Cape Peninsula University of Technology

CHEMOMETRICS, PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS OF PEARL MILLET BEVERAGE PRODUCED WITH BIOBURDEN LACTIC ACID BACTERIA PURE CULTURES

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DECLARATION

I, **Mmaphuti Abashone Ratau**, declare that the contents of this thesis represent my own unaided work, and that the thesis 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**

ABSTRACT

The aim of this study was to evaluate the physical, chemical and sensory characteristics of non-alcoholic pearl millet beverage produced using isolated and purified cultures of bioburden lactic acid bacteria (LAB). Traditional non-alcoholic pearl millet beverage (TNAPMB) was produced through spontaneous fermentation. The slurry was fermented for 36 h at 37°C while monitoring the microbial growth at 3 h interval. LAB were grown on deMan, Rogosa and Sharpe agar and identified using Vitek 2 system. The initial numbers of LAB were 7.04 log cfu/ml and increased to 8.00 log cfu/ml after 21 h. The beverage was dominated by LAB and contaminants and their survival was in succession. LAB from the genera *Leuconostoc, Pediococcus, Streptococcus* and *Enterococcus* were the main fermenting species in TNAPMB. Pearl millet extract (PME) was produced by hydrating pearl millet flour (PMF) with water (1:10, PMF:Water). To the mixture sprouted rice flour (10%), ground ginger (10%) and pectin (0.6%) were added. Stable PME was used in the production of plain non-alcoholic pearl millet beverage (PNAPMB). PME was pasteurized at 98°C for 30 min, hot filled and cooled to 25°C. The fluid was inoculated with *Leuconostoc mesenteroides, Pediococcus pentosaceus* and *Enterococcus gallinarum* each at 0.05, 0.075 and 0.1%, respectively, using factorial design and fermented for 18 h at 37°C. The pH of the beverage ranged between pH 3.32 and pH 3.90. *L. mesenteroides*, *P. pentosaceus*, *E. gallinarum,* the interaction between *L. mesenteroides* and *P. pentosaceus* and the interaction between *L. mesentoroides* and *E. gallinarum* had a significant effect ($p < 0.05$) on the pH of PNAPMB except the interaction between *P. pentosaceus* and *E. gallinarum* ($p = 0.631$). The total titratable acidity (TTA) of the beverage ranged from 0.50 to 0.72%. All cultures had a significant influence ($p < 0.05$) on the TTA of the beverage with the exception of the interaction between *L. mesenteroides* and *E. gallinarum* ($p = 0.102$). However, Monte Carlo simulation showed that *E. gallinarum* caused an increase in the pH and a decrease in the TTA of the beverage. During fermentation, the pH of the beverage is desired to decrease while the TTA increases, hence *E. gallinarum* was removed. The interaction between *L. mesenteroides* and *P. pentosaceus* at 0.05% and 0.025%, respectively produced an acceptable PNAPMB with potential for commercialization. Furthermore, moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) was produced by adding 4% of moringa (*Moringa oleifera*) leaf powder extract during the production of PNAPMB. The physicochemical, nutritional, microbial (LAB) and sensory characteristics of the PNAPMB, MSNAPMB and TNAPMB were determined. LAB were significantly ($p < 0.05$) affected by the fermentation period and increased from 3.32 to 7.97 log cfu/ml and 3.58 to 8.38 log cfu/ml in PNAPMB and MSNAPMB, respectively. The pH of

PNAPMB decreased from pH 5.05 to pH 4.14 while the pH of MSNAPMB decreased from pH 5.05 to pH 3.65 during the 18 h fermentation. The growth of LAB during fermentation had a significant effect ($p < 0.05$) on the pH of the beverages. The TTA increased from 0.14 to 0.22% and increased from 0.17 to 0.38%, in PNAPMB and MSNAPMB, respectively. The TTA of the beverage was affected significantly ($p < 0.05$) by the 18 h of fermentation. The protein content was 1.62, 2.17 and 1.50% in PNAPMB, MSNAPMB and TNAPMB, respectively. PNAPMB sample was deemed acceptable in comparison to the MSNAPMB. The total colour difference (ΔE) was 5.91 and 10.60 in PNAPMB and MSNAPMB, respectively in comparison to the TNAPMB. Volatile compounds with beneficial effect such as anti-inflammatory and anti-pathogenic properties were identified in the beverages. Principal component analysis indicated that the variations in characteristics of PNAPMB and MSNAPMB could be explained using total fat, saturated fat, total sugar, ash, moisture, proteins, chroma (C), hue and b*. The results showed that isolated pure cultures could be used as starter cultures in the production of non-alcoholic cereal beverages at a commercial level with predictable quality and safety properties.

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DEDICATION

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GLOSSARY

Terms/Acronyms/ Abbreviations	Definition/explanation
ΔE	Total colour difference
ANC	Anaerobic card
GC-MS	Gas chromatography mass spectrometry
GP	Gram positive cards
HPLC	High performance liquid chromatography
MANOVA	Multivariate analysis of variance
SRF	Sprouted rice flour
MSNAPMB	Moringa supplemented non-alcoholic pearl millet beverage
MUFA	Monounsaturated fatty acids
NACB	Non-alcoholic cereal beverage
NAPMB	Non-alcoholic pearl millet beverage
NIST	National Institute of Standards and Technology
OD	Optical density
OD_{corr}	Correlated optical density
PME	Pearl millet extract
PMF	Pearl millet flour
PMS	Pearl millet slurry
PNAPMB	Plain non-alcoholic pearl millet beverage
PUFA	Polyunsaturated fatty acids
r	Pearson correlation coefficient
SEM	Scanning electron microscope
SFA	Saturated fatty acids
SMEs	Small and medium-sized enterprises
TNAPMB	Traditional non-alcoholic pearl millet beverage
TTA	Total titratable acidity
X_1	*Leuconostoc mesenteroides*
X_2	*Pediococcus pentosaceus*
X_3	*Enterococcus gallinarum*

