) was regarded as the control model, and the optimised models were low inoculum (LPAB-J17) and high inoculum (HPAB-J17). The pH analyses for each sampling time are summarized in Figure 3.3 below.

3.4.1.1. pH analysis during fermentation

During the 12 h of fermentation (fig 3.3 below), the pH was analysed every 3 h. The initial pH for MFS was 6.37 ± 0.00, which decreased to 6.18 ± 0.00 after 3 h of fermentation and was not regarded as a significant drop ($p \geq 0.05$). There was a significant decrease ($p \leq 0.05$) to 5.48 ± 0.00 after 6 h; the pH did not drop after 9 h (5.48 ± 0.00) but dropped significantly to 4.75 ± 0.00 by T= 12 h of fermentation. Moodley (2015:62) obtained similar results, with the pH dropping from 6.6 to 4.2 after fermenting overnight. The decline in pH during fermentation can be attributed to the LAB population increase, which results with the production of lactic acid due to the abundance of nutrients and optimum growth temperature of 30 °C (Vera-Peña & Rodriguez, 2020:342). For LPAB-J17, the pH decreased throughout fermentation, with an observed significant difference ($p \leq 0.05$) at T= 3 h and there was no significant ($p \geq 0.05$) difference in pH at T= 6 h. However, there was a significant difference ($p \leq 0.05$) for the pH obtained at T= 9 h, the pH had dropped from 4.15 to 3.94 The pH did decrease further by T= 12 h, however the decrease was no significant difference ($p \geq 0.05$). For HPAB-J17, there was a significant difference ($p \leq 0.05$) in the pH obtained at 0 h, 3 h and 6 h, whereby the pH decreased after each sampling time. There was no significant difference ($p \geq 0.05$) between the pH obtained from T= 6 h to T= 12 h, even though the pH continued to drop.

The final pH measurements (T= 12 h) obtained by the three models (MFS, LPAB-J17 and HPAB-J17) were all below pH ≤ 5.5, which was below PAB growth range. It is noted that the optimum growth parameters for aerotolerant and anaerobic PAB are at 30°C, pH 6-7 with a 6 to 7 h generational time (Turgay *et al*., 2020). However, some PAB-strains, such as *P. freudenreichii* have been reported to be able to adapt to various environmental stress conditions, provided that the culture was pre-adapted or preconditioned (Turgay *et al*., 2020). The decrease in pH for PAB survival during fermentation could be due to the production of pyruvic acid, which can be temporarily transported to the exterior of the cell and converted to alanine and lactic acid. *P. freudenrichii* has demonstrated to produce propionic acid by utilizing lactic acid as the core carbon source, whereby the lactic acid is converted to pyruvic acid, which is metabolised via the methylmalonyl-CoA pathway (Turgay *et al*., 2020:5-6). *P. freudenrichii* is reported to have the ability to endure stress encountered during various

successive stresses such as acidification, heating to °C ≥ 50 °C and osmotic stressed caused by the addition of NaCl. During spontaneous cereal fermentation, acid stress can inhibit and/or delay PAB growth, with *P. freudenrichii* demonstrating to tolerate moderate acidic conditions (pH 4-5). The acid tolerance respond is dependent on the over-expression of enzymes involved in DNA-synthesis and repair (Turgay *et al*., 2020:7).

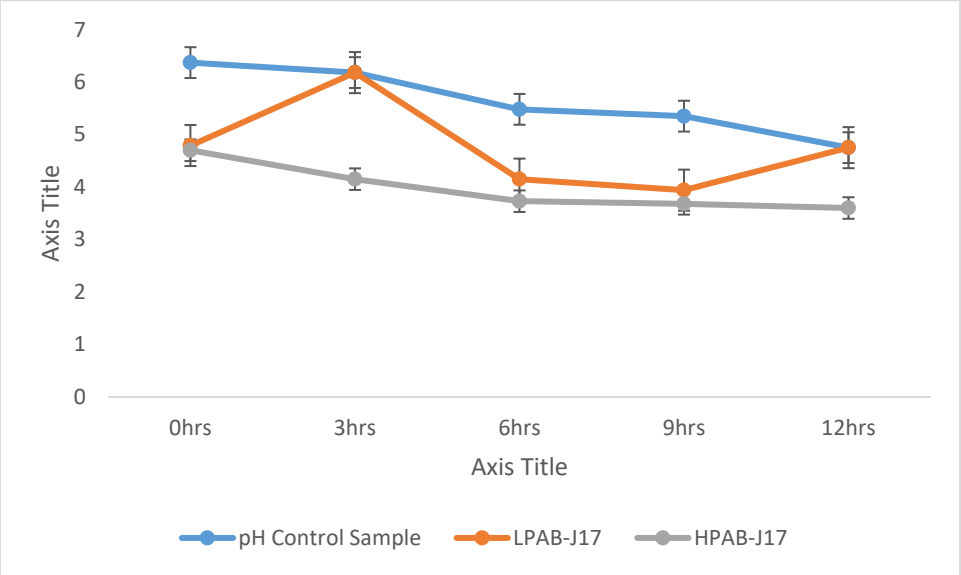


Figure 3.2: The pH levels during fermentation for the control (MFS), low (LPAB-J17) and high (HPAB-J17) cell inoculum of PAB-17

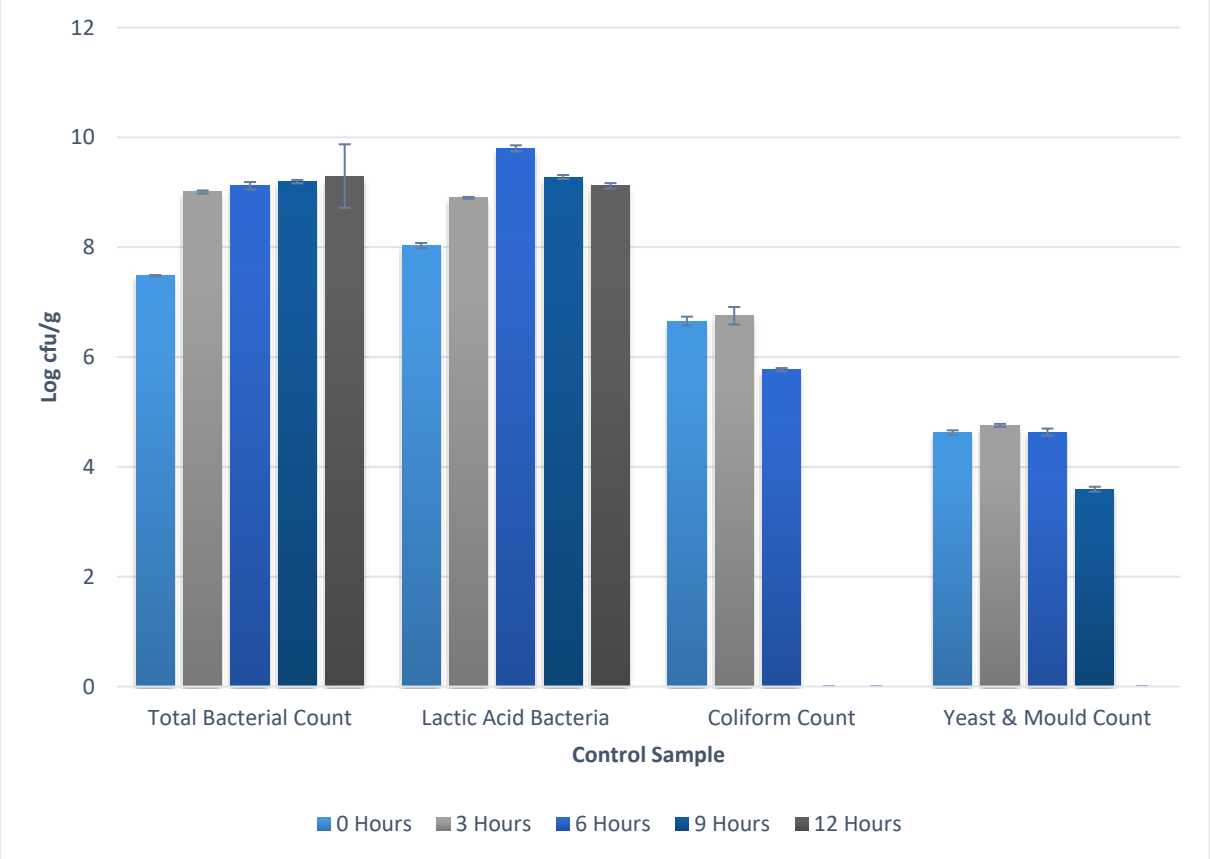


Figure 3.3: Overall microbial succession during fermentation for the control (MFS) as observed in different culture media; PCA (Total Bacterial Count), MRSA (Lactic Acid Bacteria), VRBA (Coliforms), RBCA (Yeasts and moulds).

Table 3.1: Mean microbial succession (log cfu/g) of the three models of motoho

Sample	Time (h)				
	0 h	**3 h**	**6 h**	**9 h**	**12 h**
			Total Viable Count (log cfu/g)		
MFS	7.48 ± 0.01^{a}	9.01 ± 0.03^{b}	9.12 ± 0.07^{b}	9.19 ±0.03^{b}	9.3 ± 0.58^{b}
LPAB-J17	7.23 ± 0.12^{a}	8.14 ± 0.02^{b}	8.28 ± 0.08^{c}	8.28 ± 0.03^{c}	8.2 ± 0.06b^{b}
HPAB-J17	7.23 ± 0.6^{a}	5.78 ± 0.06^{b}	9.31 ± 0.02^{c}	8.86 ±0.03^{d}	9.24 ± 0.02^{c}
			Coliforms (log cfu/g)		
MFS	6.65 ± 0.08^{a}	6.75 ± 0.12^{a}	5.77 ± 0.03^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
LPAB-J17	3.65 ± 0.09^{a}	3.74 ± 0.03^{a}	4.72 ± 0.06^{b}	5.82 ± 0.05^{c}	4.88 ±0.05^{d}
HPAB-J17	3.7 ± 0.13^{a}	3.77 ± 0.04^{a}	3.72 ± 0.09^{a}	6.05 ± 0.07^{b}	4.82 ± 0.09^{c}
			Lactic acid bacteria (log cfu/g)		
MFS	8.03 ± 0.05^{a}	8.9 ± 0.02^{b}	9.8 ± 0.06^{c}	9.28 ± 0.04^{d}	9.12 ± 0.60^{e}
LPAB-J17	7.44 ± 0.02^{a}	7.40 ± 0.02^{a}	8.36 ± 0.03^{b}	9.05 ± 0.05^{c}	9.34 ± 0.02^{d}
HPAB-J17	7.27 ± 0.04^{a}	5.83 ± 0.04^{b}	6.96 ± 0.01^{c}	8.90±0.05^{d}	9.96 ± 0.03^{e}
			Yeast (log cfu/g)		
MFS	4.62 ± 0.04^{a}	4.76 ± 0.03^{b}	4.53 ± 0.06^{a}	3.59 ± 0.05^{c}	0.00 ± 0.00^{d}
LPAB-J17	5.05 ± 0.06^{a}	5.14 ± 0.03^{a}	5.02 ± 0.05^{a}	5.84 ± 0.07^{a}	5.02 ± 0.09^{b}
HPAB-J17	5.16 ± 0.11^{a}	5.25 ± 0.08^{a}	3.98 ± 0.11^{b}	5.21 ± 0.02^{a}	4.29 ± 0.02^{c}

The values are the means ± standard deviations of triplicate determination—three independent trials of motoho; traditional method and optimization with PAB-J17 at low concentration (LPAB-J17) and high concentration (HPAB-J17). There is no significant difference ($p \geq 0.05$) for the values with the same letter in the same row. MFS, Motoho fermented slurry; LPAB-J17, low inoculum cell concentration HPAB-J17, high inoculum cell concentration.