CHAPTER ONE
MOTIVATION AND DESIGN OF THE STUDY

1.1 Introduction

Pearl millet [*Pennisetum glaucum* (L) R. Br.] is a cross pollinated cereal crop grown annually in summer (Guarino, 2012) mainly in tropical regions of Africa and Asia (Nambiar *et al*., 2011). Pearl millet grows well in areas susceptible to drought, poor soil fertility, and extreme temperature (Kajuna, 2001; Oushy, 2008; Basavaraj *et al.,* 2010). The fifth important cereal crop in the world is pearl millet after rice, wheat, maize, and sorghum (Nout, 2009; Mathur, 2012) grown in arid and semi-arid areas (Ojediran *et al*., 2010). The crop is neglected due to low demand, and or unreliable availability (Basavaraj *et al.,* 2010). Not much is known about many African foods that are prepared through fermentation of cereal crops such as pearl millet (Abegaz, 2007).

Fermentation is a metabolic process carried out by microorganisms in which an organic substance, usually, carbohydrates is broken down resulting in biochemical changes (desirable and undesirable) and significant food modification (Sahlin, 1999). Fermentation leads to lower volume of the material to be transported due to food modification; remove unwanted components; improve the nutritive value and overall appearance of the food; uses less energy for production and makes food safer (Blandino *et al*., 2003). There are two important types of commercial fermentation, namely, ethanolic and lactic acid fermentations (Abegaz, 2007; Chojnacka, 2011). Ethanolic fermentation is a process in which sugar inherent in biomass is converted into liquid fuel such as ethanol (Houghton *et al.,* 2006), a major end product of anaerobic metabolism carried out by mostly yeast but also of *Zymomonas* species. This fermentation pathway is also referred to as alcoholic fermentation (Muller, 2001; Blandino *et al.,* 2003). The major end product of lactic acid fermentation is lactate (lactic acid) caused by the breakdown of sugar (usually glucose) by lactic acid bacteria (Muller, 2001). Lactic acid has applications in food products as a preservative, acidulant and flavourant (Liu, 2003). Lactic acid fermentation processes are the ancient method used to preserve food for consumption (Nyanzi & Jooste, 2012). Cereal grains such as maize, sorghum and millet are commonly used as substrate in Africa for the production of many fermented products such as non-alcoholic beverages. The most popular non-alcoholic beverage in southern Africa is *Mahewu* (Bvochora *et al*., 1999; Gadaga *et al.,* 1999).

The preparation of many fermented traditional foods especially non-alcoholic beverages (NAB) is carried out by a mixed population of bacteria and yeast (Gotcheva *et*

al., 2000). Lactic acid bacteria (LAB) such the genera *Lactobacillus, Streptococcus*, *Pediococcus* and *Lactococcus* produce lactic acid whilst *Leuconostoc, Oenococcus, Weissella* and *Lactobaccilus* can produce lactate, CO_2 and ethanol/acetate (Tanguler & Erten, 2011). LAB are labelled as Generally Recognised as Safe (GRAS) microorganisms (Chagnaud *et al.,* 2001; Macwana & Muriana, 2012); they are found in fermented and non-fermented foods and are naturally present as human commensal microflora (Rossetti & Giraffa, 2005). Their most importance is associated with physiological features that include substrate utilisation, metabolic capabilities and probiotic properties (Liu *et al.,* 2011). LAB are divided into two groups depending on the end product of glucose metabolism, namely, homolactic and heterolactic fermentation. In homolactic fermentation, lactate is the sole product from glucose metabolism whilst in heterolactic fermentation; lactates, carbon dioxide and ethanol in equal molar are the end products (Halasz, 2011; Ongol, 2012). Although different technologies such as cooking, sprouting and milling are used during cereal processing, fermentation still remains the preferred method for enhancing the nutritional, sensory and shelf-life properties of food (Coda *et al*., 2011).

Chemometrics is the science that design or select the best measurement procedure and experiments using mathematical and statistical information, to give maximum chemical information by analyzing multivariate chemical data, and to represent and show chemical data (Rodionova & Pomerantsev, 2006; Otto, 2007). Chemometrical methods are applied to develop quantitative and qualitative structural activity relationships between chemical structure and biological activity (Rodionova & Pomerantsev, 2006; Otto, 2007). Multivariate data analysis refers to the analysis of information with many variables measured from a number of samples. Thus, chemometric tools are used to find the link between the samples and variables in a given set of information and convert new variables not directly observed (Kumar *et al*., 2014).

1.2 Statement of the Problem

Many of the indigenous foods and non-alcoholic beverages are produced by natural fermentation (Gotcheva *et al*., 2000) and the preparation remains household art (Blandino *et al*., 2003). This age-long chance inoculation and uncontrolled fermentation process leads to variations in quality and stability (Sanni e*t al*., 1999; Abegaz, 2007; Ali & Mustafa, 2009; Agarry *et al*., 2010; Omemu, 2011; Mukisa e*t al*., 2012; Temitope, 2012). Traditionally, the beverage is prepared in poor hygienic conditions and due to its nutritional contents is susceptible to microbial growth and metabolism caused by mixed

microflora comprising of lactic acid bacteria (LAB), coliforms, yeast and mould (Abegaz, 2007; Ojimelukwe *et al*., 2013). Consequently, pasteurisation as a feasible preservative method has been studied (Maji *et al*., 2011; Ratau, 2011). This has limitations associated with destruction of probiotic (LAB) microorganisms, which exert health benefits beyond inherent general nutrition (Prado *et al.,* 2008; Nyanzi & Jooste, 2012). A lot has been reported in the literature on the types of organisms found in naturally fermented millet beverage but nothing on the effect of isolated and purified organisms on the properties of beverage. Hence, the use of isolated and purified cultures of LAB from the chance fermented beverage will benefit consumers who pay attention to food with health promoting properties (functional food).

1.3 Research Objectives

1.3.1 Broad objectives

The aim of this study was to evaluate the physicochemical, nutritional and sensory characteristics of non-alcoholic pearl millet beverage produced using pure cultures of bioburden lactic acid bacteria.

1.3.2 Specific objectives

The specific objectives of the project were:

1. Isolation and identification of the microorganisms involved in the fermentation of pearl millet beverage.
2. Obtain pure cultures of lactic acid bacteria involved in the natural fermentation of pearl millet beverage.
3. Produce a beverage using isolated and purified lactic acid bacteria.
4. Establish the physical, chemical and viscosity properties of the beverage.
5. Establish the sensory properties of the beverage.

1.4 Research Hypotheses

It was hypothesised that:

1. Different types of microorganisms will be involved in the traditional fermentation of pearl millet.
2. The naturally occurring microorganisms will produce a desirable non-alcoholic pearl millet beverage.
3. The non-alcoholic pearl millet beverage will be acceptable to consumers.

1.5 Delimitations of the Research

Only pearl millet will be used in this study.

1.6 Importance of the Study

Climate change threaten less rain, more heat (projected $2^{o}C$ rises annually), reduced water and malnutrition. Pearl millet is a crop that can withstand these challenges, survive and flourish (MINI, 2009). The production of the non-alcoholic beverage using pearl millet could provide food for millions of people. There is a widespread level of consumption, popularity and high demand of the beverage (Gaffa *et al*., 2002) not only in rural areas but also urban centres as a result of traditions, commuting and rural migration (Marshall & Mejia, 2012). The beverage could address the need for non-dairy fermented functional food ideal for health conscious people. This could expand the growth of small and medium-sized enterprises (SMEs) through the expansion of probiotic markets and competitiveness. Other sectors may also benefit by supplying other raw materials and services such as sugar, spices, transportation etc. Furthermore, the use of indigenous crops would accelerate growth of the South African market on new brand of fermented foods and open exports of these products in Africa and all over the world. The production of the beverage would provide income to the cereal farmers and also offer employment. The farm employee would also purchase goods within South Africa thus boosting the economy through tax payments of goods. This would have positive implication on the country's food security system. The beverage would serve as a source of fluids, proteins and energy required for daily manual work. The cereal beverages are popular in Africa and have potential for export due to migration of African people. This would lead to the South African economy earning African and international currencies. The availability of the nutritious cereal beverage on the shelf would also support the convenient life in urban areas where time and space does not allow the preparation of these indigenous drinks. Overall, the study will give a better understanding of the beverage fermentation process.

1.7 Expected outcomes

The expected outcomes of this study were:

1. Innovative method for the production of non-alcoholic pearl millet beverage.
2. Non-alcoholic pearl millet beverage produced.
3. A better understanding of the beverage fermentation process.
4. A Master's degree obtained.
5. At least one manuscript sent for publication in an accredited journal.

1.8 Ethical Statement

Ethical clearance was obtained from the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology.

1.9 Thesis Outline

The chapters in this thesis are individual entities structured in an article format; hence, some repetitions between chapters have been unavoidable. Figure 1.1 shows the structure of the thesis. Chapter one presents an introduction to the problem, the objectives, hypothesis, delineation and the outcomes expected at the end of the study. The evaluation of the available relevant literature is summarised in Chapter two. A background on pearl millet, the growth conditions of millets, nutritional contents, their utilisation, trends in their production and the varieties found in South Africa are described in this section. In addition, fermentation which is used to produce non-alcoholic cereal beverages (NACB) is discussed focusing on the classification and the modes of fermentation. The different types of microorganisms found in fermented cereal beverages are also presented. Finally, the different types of fermented NACB in Africa, the cereals used during the production of NACB and the economic benefits of fermented cereal beverages are highlighted.

The first research chapter (Chapter 3) focuses on the isolation, identification and purification of lactic acid bacteria from pearl millet slurry during the production of NACB. The alpha amylase activity of sprouted rice flour used as a source of hydrolytic enzymes was evaluated. Lactic acid bacteria (LAB) were isolated and purified as the main fermenting species of NACB. During fermentation for the production of NACB the pH, total titratable acidity, total sugar contents and optical density of the beverage were evaluated.

In chapter four, a new beverage was developed using pearl millet extract fermented using purified cultures of LAB isolated in Chapter three.

Chapter five is the last research chapter in which the new beverage produced in chapter four was evaluated in terms of proximate analysis, colour, viscosity, sensory and total sugars. Finally, Chapter six summarises the entire work and the conclusions reached.