3.4.1.2. Total Viable Count (TVCs)

For the MFS (Table 3.1 and Figure 3.3 above), there was a significant difference ($p \leq 0.05$) in TVC after 3 h (9.01 ± 0.03 log cfu.g^{-1}) of fermentation, there was no significant difference ($p \geq 0.05$) in TVC from (6 h) 9.12 ± 0.07 cfu.g^{-1} to the fermentation end-stage (12 h) 9.3 ± 0.58 log cfu.g^{-1}. The study conducted by Moodley (2015:59) resulted in a final overnight fermentation count of 7.9 log cfu.g^{-1} for the TVC, which is lower than what was obtained in this study. For LPAB-J17 (Table 3.1 and Figure 3.4), there was a significant difference ($p \leq 0.05$) between 0 h and 3 h, where the initial counts increased from 7.23 ± 0.12 log cfu.g^{-1} to 8.14 ± 0.02 log cfu.g^{-1}, and there was no significant difference ($p \geq 0.05$) between (3 h) 8.14 ± 0.02 and (12 h) 8.2 ± 0.06b log cfu.g^{-1}. There was also no significant difference ($p \geq 0.05$) in cell counts obtained at (6 h) 8.28 ± 0.08 log cfu.g^{-1}, (9 h) 8.28 ± 0.03 log cfu.g^{-1} and (12 h) 8.2 ± 0.06 log cfu.g^{-1}. For HPBA-J17 (Table 3.1 and Figure 3.5), there was a significant difference ($p \leq 0.05$) in cell counts, there was a decrease from 7.23 ± 0.6 log cfu.g^{-1} (0 h) to 5.78 ± 0.06 log cfu.g^{-1} (3 h), where the initial pH decreased from 4.70 ± 0.00 to 4.15 ± 0.00. There was no significant difference ($p \geq 0.05$) between (6 h) 9.31 ± 0.02 log cfu.g^{-1} and (12 h) 9.24 ± 0.02 log cfu.g^{-1} but there was a significant difference ($p \leq 0.05$) between 9 h and 12 h; the TVCs decreased from 9.31 ± 0.02 log cfu.g^{-1} to 8.86 ± 0.03 log cfu.g^{-1} by 9 h. But increased to 9.24 ± 0.02 log cfu.g^{-1} by 12 h of fermentation. For the overall total viable counts obtained after fermentation, there was no significant difference ($p \geq 0.05$) between LPAB-J17 8.03 ± 0.42 log cfu.g^{-1} and HPAB-J17 8.08 ± 1.43 log cfu.g^{-1}, but MFS 8.82 ± 0.73 log cfu.g^{-1} was significantly higher ($p \leq 0.05$) than LPAB-J17 and HPAB-J17.

3.4.1.3. Coliforms

During the 12 h fermentation of MFS (Table 3.1 and Figure 3.3), there was no significant increase ($p \geq 0.05$) in the total coliform count. For MFS, the counts obtained for 0 h and 3 h of fermentation were 6.65 ± 0.08 log cfu.g^{-1} and 6.75 ± 0.12 log cfu.g^{-1}, which were not significantly different ($p \geq 0.05$) from each other. At 6 h, the cell count had decreased to 5.77 ± 0.03 log cfu.g^{-1} and was significantly different ($p \leq 0.05$) to 0 h and 3 h; however, there was no growth observed at 9 h and 12 h. For LPAB-J17 (Table 3.1 and Figure 3.4), there was no significant difference ($p \geq 0.05$) between 0 h to 3 h, but there counts increased to 4.72 ± 0.06 log cfu.g^{-1} by 6h and was significantly different ($p \leq 0.05$) from 0h and 3 h. The there was also a significant increase ($p \leq 0.05$) to 5.82 ± 0.05 log cfu.g^{-1} at 9 h. At 12 h, there was a decrease 4.88 ±0.05 log cfu.g^{-1}, which was significant different ($p \leq 0.05$) to 9 h. The total coliform count obtained with the HPAB-J17 (Table 3.1 and Figure 3.5) were relatively low from 0 - 6 h and gradually increased from 6 to 12 h ($p \leq 0.05$). The overall total coliform counts obtained after fermentation, MFS was significantly lower ($p \leq 0.05$) than HPAB-J17 and LPAB-J17, though

higher values were obtained from 0 to 6 h. There was no significant difference ($p \geq 0.05$) between LPAB-J17 and HPAB-J17 in total coliform counts by 12 h fermentation.

The pH conditions of the three models created a hostile environment for coliforms. For MFS, the pH decreased from 5.48 from 6 h to 9 h, and decreased again to 4.75 after 12 h. For LPAB-J17, the pH fluctuated throughout fermentation, the pH at 0 h was 4.79, with 6.18 at 6 h and the final pH was 4.75 (12 h). For HPAB-J17, the pH decreased throughout fermentation, with an initial pH of 4.70 and a final pH of 3.60. The pH conditions of the three models did not favor the survival and growth of coliforms. The ideal pH for the survival and growth of coliforms is pH 5.5 to 7.5; fermentation is known to improve the quality of food products by inhibiting the growth of microorganisms that can lead to poisoning and spoilage (Achi and Ukwuru, 2015:72).

3.4.1.4. Lactic Acid Bacteria (LABs)

The LAB cell count for MFS (Table 3.1 and Figure 3.3) increased throughout fermentation, 8.03 ± 0.05, 8.9 ± 0.02, 9.8 ± 0.06, 9.28 ± 0.04 and 9.12 ± 0.60 log cfu.g^{-1}. There was a significant difference ($p \leq 0.05$) in LAB counts between 0 h and 12 h. There was a significant difference ($p \leq 0.05$) between 6 h and 9 h; there was a decrease in counts obtained between 9 h and 12 h, and it was significantly different ($p \leq 0.05$). Moodley (2015:59) obtained 7.7 log cfu.g^{-1} after 12 h of fermentation. The traditional method of production adopted in this study is different from the one used by Moodley (2015:53). The increase in fermentation time resulted in the increase of LABs, whereby the coliforms, decreased as fermentation time increased. The LABs were the most dominant during fermentation; the abundant availability of nutrients and temperature of 30 °C facilitated their optimum growth over the 12 h of fermentation.

There was no significant growth ($p \geq 0.05$) for LPAB-J17 (Table 3.1 and Figure 3.4) within the first 3 h of fermentation. However, there was significant growth ($p \leq 0.05$) after 3 h of fermentation, growth went from 7.40 ± 0.02 log cfu.g^{-1} to 8.36 ± 0.03 log cfu.g^{-1} by 6 h of fermentation. There was also significant increase ($p \leq 0.05$) in growth from 9.05 ± 0.05 log cfu.g^{-1} to 9.34 ± 0.02 log cfu.g^{-1} from 9 h to 12 h of fermentation. For HPAB-J17 (Table 3.1 and Figure 3.5), there was a significant decrease ($p \leq 0.05$) in growth by 3 h of fermentation, where initial count decreased from 7.27 ± 0.04 log cfu.g^{-1} to 5.83 ± 0.04 log cfu.g^{-1}. However, cell counts increased significantly ($p \leq 0.05$) by 6 h of fermentation to 6.96 ± 0.01 log cfu.g^{-1}. The LABs continued to increase significantly to 8.90 ± 0.05 log cfu.g$^{-1,}$ (9 h) with a final cell count of 9.96 ± 0.03 log cfu.g^{-1} by 12 h of fermentation. The LABs had the highest overall microbial counts during fermentation; the overall LABs production from the MFS 9.02 ± 0.6 log cfu.mL^{-1} was significantly higher ($p \leq 0.05$) than that of LPAB-J17 8.32 ± 0.83 log cfu.mL^{-1} and HPAB-J17 7.78 ± 1.52 log cfu.mL^{-1}. However, LPAB-J17 was also higher ($p \leq 0.05$) than HPAB-J17.

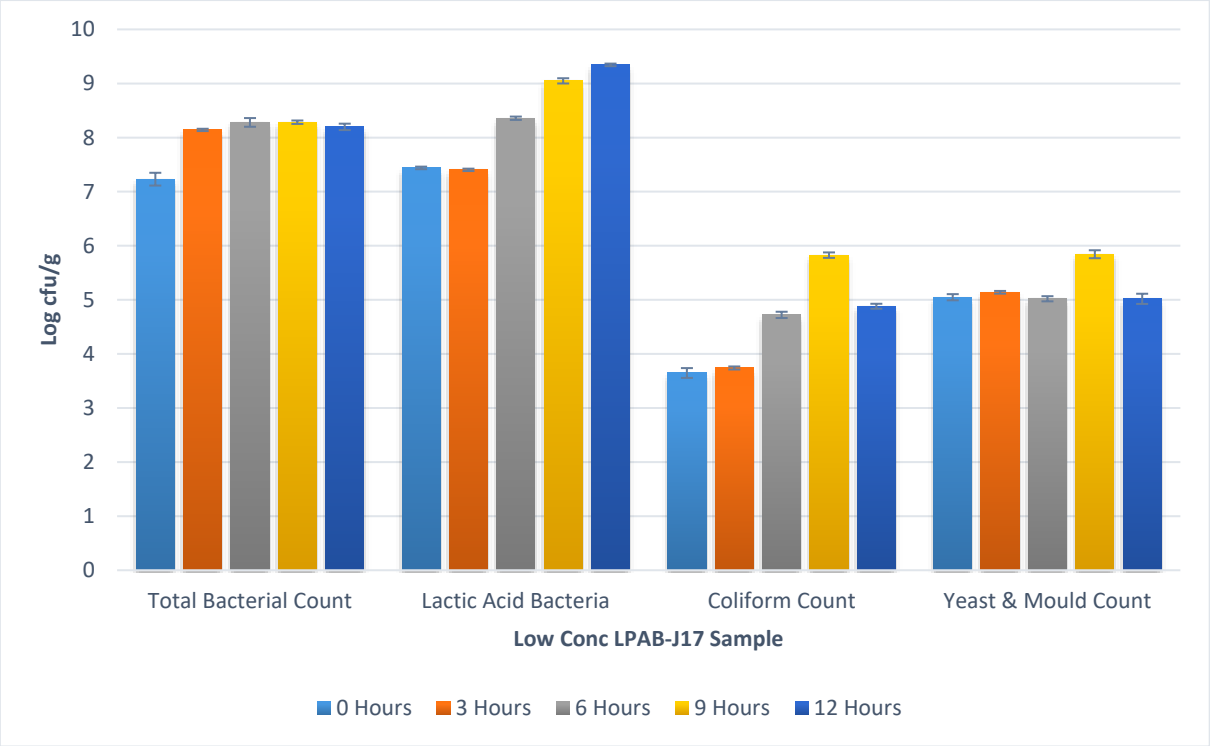


Figure 3.4: Low inoculum (LPAB-J17) overall microbial succession during fermentation as observed on different media; PCA (Total Bacterial Count), MRSA (Lactic Acid Bacteria), VRBA (Coliforms), RBCA (Yeasts and moulds).

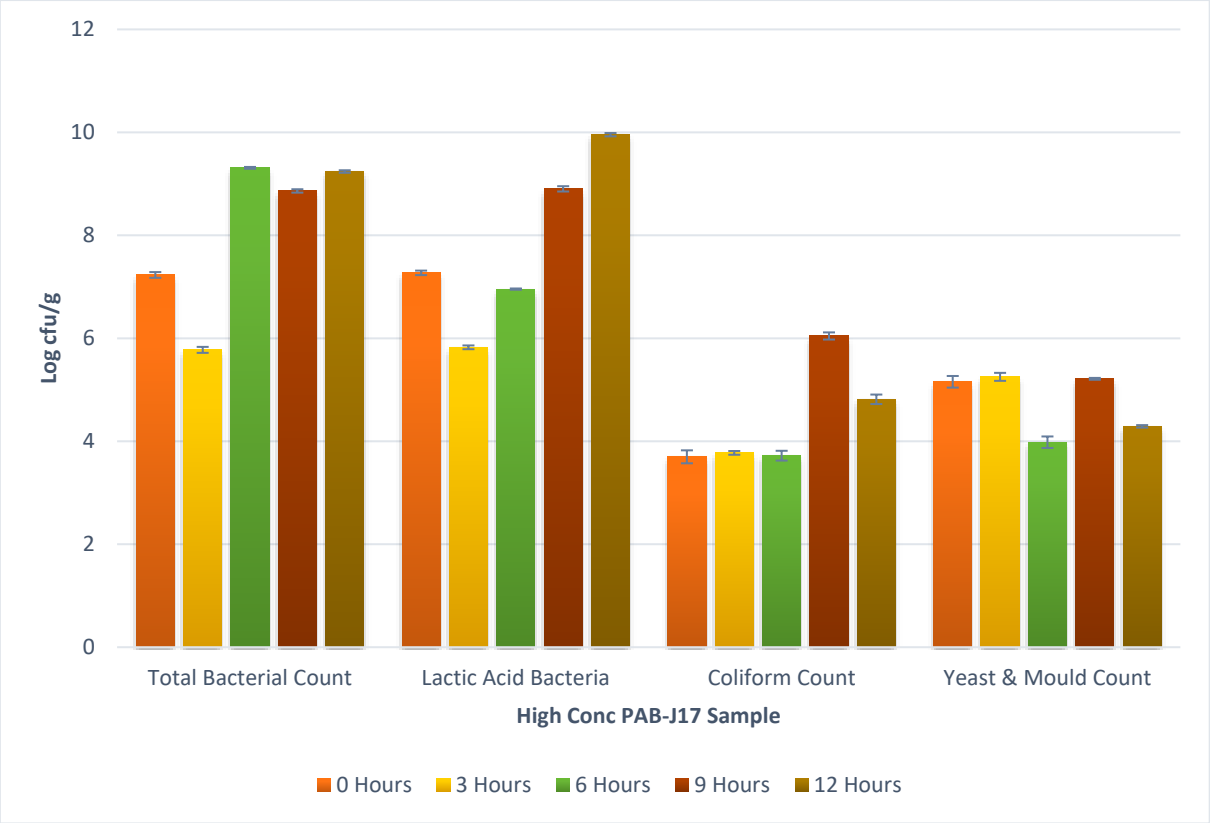


Figure 3.5: High inoculum (HPAB-J17) overall microbial succession during fermentation as observed on different media; PCA (Total Bacterial Count), MRSA (Lactic Acid Bacteria), VRBA (Coliforms), RBCA (Yeasts and moulds).

The dominance of LABs in sorghum fermentation can be attributed to one or more of the following: (i) superior utilization of starchy sorghum substrates, (ii) versatile carbohydrate metabolism and (iii) tolerance of low pH (Gänzle, 2018:106-107; Adebo, 2022:8). In spontaneous fermentation, it has been determined that LABs and yeast have a symbiotic relationship, with LABs being the most ascendant microorganisms and yeast being inferior over time with a constant temperature (Ukwuru *et al.*, 2018:25-26; Adebo, 2020:11). The LABs for the MFS bacterial counts increased throughout fermentation as the pH dropped, whereas for LPAB-J17 the counts remained insignificant after 3 h and then increase up until 12 h.

The interaction of PABs and LABs has been investigated. It was determined that the fermentation of propionic acid by some PAB- strains was highly affected by the type of LABs present during the fermentation process.; the most commonly found LABs being lactobacilli and streptococci. The ability for PAB to survive interaction with some LABs such as lactobacilli facilitates for the stimulation of PAB growth by means of releasing peptides. Carbohydrates are oxidised to produce pyruvic acid via glycolysis or the pentose phosphate pathway (Turgay *et al.*, 2020:9).

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3.4.1.5. Yeast

During fermentation, it was observed that yeast counts for MFS (Table 3.1 and Figure 3.3) obtained significant growth ($p \leq 0.05$) after 3 h and 6 h of fermentation. However, there was no observable significant growth ($p \geq 0.05$) between counts obtained at the start of fermentation and by 6 h of fermentation. There was significant growth ($p \leq 0.05$) by 9 h, but there was no cell growth by 12 h fermentation. The cell counts obtained for MFS were 4.62 ± 0.04, 4.76 ± 0.03, 4.53 ± 0.06, 3.59 ± 0.05 and 0.00 log cfu.g^{-1}. Lactic acid bacteria and yeasts are known to dominate spontaneous indigenous cereal fermentation. When LPAB-J17 (Table 3.1 and Figure 3.4) was analysed, it was observed that there was no significant difference ($p \geq 0.05$) in the yeast growth from 0 h to 9 h, 5.05 ± 0.06, 5.14 ± 0.03, 5.02 ± 0.05 and 5.84 ± 0.07 log cfu.g^{-1}. There was significant difference ($p \leq 0.05$) after 9 h, the cell count decreased to 5.02 ± 0.09 log cfu.g^{-1} by 12 h. It was noted that the cell counts obtained after 9 h were the only ones significantly different from the other sampling times. For HPAB-J17 (Table 3.1 and Figure 3.5), there was no significant difference ($p \geq 0.05$) in cell counts for 0 h (5.16 ± 0.11 log cfu.g$^{-1)}$, 3 h (5.25 ± 0.08 log cfu.g^{-1}) and 9 h (5.21 ± 0.02 log cfu.g^{-1}). There was a significant decrease ($p \leq 0.05$) between 3h and 6 h, with counts dropping from 5.25 ± 0.08 to 3.98 ± 0.11, the cell counts then increased ($p \leq 0.05$) by 9h of fermentation. By 12 h of fermentation, there was a significant difference ($p \leq 0.05$) in cell counts, they dropped to 4.29 ± 0.02 cfu.g^{-1}. The overall counts obtained at the end of fermentation indicated that MFS 3.52 ± 1.87 log cfu.g^{-1} was significantly lower ($p \leq 0.05$) than LPAB-J17 5.21 ± 0.33 log cfu.g^{-1}, and HPAB-J17 4.78 ± 0.56 log cfu.g^{-1} was significantly higher than LPAB-J17 and MFS. The yeast counts obtained in the study conducted by Moodley (2015:59) were 7.5 log cfu.g^{-1} and were higher than those obtained in for this study MFS (3.52 ± 1.87 log cfu.g^{-1}), LPAB-J17 (5.21 ± 0.33 log cfu.g^{-1}) and HPAB-J17 (4.78 ± 0.56 log cfu.g^{1}).

Yeasts are known for their ability to ferment maltose, glucose and fructose, with the ability to tolerate low pH-environments (pH ≤ 3.5). Non-*Saccharomyces* yeasts have been reported to be abundant in the initial stages of spontaneous fermentation (Maicas, 2020:3). In the study conducted by Adebo *et al.* (2017:8), it was stated that sorghum fermentation favoured the growth of LABs over other microorganisms such as yeasts and moulds; this is due to the

abundant growth of LABs during fermentation. Yeasts and LABs are the foremost microorganisms involved in traditionally fermented foods (Guyot, 2012:1111; Mokoena *et al.*, 2016:1)

3.4.2. Isolate characterisation and identification using Vitek

Dominant colonies from the MFS, coded MFS1, were small, light cream in colour, and had smooth surfaces with entire edges. They stained as gram-positive (Figure 3.6 below), short rods with a central endospore.

The gram-positive (GP) Vitek card was utilised for characterisation of the isolate using the Vitek2 system. The results of the biochemical reactions analysed are shown in Table 3.2 below. The positive biochemical reactions were for D-amygdaline (AMY), arginine dihydrolase 1 (ADH1), beta-glucuronidase (BGURr), alanine arylamidase (AlaA), D-galactose (dGAL), N-acetyle-D-glucosamine (NAG), D-mannose (dMNE), methyl-B-D-glucopyranoside (MBdG), D-raffinose (dRAF), salicin (SAL), saccharose/sucrose (SAC), D-trehalose (dTRE), arginine dihydrolase 2 (ADH2s) and optochin resistance (OPTO). This analysis gave a low discrimination result, which was inconsistent with PAB. Furthermore, the results for the PCR identification for PAB were inconclusive, which disqualified the isolate as PAB.