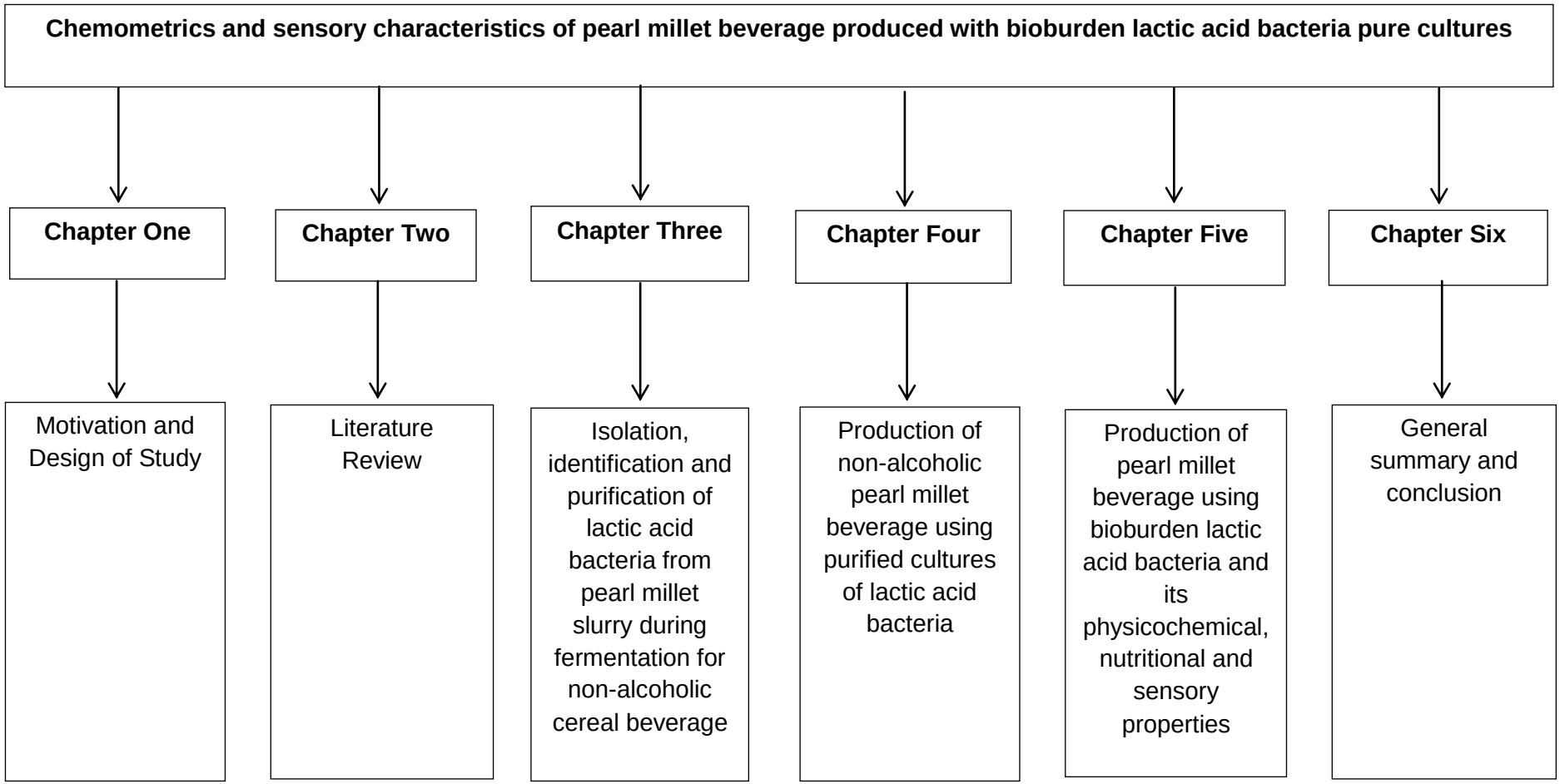

Figure 1.1 Thesis outline

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CHAPTER TWO
LITERATURE REVIEW

2.1 Description of millets

Millets are cereal crops or grains among the small-seeded species that belong to the family Poaceae and are usually grown worldwide for food and fodder (Newman *et al*., 2010; Ojediran *et al*., 2010). The crops grow in a wide range of ecological environments where there is less water (25%), infertile soil and vast dry-land making them the crop suitable for the changing climate (MINI, 2009). The species of millet that grow widely in order of cultivation worldwide are pearl millet (*Penisetum glaucum*), foxtail millet (*Setaria italic*), proso millet (*Panicum miliaceum*) and finger millet (*Eleusine coracan*) [Ojediran *et al*., 2010]. Millet production per species globally is projected at 50% pearl millet, 30% proso & foxtail, 10% finger millet and 10% for others including Barnyard and kodo millet (Gramene, 2014).

2.2 Description of pearl millet

Pearl millet is hard in texture when compared to wheat and rice and is grown in areas with low rainfall (300 – 500 mm) and high temperature (>30^{0}C) because of its ability to grow and survive under continuous or intermittent drought (Jain & Bal, 1997). The crop is similar to sorghum in terms of development and structure with exceptions that it grows straight upward, short and an annual crop (Kajuna, 2001). The crop is planted in the environments of the arid and semi-arid tropical regions of sub-Saharan Africa and Asia (Sharma *et al*., 2014). Grains of pearl millet are shaped like a liquid drop. It is smaller in size when compared to other cereal crops (Obilana, 2013). Figure 2.1 shows pearl millet crops and grains.

Pearl millet, *Pennisetum glaucum* (L.) R. Br. belongs to the tribe Paniceae of family Poaceae (Himeno *et al*., 2009; ICRISAT, 2014). The crop may grow from 50 to 400 cm tall in size (DPP, 2013). The crop has high yield of grains in comparison to foxtail millet and can re-grow after harvest if sufficient stubble is left (Lee, 2014). Vernacular name of the crop include leotja (Pedi - South Africa), mexoeira (Mozambique), mhunga (Zimbabwe), lebelebele (Botswana), bajra (India), gero (Hausa – Nigeria), hegni (Djerma – Niger), sanyo (Mali), dukhon (Arabis – Sudan) and mahangu (Namibia – Hausa), cattail or bulrush (English) [Ratau, 2011; Guarino, 2012; Andreas, 2013; Chitalu, 2013; DPP, 2013]. Pearl millet originated in areas with a lot of grasses and parks at the edge of the

Savanna-Sahel desert of West Africa 2500 BC and quickly spread around 3000 BC due to increasing dessication of Saharan desert (del Rio & Simpson, 2014).

a

b

Figure 2.1 Pearl millet (a) crops (RS, 2014) and (b) grains

The crop is consumed as a staple food by more than 500 million people in the sub-Saharan Africa and Asia where it is grown over 28 million ha of land (DPP, 2011). Pearl millet is usually known in areas prone to famine since it is reliable to always produce during harvest although at times the yield may be low (Kajuna, 2001).

Pearl millet is able to grow in many zones of ecological environments with low precipitation and relative humidity during seed ripening and maturation (Upadhyaya *et al*., 2008). It produces the best yield on (i) fertile soil, (ii) well drained acidic and sandy soils and (iii) hot environment (Oushy, 2008) as compared to maize or wheat. Pearl millet is usually cultivated without or with little fertilisers, hence it normally produces low grains (300 – 800 kg/ha grain yield). However, the crop is capable of producing about 4000 – 5000 kg/ha of grain yield during hot season when irrigated with 60 - 80 kg/ha of applied nitrogen (Khairwal *et al*., 2007). Figure 2.2 shows the amount of water required by pearl millet in comparison to other cereal crops.