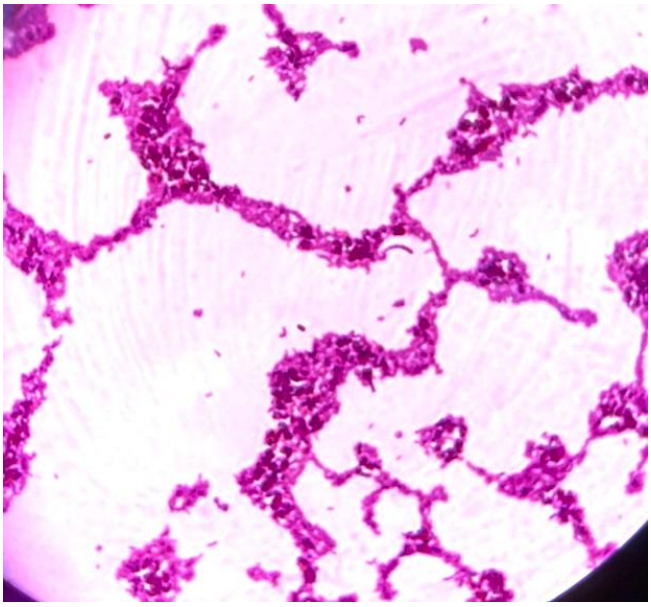

Figure 3.6: Gram-staining results for isolate (MFS1) obtained from Yel-agar

Table 3.2: Vitek biochemical results of isolate MFS1

2	AMY	+	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	+	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	+
38	dRIB	-	39	ILATk	-	42	LAC	-	44	NAG	+	45	dMAL	-	46	BACI	-
47	NOVO	-	50	NC6.5	-	52	dMAN	-	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	-	59	SAL	+	60	SAC	+	62	dTRE	+	63	$ADH2_2$	+
64	OPTO	+															

The main significance of these biochemical products is discussed below in turn: D-amygdaline is regarded as a primary ingredient found in *Rosaceae* species and various plant seeds (almonds, apple seeds and apricots); hydrogen cyanide is a byproduct of amygdalin enzymatic hydrolysis and can be poisonous. The identified positive functions of D-amygdaline are immunomodulatory effects, antibacterial, antioxidant and anti-inflammatory effects (Barakat *et al.,* 2022). Beta-glucuronidase is a lysosomal enzyme that participates in the degradation of complex carbohydrates, glucoronate-containing glycosaminoglycan (Sui *et al.,* 2021). Alanine is an amino acid that is utilised for protein formation and is also an essential substance for the synthesis of peptidoglycan, an important cell wall component especially for Gram positive organisms. It is important to note that alanine arylamidase can be synthesised from pyruvate and branched chain amino acids such as isoleucine, leucine and valine (NCBI, 2023a). N-acetyle-D-glucosamine is the D-isomer of N-acetylglucosamine and is plays a role as bacterial metabolite. It is produced by *Escherichia coli* (K2, MG1655) and is a natural product found in *Streptomyces alfalfa*, and *Euglen gracilis* (NCBI, 2023b). D-raffinose is a trisaccharide that is composed of alpha-D-galactopyranose, alpha-D-glucopyranose and beta-D-fractofuranose joined in sequence by means of glycosidic linkages. It participates as a metabolite for plants and *Saccharomyces cerevisiae* (NCBI, 2023c). The disaccharide trehalose plays a role as a metabolite for *S. cerevisiae*, *E. coli* and humans; it is identified as having both glucose residues having alpha-configuration at the anomeric carbon (NCBI, 2023d). Optochin (an antimicrobial derivative of quinine) resistance is a susceptibility test done in the detection of *Streptococcus pneumonia* (Pinto *et al.*, 2013).

3.4.3. Polymerase chain reaction (PCR) analysis

The agarose gel (Figure 3.7 below) was loaded as follows: loading dye (well 1), empty (well 2), blank (well 3), PAB-J15 (well 4), PAB-J17 broth culture (well 5), PAB-J17 colony (well 6), MFS1 colony (well 7) and MFS1 broth (well 8). The PAB DNA fragments were amplified at 750 base pairs (bp) region in the agarose gel, however, MFS1 was not amplified at the same base pair region as PAB. In well 8, MFS1 DNA remained in the well and did not move through the agarose gel. The non-amplification of MFS1 in well 7 and 8 resulted in the conclusion that it was not Propionibacterium which was isolated from motoho.

The polymerase chain reaction (PCR) yielded results that confirmed that the isolate (MFS1) obtain was indeed not a PAB-strain. Based on the Gram-staining, Vitek and PCR analysis it was confirmed that MFS1 was not PAB or related to it.

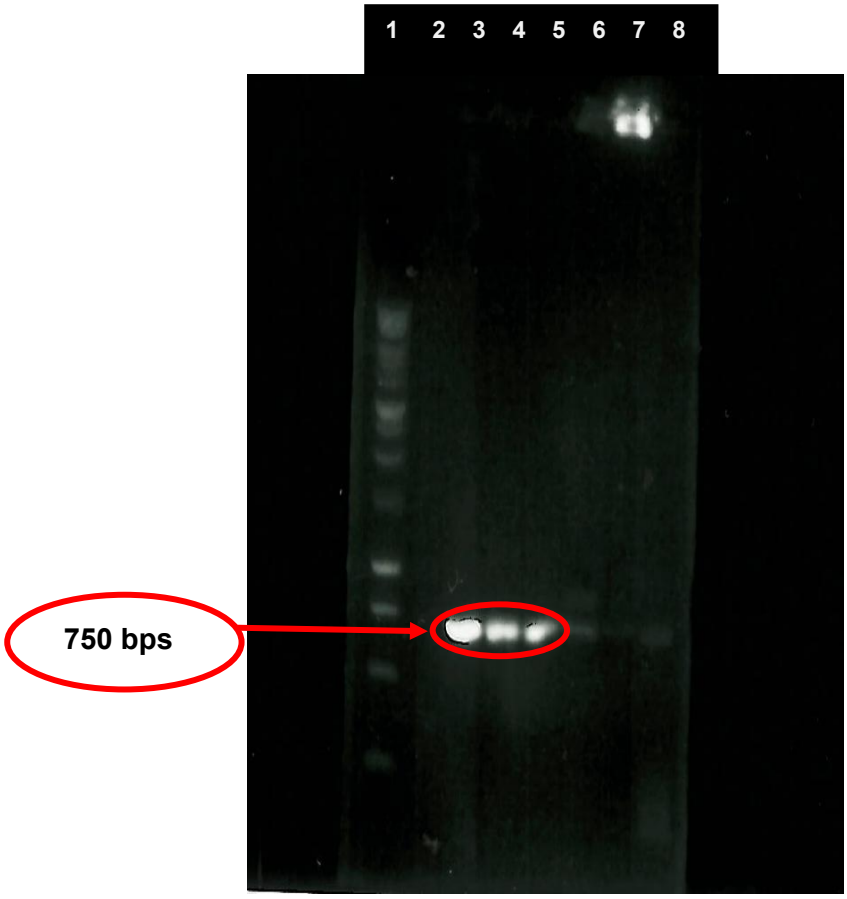

Figure 3.7: PCR analysis of MFS1; loading of wells: 1- loading dye, 2-empty, 3-blank, 4- PAB-J15, 5-PAB-J17 broth, 6- PAB-J17 colony, 7- MFS1 colony, 8- MFS1 broth.

3.4.4. Phylogenetic identification

The isolate was further characterized in order to determine its exact taxonomic position. Sequencing of the 16S rRNA region revealed 1407 base pairs (bp). A BLAST analysis was performed on the NCBI Bioinformatics Resource website (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_1847773583, [19/11/2023]) to compare and identify regions of similarity between sequences. Even though isolate MFS1 was related to seven strains at 99.5 % similarity (Table 3.3 below), it was closely related to the Type Strain of *Bacillus rugosus*. *B. rugosus sp. nov.* is an emerging organism of the *Bacillus* genus that has been isolated from sea sponges (*Spongia officinalis L.*) (Bhattacharya *et al*., 2020:1676). *B. rugosus* is reported to produce diketopiperazine, which is a cyclic peptide naturally synthesised from amino acids by humans, marine organisms, fungi as well as a large group of gram-negative and gram-positive microorganisms (Carvalho and Abraham, 2012:3554). Diketopiperazine is possesses anticancer antithrombotic, neuron protective, analgesic pharmacological activities as well as antimicrobial activities (Carvalho and Abraham, 2012:3554; Bhattacharya *et al*., 2020:1676)

In order to determine the exact classification of isolate MFS1, phylogenetic trees (Figure 3.8 below) were constructed according Meier-Kolthoff *et al.* (2021), using the DSMZ single-gene phylogeny server (https://ggdc-test.dsmz.de/phylogeny-service.php#). Treeing methods used were the Maximum-Likelihood and the Maximum Parsimony methods considering pairwise similarities. Isolate MFS1 clustered with the type strain for *Bacillus inaquosorum* forming a relatively stable phylogeny at 68 %. This cluster was located in a clade that was 99 % stable, giving further testimony to the validity of the phylogeny. *B. inaquosorum* exhibits anti-bacterial activity and was demonstrated to be active against pathogens such as *Vibrio parahaemolyticus* and other food pathogens (Avery *et al*., 2020:2). Thus, isolate MFS1 is likely to also be beneficial in a similar way and improving the safety of motoho but this requires further investigation.

Table 3.3: BLAST analysis of isolate MFS1. NCBI Bioinformatics Resource (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_1847773583, [19/11/2023])

Select for downloading or viewing reports	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Select seq MT554518.1	Bacillus rugosus strain SPB7 16S ribosomal RNA gene, partial sequence	Bacillus rugosus	2556	2556	100%	0.0	99.50%	1548	MT554518.1
Select seq MT326211.1	Bacillus tequilensis 16S ribosomal RNA gene, partial sequence	Bacillus tequilensis	2556	2556	100%	0.0	99.50%	1417	MT326211.1
Select seq MN889329.1	Bacillus subtilis strain OsEnb_ALM_B18 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2556	2556	100%	0.0	99.50%	1422	MN889329.1
Select seq MT126334.1	Bacillus sp. (in: Bacteria) strain 79 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: firmicutes)	2556	2556	100%	0.0	99.50%	1480	MT126334.1
Select seq MT126331.1	Bacillus sp. (in: Bacteria) strain 75 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: firmicutes)	2556	2556	100%	0.0	99.50%	1475	MT126331.1
Select seq MN704546.1	Bacillus subtilis subsp. stercoris strain EGI160 16S ribosomal RNA gene, partial sequence	Bacillus stercoris	2556	2556	100%	0.0	99.50%	1459	MN704546.1
Select seq MN704534.1	Bacillus subtilis subsp. stercoris strain EGI13 16S ribosomal RNA gene, partial sequence	Bacillus stercoris	2556	2556	100%	0.0	99.50%	1461	MN704534.1