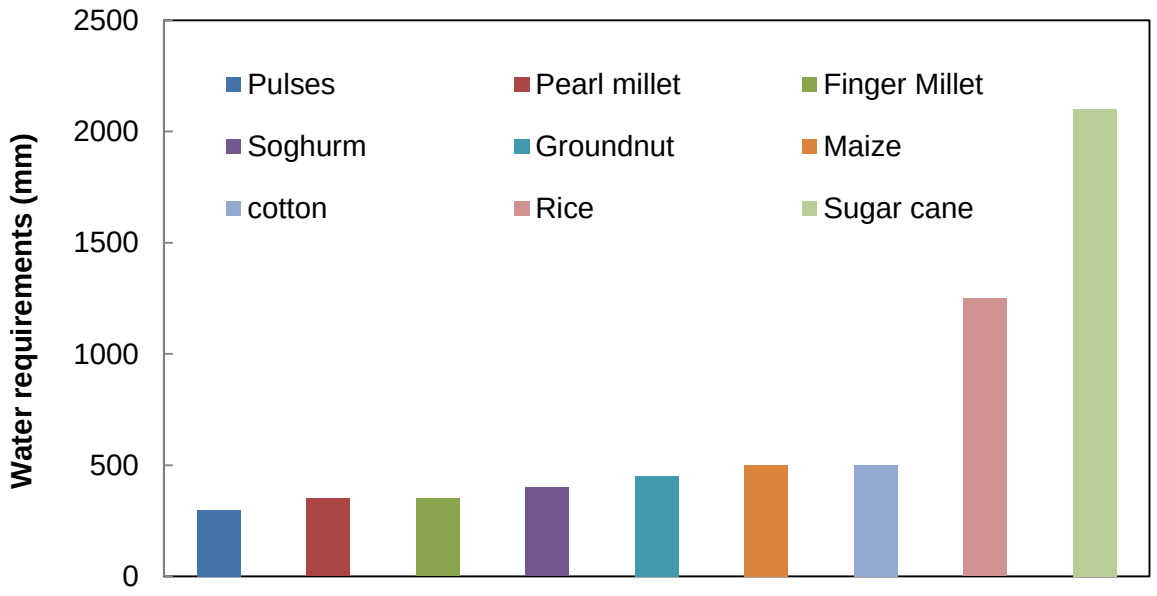

Figure 2.2 Rainfall requirements of various crops (MINI, 2009)

2.3 Utilisation and health benefit of pearl millet

Millet is mostly planted to feed only the farmer and family (0.3 – 5.0 ha farm size) as a staple food and for animal (Obilana, 2003). Nearly 80% of millet produced worldwide is used as food, while the rest is used for stock feed, beer and others (15%) [Arunachalam, 2010]. The crop is still underutilised and/or studied in science, agriculture and policies even though its becoming popular globally (Gari, 2001). Thus, only less than 2% of

globally cereal production is millet (Prasad & Staggenborg, 2009). The consumption of millet by humans has increased slightly in the past years in comparison to the increase in consumption of other cereals (FAO, 1996). About 90% of the world millet (30 million tonnes) is used in developing countries and a small portion is used in Russia. Africa has recorded the highest per capita food consumption of millet among other cereal producing countries. The consumption of millet differs between countries, with the highest consumption in African countries where it is used as staple food and is important in parts of India, China and Myanmar (FAO, 1996). Figure 2.3 shows the global millet consumption pattern. Pearl millet utilisation pattern is changing in developing countries where it is used in feed, making beer and food processing industries (Basavaraj *et al*., 2010).

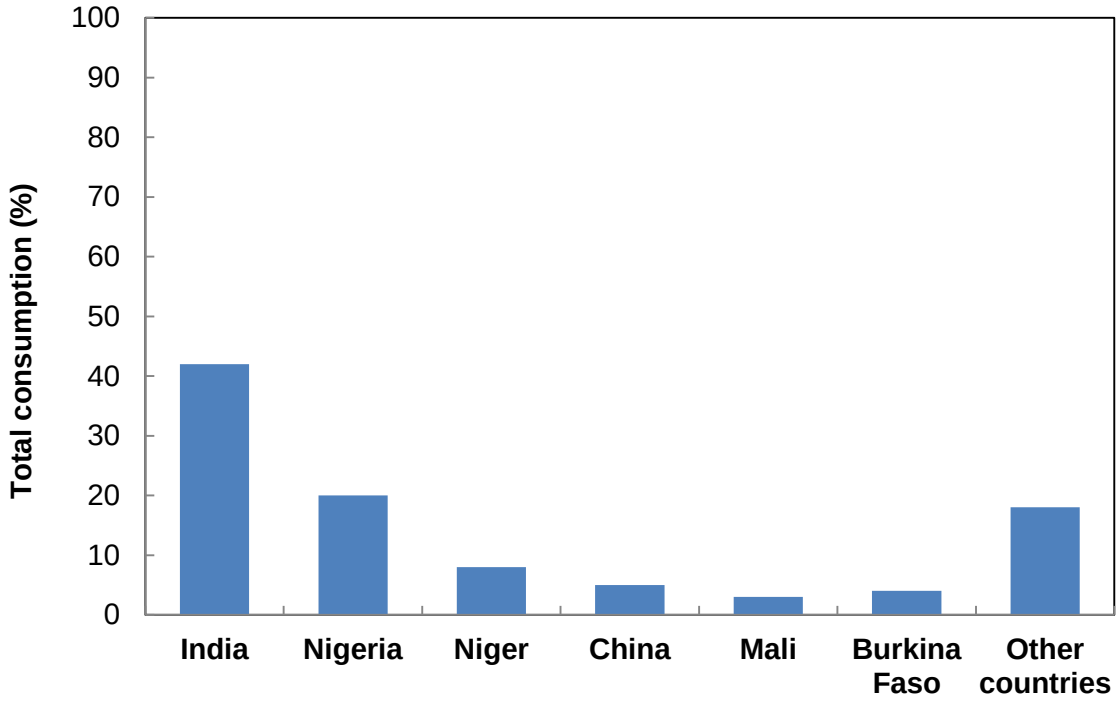

Figure 2.3 Global millet consumption pattern (MINI, 2009)

The decline in pearl millet consumption is due to various factors such as (i) the change in food habits, (ii) long processing time of the crop into food, (iii) a change in taste preferences among medium to high income consumers and (iv) the easy availability of rice and wheat at lower prices due to technological advances (Amarender-Reddy *et al*., 2013). In addition, other factors that influence the decline as reported by Arunachalam (2010) are: (i) few producers, processors and consumers use pearl millet grains, (ii)

storage costs due to limited usage, (iii) reaping, winnowing and processing by women at household level, and (iv) slow and emerging trend in industrial use. However, pearl millet has medicinal benefits compared to other cereal grains such as wheat, sorghum or maize that includes: (i) suitability for gluten intolerant and diabetic individuals, (ii) relief of severe constipation and stomach ulcers, (iii) lowering of cholesterol level due to its phytic acid and niacin content, (iv) lowering the risk of some cancer (inhibit tumour development), (v) rich in fibre content, and the wholegrain helps support weight loss, (vi) help in bone development due its phosphorus content, (vii) have antioxidant activity due its lignin and phytonutrients content (help with heart health) and (viii) has magnesium which helps alleviate respiratory problems such as asthma (Malik, 2015).

2.4 World production of millet in comparison to maize and sorghum

The highest global producer of millet is India followed by Nigeria, Niger, China, Mali, Burkina Faso, Uganda, Ethiopia, Chad and Senegal. Trends in the global cereal in terms of area harvested, yield and production between 1992 and 2013 are shown in Table 2.1 (FAOSTAT, 2015). Millets are grouped together in global millet production since is difficult to determine the amount of production for each genus. During this period (1992 – 2013), the land used to grow millet and sorghum globally has decreased by 8% each in comparison to maize which grew by 27%. However, during this period Africa has seen a growth in the land to grow millet by 11%. Meanwhile, the biggest decline in land to grow millet was in Europe accounting for 59%. The global yield for millet has increased by 18% of which Africa contributed an increase of 10%. In the same period, maize production increased globally by 31% and sorghum by 4%. Africa has increased the yield of maize and sorghum by 34% and 22%, respectively. The production for millet globally and in Africa increased by 8% and 22%, respectively. Maize production has increased globally and in Africa by 66% and 77%, respectively. Meanwhile, sorghum production decreased globally by 5% while there was an increase in production in in Africa by 38%. Africa has decreased the area of harvest for production of underutilised millet and sorghum in comparison to maize which grew. However, the production and yield of millet and sorghum has increased. As stated by Masuda & Goldsmith (2009), an increase in the harvested area has historically been the way of boosting the crop output. In future the available area for harvesting cereal for production could be reduced as a result of a decrease in the area not yet used for farming mainly due to urbanisation and population

Table 2.1 World production of millet in comparison to maize and sorghum (FAOSTAT, 2015)

Continent	Cereal	1992 - 1997	1998 - 2003	2004- 2009	2010 - 2013	1992 - 1997	1998 - 2003	2004- 2009	2010 - 2013	1992 - 1997	1998 - 2003	2004- 2009	2010 - 2013
		Area harvested (x 10^4 Ha)				Yield (x 10^3 Hg/Ha)				Production (x 10^4 tonnes)			
World	Maize	1371.07	1388.13	1536.75	1747.58	3.97	4.42	4.97	5.19	5452.41	6135.05	7650.72	9071.63
Africa	Maize	256.47	252.67	288.00	339.35	1.51	1.71	1.81	2.02	388.74	430.94	523.48	686.20
Americas	Maize	572.32	565.63	608.94	664.70	5.16	5.91	6.79	6.86	2962.88	3342.59	4139.14	4561.02
Asia	Maize	408.88	433.66	495.82	572.95	3.55	3.79	4.31	4.88	1451.44	1644.70	2141.84	2798.62
Europe	Maize	132.67	135.27	143.09	169.66	4.85	5.28	5.86	6.03	644.88	711.36	840.40	1019.49
Oceania	Maize	0.73	0.90	0.90	0.91	6.07	6.09	6.51	6.91	4.48	5.46	5.86	6.30
World	Millet	366.57	359.10	352.33	335.77	0.75	0.80	0.88	0.89	276.63	286.19	311.04	299.69
Africa	Millet	185.91	198.42	206.05	206.25	0.63	0.70	0.80	0.70	117.68	138.39	165.66	143.78
Americas	Millet	1.71	2.08	2.01	1.65	1.55	1.37	1.59	1.46	2.66	2.95	3.13	2.52
Asia	Millet	165.49	147.87	136.85	122.08	0.87	0.90	0.97	1.20	144.60	134.18	133.24	145.91
Europe	Millet	13.14	10.37	7.08	5.43	0.87	0.99	1.22	1.26	11.35	10.21	8.65	7.07
Oceania	Millet	0.31	0.37	0.34	0.37	1.07	1.24	1.04	1.09	0.33	0.46	0.36	0.41
World	Sorghum	446.92	426.33	436.17	410.43	1.39	1.37	1.38	1.44	622.66	582.96	602.99	591.78
Africa	Sorghum	224.18	229.40	261.82	254.77	0.80	0.88	0.93	0.97	179.31	201.64	242.24	247.59
Americas	Sorghum	72.14	70.32	62.78	61.96	3.57	3.36	3.56	3.52	259.11	236.71	224.20	217.59
Asia	Sorghum	143.15	118.30	102.10	85.24	1.15	1.02	1.05	1.15	164.84	120.35	106.84	97.61
Europe	Sorghum	1.61	1.70	1.84	2.44	4.46	4.02	3.80	3.75	7.10	6.71	6.80	8.94
Oceania	Sorghum	5.83	6.62	7.64	6.02	2.09	2.66	2.93	3.33	12.30	17.55	22.91	20.05

increase. Worldwide, the population is increasing resulting in more land used for settlement rather than agriculture. In addition the weaker rights to properties in regions such as Africa have limited the use of modern agricultural methods. Therefore, investment into research and development to increase cereal yields to meet the growing demand and compensate the decline in available farming land is necessary.