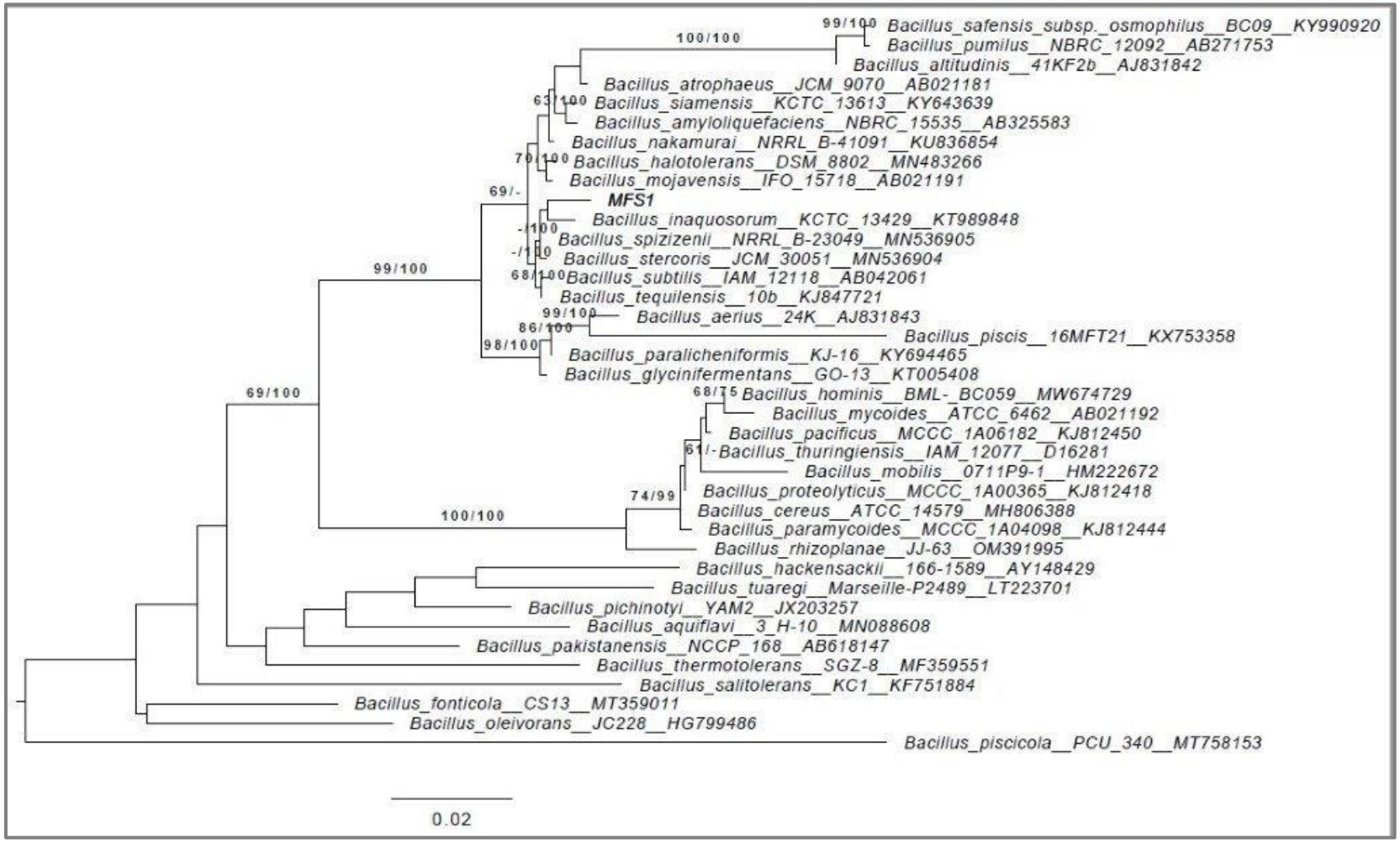

Figure 3.8: Phylogenetic trees for MFS1

DSMZ single-gene phylogeny server (https://ggdc-test.dsmz.de/phylogeny-service.php#)

3.5. Conclusion

The microbial succession of motoho was evaluated in TFM and the two optimized models LPAB-J17 and HPAB-J17, which were co-inoculated with tomoso. There was no presence of *Propionibacterium* sp. strains, but *Bacillus rugosus* was isolated from motoho.

The effect of pH on the microbial population was evident in MFS, whereby there was an observable significant difference ($p \leq 0.05$) in the coliform growth after 6 h of fermentation. The final pH for the three models was pH ≤ 5.5, which made it difficult for the survival and growth of coliforms. The LABs were the most dominant organisms for the three models, this is in accord to what has been stated in literature. The counts obtained for MFS being the highest of the three models. MFS LAB counts were significantly ($p \leq 0.05$) higher than LPAB-J17 and HPAB-J17. However, the yeasts in MFS decreased throughout fermentation for the three models. There was significant difference ($p \leq 0.05$) in the overall yeast counts for the three models.

Bacillus rugosus was isolated from motoho as isolate MFS1. The yeast extract-peptone-lactate medium which is employed for the isolation of dairy propionibacteria was able to isolate *B. rugosus*. The isolated colonies of *B. rugosus* were similar to how PAB presents itself on Yel-agar. Gram-positive staining provided the visualization of *B. rugosus* with an endospore, which dairy-PABs do not have as they are non-spore forming organisms. It is vital to analyse the cell morphology of MFS1, to measure the cell shape and size in order to build-up data for the isolate. The Vitek GP-card used for isolate-MFS1 was incapable of identifying the isolate, therefore it is of interest to perform Vitek analysis of the isolate with GP-cards which are suitable for identification of *Bacillus* sp. The DNA-extraction and PCR analysis method employed for this study was designed to target PAB strains, and therefore resulted in inadequate PCR bands.

The position and distance of isolate MFS1, together with the inconclusive Vitek 2 results which gave a unique phenotypic fingerprint, point to the likelihood that isolate MFS1 represents a new species of the genus *Bacillus*. However, more tests under the polyphasic taxonomical approach (e.g. chemotaxonomy and further genotypic analyses like DDH, ANI, AAI and WGS) still need to be performed to verify this status. These tests are outside the scope of the current study.

3.6. References

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CHAPTER 4
DETECTION, QUANTIFICATION AND THE OPTIMISATION OF VITAMIN B12 BY CO-INOCULATION WITH *PROPIONIBACTERIUM FREUDENREICHII* subsp. *SHERMANII*

4.1. Abstract

Cereal fermentation is an inexpensive method of improving the nutritional, shelf life and organoleptic characteristics of foodstuffs. Vitamin B12 (vit B12) is an essential vitamin, with an RDA of 2.4 mg/day. *Propionibacterium freudenreichii* has Generally Recognised as Safe (GRAS) status, and it is well studied for its natural synthesis of vit B12. The aim of this study was to detect and quantify vit B12 in motoho and optimise its production by co-inoculation with *P. freudenreichii* subsp. *shernanii* (strain J17). Three models of motoho were produced, namely traditional (TFM), and optimised LPAB-J17 and HPAB-J17. Optimisation was performed by co-inoculating tomoso with PAB-J17 culture as described in Chapter 3. Vitamin B12 was extracted from samples with KCN-buffer while working in the dark, due to the photosensitivity of the vitamin. For extraction, samples were autoclaved, rapidly cooled, centrifuged at 4 °C and then filtered through 0.45 µm syringe filters. The samples were purified through SPE- columns, flash evaporated under vacuum using the rotary evaporator, and then 3 mL of Milli-Q water was added before HPLC-analyses. Pure vit B12 was used as the external standard. The analytical parameters were determined.

The limit of detection (LOD) was 0.0054 µg.100 mL^{-1} and the limit of quantification (LOQ) was 0.018 µg.100 mL^{-1}. The vit B12 concentration for TFM was 9.11 ± 1.81 µg.100 mL^{-1} which was significantly higher ($p \leq 0.05$) than LPAB 3.41 ± 0.68 µg.100 mL^{-1}, however, HPAB 19.55 ± 3.45 µg.100 mL^{-1} was significantly higher ($p \leq 0.05$) than TFM and LPAB. Some lactic acid bacteria are known to synthesise vit B12, but it is inactive for humans. The pH for LPAB was 4.79 ± 00 at 0 h and fluctuated throughout fermentation, with a final pH of 4.75 ± 00 at 12 h that proved to be unfavourable for high vit B12 synthesis. The co-inoculation demonstrated to be successful for the HPAB-model, but the type of vit B12 present requires to be analysed to determine if it is active or inactive. There was no significant difference ($p \geq 0.05$) in the proximate analyses obtained for the three models. The protein content 4.33 ± 0.36 g.100 mL^{-1} (TFM), 4.19 ± 0.42 g.100 mL^{-1} (LPAB) and 4.88 ± 0.06 g.100 mL-1 (HPAB) of the models were higher than obtained in previous studies.

KEYWORDS

GRAS status, Proximate analysis, Vitamin B12, HPLC, vitamin B12 deficiency, LOD.

4.2. Introduction

Malnutrition has serious health and child-development implications, with vitamin deficiencies being said to be most prevalent in South Saharan Africa (SSA) (FAO-UN, 2018:8). Micronutrient deficiencies of minerals and vitamins can result in weakened immune systems,

blindness, birth defects and increased mortality (Brinch-Pedersen *et al.*, 2007:308; Micronutrient Forum, 2015:2). Studies have proven that vit B12 deficiency can lead to various negative health-effects. The recommended daily intake of vit B12 is 2.4 mg/day for adults. Humans are unable to synthesize vit B12 and therefore need to obtain it from their diets (Chamlagain, 2016:15-16, Walther & Schmid, 2017:150). Vitamin B12 is synthesized by a few microorganisms (Deptula *et al.*, 2015:2). *Propionibacterium freudenreichii* is widely utilised for its biosynthesis of vit B12 to quantities of 15 $\mu g.mL^{-1}$ and its GRAS status in food production (Deptula *et al*., 2015:2; Chamlagain *et al*., 2016:117). The subspecies of *P. freudenreichii* are differentiated on their ability to either ferment lactose (subsp. *shermanii*) or reduce nitrate (subsp. *freudenreichii*) (Turgay *et al*., 2020:2).

A method proposed to address vit B12 deficiency is mandatory fortification of food products or the development and promotion of fortified fermented food products with microbial cultures which specifically synthesize the vitamin in required quantities (Deptula *et al.*, 2017:2; Chamlagain *et al.*, 2018:67). Cereal grains possess adequate levels of vitamins, minerals and fermentable sugars which can be utilized by microorganisms during fermentation (Achi & Ukwuru, 2015:72-73). Sorghum is an indigenous African cereal grain that is ideal to cultivate in the SSA climate (Hadebe *et al*., 2016:178-179). Many fermented foods and beverages have been produced from sorghum throughout Africa (Debabandya *et al*., 2017:179). Motoho is a snack-beverage that is consumed daily in Lesotho and is produced from sorghum. Motoho is produced by spontaneous fermentation by back-slopping with tomoso from a previous batch (Bajpai & Tiwari, 2013:2387; Moodley, 2015:24). The vit B12 content in motoho is unknown, and the investigation of it can be beneficial for consumers. This can provide an opportunity for vitamin fortification by means of fermentation to address vit B12 deficiency. The study aimed to (1) detect and quantify the vit B12 content in motoho (2) optimize the vit B12 content by co-inoculating with two concentrations of PAB-J17.