2.4.1 Pearl millet production in Africa

Pearl millet is the most grown millet species accounting for almost half of the global production, cultivated over 60% in Africa, 35% in Asia, 4% in Europe and 1% in North America (Moreta *et al.,* 2013). Most millet (50% projected as pearl millet) in Africa is grown in Nigeria and Niger as shown in Figure 2.4. However, Nigeria and Uganda has seen a decline in millet production between the last decades (2005 – 2014) in comparison to 1995 – 2004 decade while the other countries had an increase in millet in the same period. The increase in competition from crops such as maize has resulted in low pearl

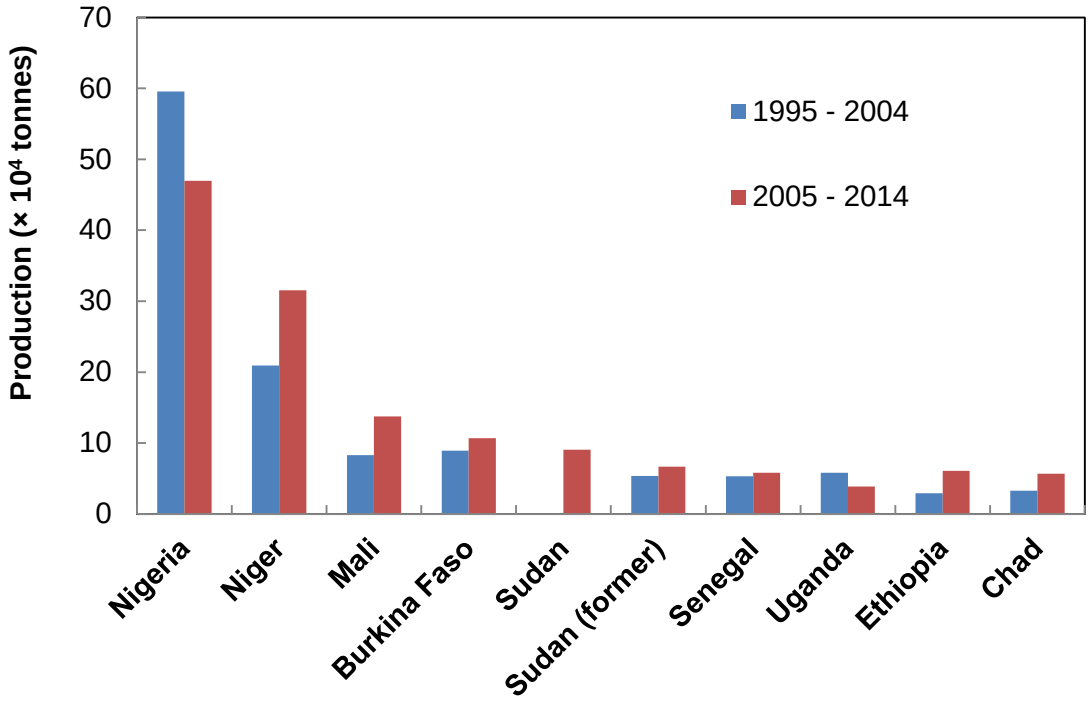

Figure 2.4 Production quantities of different millet types across African countries (50% projected as pearl millet) [Gramene, 2014; FAOSTAT, 2015]

millet production (CFC & ICRISAT, 2004). Figure 2.5 shows the average quantities of millet produced in Africa per region between 1992 and 2013 (FAOSTAT, 2015).

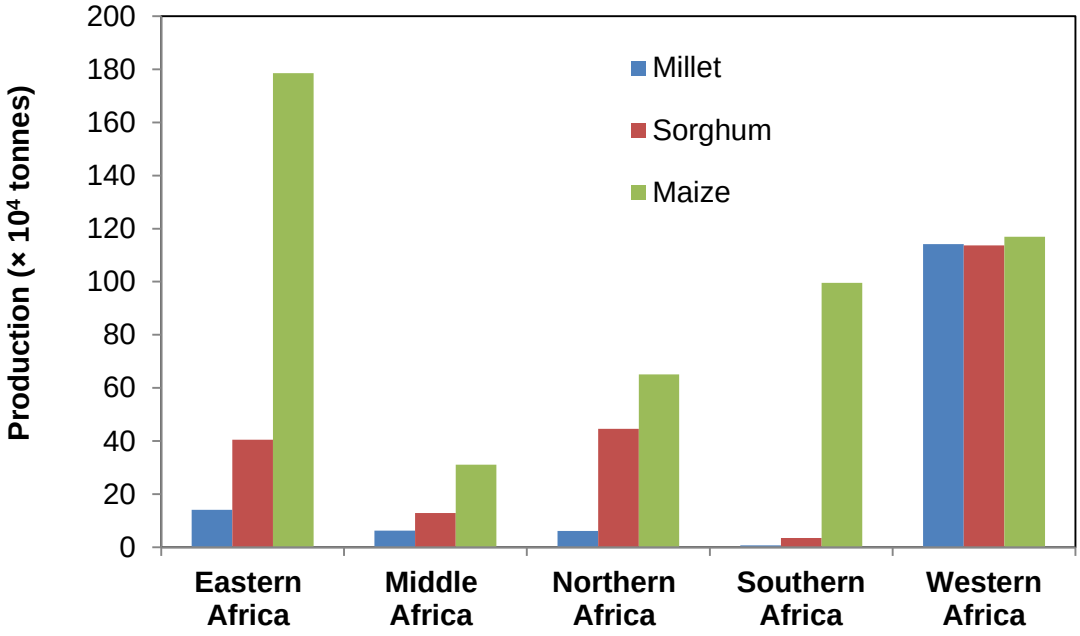

Figure 2.5 Average production of cereals in Africa divided by regions between 1992 - 2013 (FAOSTAT, 2015)