4.3. Materials and methods

4.3.1. Preparation of motoho

Motoho was prepared as described in Chapter 3.

4.3.2. Chemical reagents and other chemicals

Unless stated otherwise, all the chemicals employed in the study were of Analytical-grade and therefore prepared according to standard analytical methods.

4.3.3. Determination of cyanocobalamin (B12)

Analytical standards

The vitamin B12 (cyanocobalamin) (Sigma Aldrich, South Africa) was used as an external standard for vit B12 determination.

4.3.4. Preparation of the calibration standards

The vit B12 method employed for this study was described by Morkel (2016).

Cyanocobalamin (B12) was employed as the calibration standard. Due to the photosensitive nature of B12, it was prepared in a dark room dimly lit using LED-light. The 100 mL standard was prepared by weighing 2 mg of cyanocobalamin into an amber volumetric flask containing 100 mL KCN-acetate buffer. The KCN-buffer constituted of 20 g KCN potassium cyanide (Univar, Saarchem), 544.32 g sodium-acetate-trihydrate (Sigma Aldrich, South Africa) and 1 L Milli-Q water. The mixture was mixed until all solids had dissolved. Once dissolved, the solution was degassed in a sonicator for 5 min. The pH of the solution was adjusted to pH 4.5 using glacial acetic acid (Sigma Aldrich, South Africa). The solution was flushed with nitrogen gas and stored at 4 °C for a maximum of 2 weeks.

4.3.5. Sample extraction for vitamin B12 determination

KCN-acetate buffer was employed for the vit B12 extraction. Due to the photosensitivity of vit B12, the sample tubes were wrapped with aluminium foil. Four mL of each sample (motoho) was added to the sample tubes, respectively, containing 10 mL of KCN-acetate buffer, which represents a ratio of 4:10 ($v.v^{-1}$). The samples were homogenized, autoclaved for 25 min at 121 °C followed by rapid cooling in an ice-bath. Once cooled, the samples were centrifuged (Avanti J-E, Beckman) for 10 min at 4 °C and 15000 × g. After centrifugation, the supernatant was filtered through 0.45 µm syringe filters (Ministart, National Separations) into amber HPLC sample vials with septa screw-caps (Science World, South Africa). Samples, which were not analysed immediately, were flushed with nitrogen gas and stored in the ultra-freezer set at –80 °C in a dark environment for a maximum of 30 d. The storage conditions were to ensure complete retention of the vit B12 activity.

4.3.6. Sample purification and concentration

To improve the sample concentration and purification for analysis, Solid Phase Extraction (SPE) columns were employed. A preconditioned SPE column (Chromabond SB/ 3 mL/ 500 mg, Macherey-Nagel, Düren, Germany) were utilised to purify the samples. 9-12 mL sample extract and the eluate (E1) was collected in clean sample vials. After eluting the purified vit B12, the SPE column contained a high concentration of impurities with some retained vit B12 residue. Therefore, one column volume (c.v) of 400 $mL.L^{-1}$ HPLC-grade methanol (Science World) was employed to elute the remaining vit B12 residue. The second eluate (E2) was collected separately from E1. The samples were concentrated by preconditioning the SPE column (Chromabond C18 EC/ 6 mL/ 1000 mg column, Macherey-Nagel). It entailed applying eluate E1 to the column. The vit B12 in the column was then eluted using E2 followed by 1 mL of 900 $mL.L^{-1}$ methanol. The methanol in the sample was removed by means of flash

evaporation under vacuum using the rotary evaporator set at 40 °C to concentrate the samples. Three mL of Milli-Q water was added to the concentrated samples in a volumetric flask, and then filtered into amber vials prior to HPLC analysis.

The HPLC separation of the samples and standard was performed with the aid of an Agilent 1100 HPLC system. The HPLC-system consisted of a vacuum degassing unit (G1322A), G1311A quaternary pump, a compartment for the thermostat column set at 30 °C, an auto sampler which was set at a volume of 20 µL as well as a G1315C Diode Array Detector (DAD) set at 360.4 nm with a reference wavelength of 360.2 nm which was employed during analyses. The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) was used to integrate peak-areas as well as to record and store the data.

4.3.7. HPLC assay

The cyanocobalamin was determined under the following conditions: the analytical column employed for the reverse phase was a 3 µm C18 column (Luna 250 mm X 4.6 mm; Phenomenex Inc., Torrance, CA, USA), and a reversed-phase C18 guard cartridge (ODS 4.0 mm X 4.0 mm; 5 µm; Phenomenex Inc). The guard cartridge required to be replaced after every 150 injections. The mobile phase used was 95:5 to 50:50 (v/v) acetonitrile-water at a linear gradient over 35 min with a flow rate of 0.5 $mL.min^{-1}$.

The Agilent Chemstation software was utilized for the calibration function, whereby the constructed calibration curve and the quantified vit B12 concentration in the samples were performed by the external standard method. Concerning quantification, the standard solution was diluted from 20 $\mu g.mL^{-1}$ to 0.01 $\mu g.mL^{-1}$, and analysed in duplicate at the beginning of each working day. The software was programmed to utilize the resultant peak-area to determine the concentration of B12 in the samples. The column was flushed with 100% acetonitrile for 5 min, then by 100% acetonitrile for 5 min and then recycled to initial conditions for 5 min. This was done to store the column for future use.

4.3.8. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

A linearity curve required to be construed using B12 standard solutions at 0.01, 0.03, 0.10, 0.25, 0.50, 1.0, 1.50, 2.0, 2.50, 5.0, 10.0 and 20.0 $\mu g.mL^{-1}$. Twelve samples per concentration (n=12) were analysed and the various correlation coefficients (R2) and regression coefficients (R) were used to determine if the peak area plotted was linear over the concentration range. The linearity curve was evaluated to determine if the linear region was suitable to be used to quantify the B12 concentration in motoho. The LOD and LOQ were respectively determined based on the signal noise ratios of 3 and 10 (S/N=3 and 10). The standard deviation (SD) of the slope (S) and response of the calibration curve were utilized to approximate the LOD according to the formula:

LOD = 3 (SD/S)

The LOQ was determined using the response SD and the slope of the calibration curve according to:

LOQ = 10 (SD/S)

It was then possible to determine the standard deviation of the response, by using the standard deviation of the y-intercepts of the regression line. The SD and slope values were determined from the LINEST function when the calibration curve was created using SigmaPlot® (Systat Software, San Jose, USA). The SD of the y-intercepts was then utilized as the standard deviation for LOD and LOQ calculation.

4.4. Results and discussion

4.4.1. Linearity and peak identification

A linear curve was constructed to determine the response variation with that of the vit B12 concentration variation. The vit B12 linearity curve illustrated acceptable linearity (R^2=0.982) between the vit B12 peak area and the concentration of the injected analyte for the concentration range between 0.01 µg.100 mL^{-1} and 20 µg.100 mL^{-1}, with 0.0054 µg.100 mL^{-1} as the LOD and 0.018 µg.100 mL^{-1} as the LOQ (Figure 4.1 and Table 4.1). The correlation coefficient (R) was 0.991 and multiple correlation coefficient (R^2) was 0.982. The calibration method employed for vit B12 was conducted in accordance the AOAC method (Anon., 2002; Morkel, 2016). The results obtained indicated that the concentration range with 12 points with equal spacing was ideal for the calibration pattern. A high correlation coefficient > 0.99 and multiple correlation coefficients (R^2) served as evidence for a good linear (Anon., 2002; Morkel, 2016).

The identification of vit B12 was accomplished by analysing the retention time (t_R), with the quantification of the vitamers accomplished by measuring the peak areas of the samples in relation to those of the standards. The t_R for the vit B12 was determined by analysing the t_R of sample standards during (n= 3) three sessions, coupled with the calculation of the means and standard deviation. The vit B12 retention time (min) obtained was 16.61 ± 0.10.

4.4.2. Vitamin B12 detection and quantification in motoho

The vit B12 concentration detected in traditionally fermented motoho (TFM) (Figure 4.2 and 4.3) resulted in 9.11 ± 1.81 µg.100 mL^{-1} for the control, which is higher than the recommended daily intake of vit B12. The recommended daily allowance for vit B12 for teenagers (14-18

years) and adults is 2.4 µg/day, for pregnant teenagers and women is 2.6 µg/day, and with 2.8 µg/day for breastfeeding teenagers and women (National Institutes of Health, 2021:1)

Cereal grains such as sorghum are deemed as ideal substrates for fermentation, due to their high content of carbohydrates, minerals, vitamins and other constituents that support microbial fermentation (Achi and Ukwuru, 2015:73; Ukwuru *et al*., 2018:23). Lactic acid bacteria are recognised for their ascendency during spontaneous cereal fermentation (Moodley *et al.,* 2019b:5; Xie, 2020:2), with the genus *Lactobacillus* being found to have the most extensive cobalamin biosynthesis capacity (Ribeiro *et al*., 2023:2). *Lactobacillus reuteri* and *Lactobacillus plantarum* were reported to be able to synthesise vit B12, however in other studies it was discovered that lactobacilli produced pseudo-vitamin B12 due to its lower ligand (adenine) which is inactive in the human body (Xie, 2020). A study conducted by Ribeiro *et al*. (2023:5) evaluated the biosynthesis capacity of vit B12 by LABs using a microbiological approach for quantification. The study yielded that some of the LABs isolated from *C. vulgaris* were able to synthesise vit B12 obtaining 6.18 pg.mL^{-1} *Lactococcus lactics* E31, 5.47 pg.mL^{-1} *Levilactobacillus brevis* G31 and 28.19 pg.mL^{-1} *Pedicoccus pentosaceus* L51, with *P. pentosaceus* L51 synthesising similar vit B12 concentration as *Furfurilactobacillus rossiae* 29.30 pg.mL^{-1}.

The production of vit B12 in motoho could be due to the LABs, which were present in the sorghum flour. It is still to be determined if the vit B12 content in motoho is active or inactive, in order to determine its suitability for human consumption.

4.4.3. Optimisation of vitamin B12: low and high inoculum

There was a significant ($p \leq 0.05$) difference in vit B12 levels between the TFM (control) and the inoculum optimised culture batches (Figure 4.2 and 4.3). The low concentration inoculum (LPAB-J17) obtained 3.41 ± 0.68 µg.100 mL^{-1} which was significantly lower ($p \leq 0.05$) than the control and was significantly lower ($p \leq 0.05$) than the high concentration inoculum (HPAB-J17), which was 19.55 ± 3.45 µg.100 mL^{-1}. The vit B12 content for HPAB-J17 was two-fold higher than the control and five-fold higher than LPAB-J17, which demonstrated that co-inoculation with a high inoculum cell concentration PAB-J17 culture and tomoso is effective in substantially increasing the vit B12 concentration in motoho.

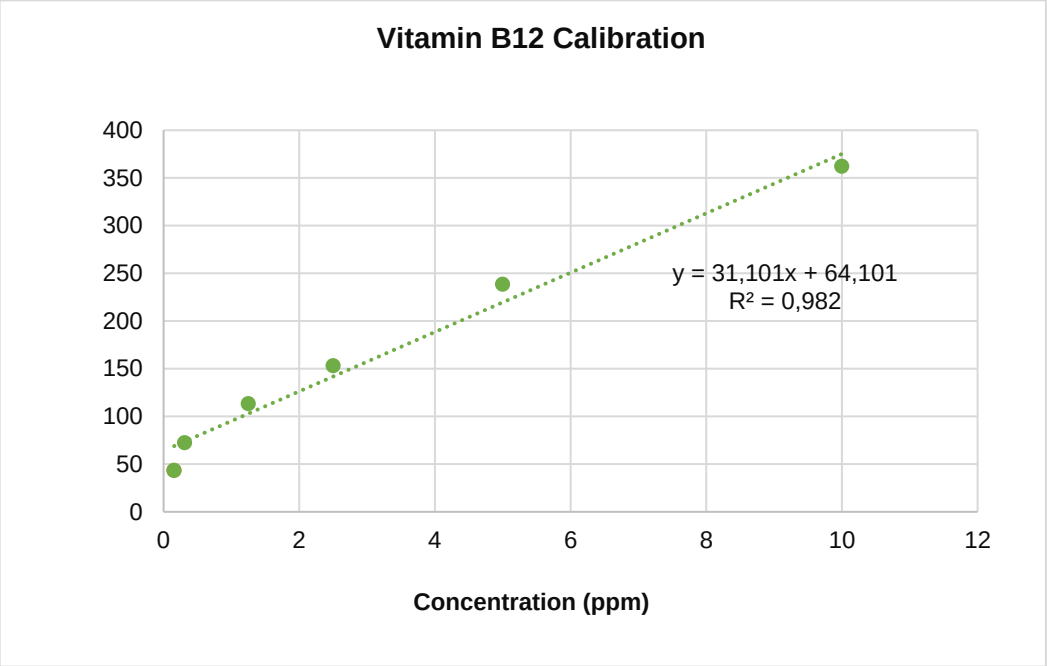


Figure 4.1: Linearity curve for the vitamin B12 standard solution

Table 4.1: The vitamin B12 standards solution linear range, the limits of detection (LOD) and quantification (LOQ)

Parameter	Vitamin B12 (µg.100 mL^{-1})
Linear range	0.01 - 20
LOD	0.0054
LOQ	0.018

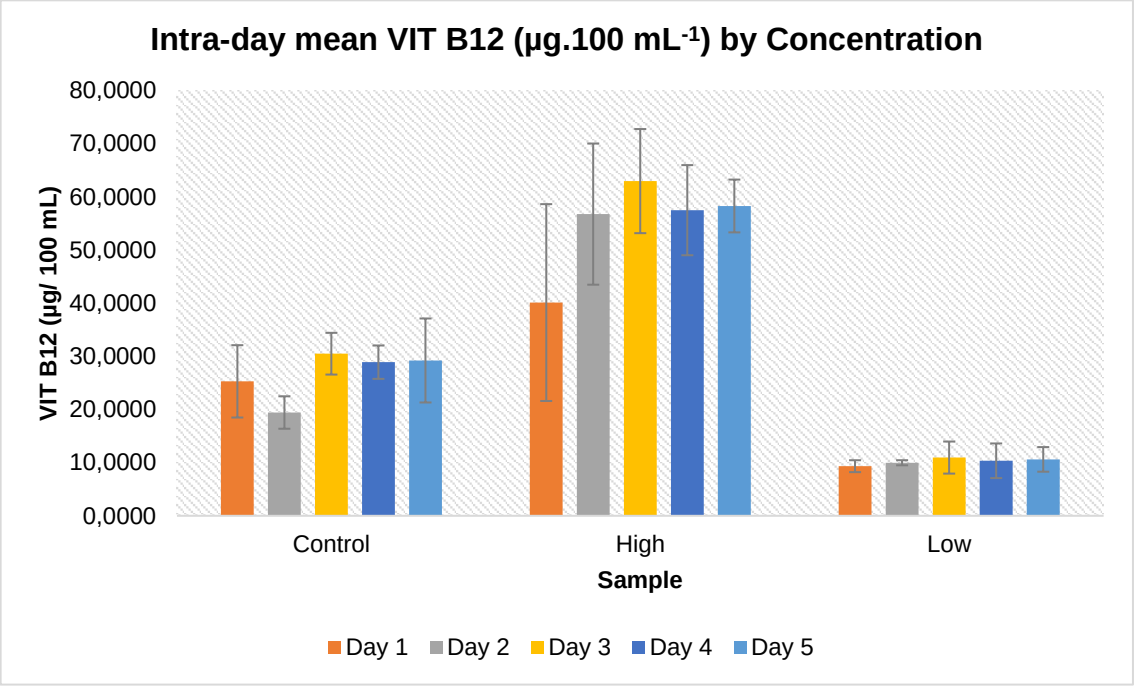


Figure 4.3: Vitamin B12 analysis over 5 days

The vit B12 content of LPAB-J17 was lower than that of TFM and HPAB-J17. LPAB-J17 was co-inoculated with tomoso and PAB culture at half the quantity spiked in the HPAB-J17. The pH fluctuated throughout fermentation but had the same final pH as MFS at pH 4.45. The low vit B12 content in LPAB-J17 could be due to the stress experienced by the PAB during fermentation. The type of LAB present in the medium can significantly affect the growth of PAB as well as the type of PAB strain used for fermentation. The biosynthesis of vit B12 requires a variety of optimised anaerobic and aerobic fermentation parameters (Chamlagain, 2016:14-15; Turgay *et al.,* 2020:6-7). Vitamin B12 is an essential vitamin required for fundamental biological processes in humans and bacteria. Certain *Lactobacillus* species have been reported to require B-vitamins for growth (Forgie *et al.*, 2023). The low vit B12 content in LPAB-J17 could be attributed to the inherent ability for LABs asserting their dominance by growing at a faster rate than the PAB-J17. The nutrient-rich environment in which the LABs present in tomoso were introduced to in combination with the introduction of PAB-culture could have affected the vit B12 concentration produced by PAB-J17. Turgay *et al*. (2020:9) stated that the presence of some hetero-fermentative mesophilic lactobacilli can inhibit the fermentation of propionic acid. *P. freudenreichii* subsp*. shermanii* is characterised to have poor nitrate reductase activity with the ability to ferment lactose. At the onset of fermentation, the pH was 4.79 ± 0.00 and with the abundance of nutrients, the LABs dominated and maintained dominance throughout by utilising the available vit B12. Therefore, there is a possibility that the vit B12 produced by PAB during fermentation of LPAB-J17 could have been utilised by LAB in competition for survival.

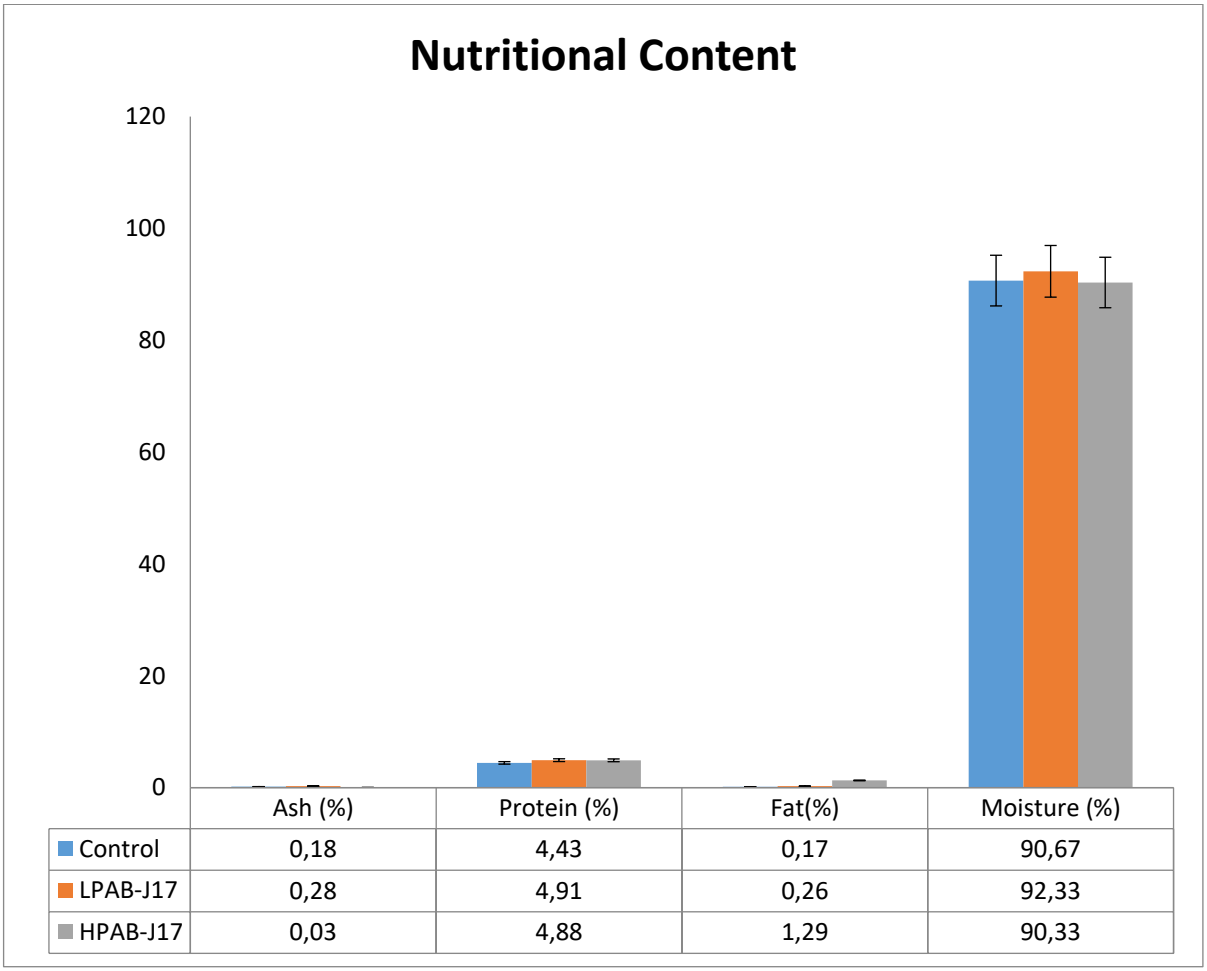
For HPAB-J17, the co-inoculated concentrations were high enough to be effective in increasing the vit B12 concentration. *P. freudenreichii* is known to have the ability to adapt to various stressful conditions, which could be encountered during fermentation. However, that is only possible if the bacteria have been pre-conditioned (Turgay *et al.,* 2020:7). From the beginning to the end of fermentation, the pH decreased from 4.70 ± 0.00 to 3.60 ± 0.00; the pH condition was not optimal for both LABs and PAB, but *P. freudenreichii* has been reported to be able to survive and grow at pH ≤ 5.5.

The optimisation of vit B12 production by means of cereal fermentation with *P. freudenreichii* has been explored. In studies where *P. freudenreichii* was employed in cereal fermentation, it was co-inoculated with LABs. The study conducted by Xie (2020:6,8) explored fermentation of various cereal flours with *P. freudenreichii* and *L. brevis* in various inoculum concentrations. The resultant vit B12 concentrations obtained were 265 ± 13, 125 ± 27 and 67 ± 14 $ng.g^{-1}$ (dw) for the three sorghum fermentation models, which is lower than the vit B12 content obtained in this study. Cichonska *et al.* (2022:10) produced rice-based fermented beverages by co-inoculating with two LAB starter-cultures and three PAB cultures, including *P.*

freudenreichii subsp. *shermanii;* both of the studies are testament to exploring cereal fermentation with microbial cultures that exhibit both qualitative and quantitative vitamin biosynthesis capacity.

4.4.4. Proximate analyses of TFM, LPAB-J17 and HPAB-J17 samples

The proximate analyses for the three models are illustrated in Figure 4.4. There was no significant difference ($p \geq 0.05$) in the protein content of motoho for the three models, 4.33 ± 0.36 g.100 mL^{-1} (dw) (TFM), 4.19 ± 0.42 g.100 mL^{-1} (dw) (LPAB-J17) and 4.88 ± 0.06 g.100 mL^{-1} (dw) (HPAB-J17). However, the protein content for the three models was significantly ($p \leq 0.05$) higher than the 0.4 ± 0.00 g.100 mL^{-1} obtained by Moodley (2015: 64). For ash content there was no significant difference ($p \geq 0.05$) between TFM 0.17 ± 0.03 g.100 mL^{-1}, (LPAB-J17) 0.28 ± 0.20 g.100 $mL^{-1}$and 0.03 ± 0.03 g.100 mL^{-1} which were the similar to what was obtained by Moodley (2015:64) 0.1 ± 0.00 g.100 mL^{-1}. There was no significant difference ($p \geq 0.05$) in the fat content between 0.17 ± 0.03 g.100 mL^{-1} (TFM) and 0.26 ± 0.05 g.100 mL^{-1} (LPAB-J17), but HPAB-J17 1.29 ± 0.33 g.100 mL^{-1} was significantly higher ($p \leq 0.05$) than TFM and LPAB-J17. For the moisture content, there was no significant difference ($p \geq 0.05$) between the models; 90.67 ± 3.21 g.100 mL^{-1} (TFM), 92.33 ± 1.15 g.100 mL^{-1} (LPAB-J17) and 90.33 ± 2.08 g.100 mL^{-1} (HPAB-J17).



	Ash (%)	Protein (%)	Fat(%)	Moisture (%)
Control	0,18	4,43	0,17	90,67
LPAB-J17	0,28	4,91	0,26	92,33
HPAB-J17	0,03	4,88	1,29	90,33

Figure 4.4: Proximate analyses of the three models of motoho

4.5. Conclusion

Vitamin B12 was detected and quantified in motoho. Co-inoculation of tomoso and a high inoculum cell concentration of PAB-J17 demonstrated to successful in optimising the vit B12 content of motoho. The co-inoculation for the low-inoculum (LPAB-J17) was unsuccessful in increasing the vit B12 content.

The detected vit B12 content of motoho (TFM which is the control) was significantly ($p \leq 0.05$) higher than the LPAB-J17 model and the daily recommended quantity of 2.4 µg/day. The analysis of the vit B12 present in motoho will facilitate declaring motoho as a source of vit B12 and allow for further exploration in commercialising motoho. The PAB-J17 inoculum concentration illustrated to have a significant ($p \leq 0.05$) effect on the quantity of vit B12 produced in the HPAB-J17 model and the LPAB-J17 model. The production of low vit B12 content IN LPAB-J17 can be attributed to the hostile environment, which prohibited the high biosynthesis of vit B12. The co-inoculated volume of tomoso was the same for both optimisation models. The only difference was the PAB-J17 inoculum cell concentrations. For LPAB-J17, the PAB-J17 could not acclimatise to the fluctuating pH throughout fermentation and other competing microorganisms present in the slurry. For HPAB-J17, the high concentration co-inoculation allowed for vit B12 production that was significantly higher than control model of motoho and LPAB-J17.

The protein content of the three models was significantly higher than that obtained in other studies. The specific variety of sorghum species used for this study could have contributed to the protein content being higher than those obtained from other previously conducted research work. HPAB-J17 obtained the highest protein and fat content than TFM and LPAB-J17, whereas the moisture content and ash contents of the three models were similar and not significantly ($p \geq 0.05$) different.

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CHAPTER 5

GENERAL DISCUSSION AND CONSLUSIONS

5.1. Discussion

Cereal fermentation is popular in Africa, with versatile cereal grains such as sorghum being cultivated primarily for human consumption. The versatility and variety of food products and beverages produced from sorghum is testament for the need to explore and promote indigenous foodstuffs produced from it (Ukwuru *et al*., 2018:23; Adebo, 1, 8). Fermented foodstuffs have been recommended to form part of consumers' daily diets due to their nutritional quality and health promoting benefits (Marsh *et al.*, 2014:121-122; Marco *et al.*, 2017:99).

Vitamin deficiency among the low socio-economic populations in Southern Africa is high, and the effects of vitamin deficiencies are evident in the general health and quality of life of the young and elderly populations (Micronutrient Forum, 2015:2; Soh *et al.*, 2020:1). Vitamin fortification by means of fermentation offers the opportunity to produce food products and beverages that have functional attributes and distinctive nutritional qualities. This is achievable by means of employing GRAS microorganisms such as PAB, which possess the ability to synthesize desired vitamins in desired quantities, and therefore foregoing the need to use synthetically synthesised food additives (Wang *et al.*, 2015:123; Chamlagain *et al.*, 2016:117, 122-123). *P. freudenreichii* is applauded for its biosynthesis of active vit B12 (Deptula *et al*., 2015:2). Research studies focused on co-inoculation with LABs are continuously being explored in cereal fermentation in order to increase the vit B12 content.

Cereal fermentation is a commonly practiced method of food production and preservation in Africa. The nutritional composition of sorghum is noteworthy and is a good substrate for microbial fermentation (Debabandya *et al.*, 2017:181; Adebo, 2020:1). Production of fermented food products and beverages produced from sorghum offers substitutes for consumers who have celiac disease, milk allergy and lactose intolerance and/or sensitivity (Taylor *et al.*, 2014:258., Tsafrakidou *et al*., 2020).

In this research study, LABs were the most dominant organisms during fermentation, with MFS 9.02 ± 0.6 log cfu.mL^{-1} obtaining significantly higher ($p \leq 0,05$) LAB counts than LPAB-J17 8.32 ± 0.83 log cfu.mL^{-1} and 7.78 ± 1.52 log cfu.mL^{-1}. The pursuit to isolate *Propionibacterium* was unsuccessful. However, isolate-MFS1 resulted in obtaining similarities (99.5 %) to seven bacterial strains. It was closely related to the Type Strain of *Bacillus rugosus* sp. nov., which is an emerging organism of the *Bacillus* genus. The detection and quantification

of vit B12 in motoho (TFM) was successful, which resulted with TFM containing 9.11 ± 1.81 µg.100 mL^{-1} of vit B12, which is significantly higher ($p \leq 0.05$) than the RDA of 2.4 µg/day. The resultant quantity of vit B12 in motoho can be utilised to encourage the production and consumption of motoho. This includes developing standardised production parameter in order to ensure product quality and safety. The demand for traditional foods such as motoho, necessitates the need for quantifying and classifying the vitamin and mineral content of the product.

The low inoculum cell concentration (LPAB-J17) obtained 3.41 ± 0.68 µg.100 mL^{-1}, which was significantly ($p \leq 0.05$) lower vit B12 than TFM and HPAB-J17. Optimisation for this model was unsuccessful. For HPAB-J17, the optimisation was successful, with vit B12 content of 19.55 ± 3.45 µg.100 mL^{-1}, which was two-fold higher than the control and five-fold higher than LPAB-J17. There protein content of the three models from the study were higher than those obtained in other studies, and there was significant difference ($p \geq 0.05$) in the proximate analyses between the three models for this study.

The interest in traditional foodstuffs as well as the increase in the popularity of cereal fermented foodstuffs necessitates that to nutritional profiles be investigated as well documented. This also includes the development of acceptable microbial standards. In conclusion, the isolation of *Propionibacterium* strain was unsuccessful; however, MFS1 from the control was identified as *Bacillus rugosus* as determined by sequencing the 16S rRNA region and phylogenetic analysis. Motoho showed to contain vit B12 and the optimisation of a acan allow for the exploration with co-inoculation with *P. freudenreichii* subsp. *shermanii* in sorghum fermentation.

5.2. Recommendations

The method employed for the isolation of MFS1 (*Bacillus rugosus*) is the one that is used for the isolation and growth of dairy PAB. Bhattacharya *et al*. (2020) isolated the organism (*B. rugosus*) by streaking on to nutrient agar. The laboratory media requirements and growth conditions for the isolate have not yet been established, the development of a standard operating procedure will assist in ensuring consistent isolation of the organism. The preservation of the isolate is paramount, therefore either preservation in trypticase soy broth (TSB) with 30 % ($v.v^{-1}$) at -80 °C (Bhattacharya *et al*., 2020:1677), or freeze drying can be explored. For DNA extraction and amplification, an appropriate protocol needs to be employed and the use of closely related *Bacillus* species such as *Bacillus spizizenni*, *Bacillus tequilensis* and *Bacillus vallismortis* for PCR-analysis as positive controls (Bhattacharya *et al*., 2020:1677). *B. rugosus* biosynthesises diketopiperazine, which is naturally present in various

food products and beverages participate in the chemesthatic effects of food. However, the presence of diketopiperazine in motoho is yet to be determined. The presence of diketopiperazine in motoho could avail opportunities for explorative research and promotion of the indigenous sorghum fermented beverage.

As motoho (TFM) contained a substantial concentration of vit B12, it is of significant value to investigate the form of vit B12 present. The vit B12 in motoho could either be the active or inactive form. Humans are unable to utilise inactive forms of vit B12 and is only able to absorb cobamides with dimethylbenzimidazole (DMBI) as the lower lignand (Chamlagain, 2016a:14). LABs have been reported to synthesise vit B12 (Ribeiro *et al*., 2023:2), therefore it is of interest to investigate if LABs in motoho are responsible for vit B12 synthesis. The inclusion of another cereal flours (millets, teff, or maize), legume flour or insect flour could assist to increase the macro nutritional composition of motoho.

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