Eastern, Northern and Southern Africa produced 14 $\times 10^6$, 6 $\times$ 10^6 and 6 $\times$ 10^6 tonnes of millet, respectively. Pearl millet is a niche crop in Eastern and Southern Africa (ESA) planted in dry small areas. In contrast, in the Western and Central Africa (WCA) and South Asia the crop is the most important cereal in contiguous areas (Mitaru *et al.,* 2012). The largest area of cultivation in Eastern and Central Africa is part of Sahelian/Northern Sudanian, Kenya and Tanzania's ecosystems where it is planted on over 1.2 million ha in areas climatically similar to ESA (Mitaru *et al.,* 2012). The Southern African Development Community (SADC) region produces an estimate of 0.5 million tons of pearl millet with South Africa accounting for only 2.5% of the total production. Tanzania and Namibia combined produces nearly 27% of pearl millet in the SADC region (Rohrbach & Mutiro, 1998). Another 27% of the total pearl millet production is shared among the remaining 10 SADC countries. Namibia has increased the available land to plant this crop while in Tanzania the land remains unchanged over the past decade (Rohrbach & Mutiro, 1998).

2.4.2 Pearl millet in South Africa

Millets are extremely vital staple and ethno-botanical crops. South Africa has 0.21 million ha of land which is semi-arid and produces only 0.04 million tons of millet. There are nine species of millets grown worldwide of which four are relatively important across the African continent. In South Africa pearl millet is mainly grown in the northern and western regions. The crop was spread to northern and western South Africa over many years of cultivation, natural growth and farmer selection. However, the cultivation of this crop is limited to areas not known to be ideal for cereal production (Bello, 2013). An unknown variety of pearl millet is grown in Limpopo, KwaZulu-Natal and the Free State (DPP, 2013). The crop is planted by few farmers since it is not in demand compared to other cereals. Residue of the crop and green plants are used as building materials for fencing, thatching and making basket (DPP, 2013).

2.5 Nutritional content of pearl millet

Pearl millet is a principal source of energy, protein, vitamins and minerals. The crop is riche in calories than wheat due to its higher oil content of 5% of which 50% are polyunsaturated fatty acids (Khairwal *et al.,* 2007). Table 2.2 shows the nutritional composition of pearl millet. Pearl millet is nutritionally better than other cereals, with high levels of calcium, iron, zinc, lipids and high quality proteins (Lestienne *et al*., 2007). Essential amino acid profile revealed that pearl millet is 40% richer in lysine and methionine, and 30% richer in threonine than in the protein of corn (Osman, 2011). Pearl millet is rich in energy (361 kcal/100 g) than wheat (346 kcal/100 g), rice (345 kcal/100 g), maize (125 kcal/100 g) and sorghum (349 kcal/100 g). The protein content in pearl millet grains is 11.6 g per 100 g comparible to wheat at 11.8 g per 100 g, higher that 6.8 g for rice, 10.4 g for sorghum and 4.7 g for maize per 100 g (Nambiar *et al*., 2011).

2.6 Socioeconomic impact of millet

Millet is an underutilized cereal compared to other cereals such as maize and rice (Gelati *et al*. 2014). Given the changing global weather pattern and increasing temperatures and drought, this cereal can be planted in large quantities and address the social and economic challenges. Although millets are the future crops due to their adaptability to African land, to date they are still grown at subsistence level for food due to the lack of market for usage. The industry only processes less than 5% (millet and sorghum combined) of annual production quantities. This results in lower investment from farmers

Table 2.2 Nutritional composition of pearl millet grains

Constituent	Range (%)	Mean (%)
Protein	5.8 - 20.9	10.6
Starch	63.1 - 78.5	71.6
Crude fibre	1.1 - 1.8	1.3
Fat	4.1 - 6.4	5.1
Soluble sugar	1.4 - 2.6	2.1

Source: Khairwal *et al*. 2007

if no reliable markets are available (Stading *et al*., 2007). This can be addressed through development of new food products that will utilise this cereal. As reported by Jones (2015), there are several initiatives that could improve the agricultural produce in Africa. Investment into plant breeding research to meet the soil requirements is essential. It is estimated that every $1 invested in plant breeding it would yield $6. Irrigation of crops would yield 90% more than rain-fed farms. Currently, only 7% of arable land in Africa is irrigated of which 3.7% is in sub-Saharan Africa (FAO, 2002). The lower yield is also due to the lack of fertilisers to boost the soil fertility. Education on the use, selection, availability and negotiation of prices for fertilisers would increase the farmer's income by 61%. The agricultural input could increase by 5% if the rural infrastructure is improved to access market where this cereal can be processed further. Arable land in Africa which is not being utilized stretch for over 202 million ha but farms occupy only a small portion. The use of available land will open more job opportunities, provide income to farmers and have economic benefit. The availability of this cereal will assure uninterrupted supply to the agrifood processors and food producers who will produce new food products such as non-alcoholic cereal beverages for commercialization.

2.7 Changes that occur during fermentation of food

Food fermentation is typically the conversion of sugars to alcohol and carbon dioxide or organic acids. These changes in food quality are related to chemical, biochemical, and physical changes such as lipid oxidation, enzymatic and non-enzymatic browning, and moisture loss, respectively (Kong & Singh, 2011). Some of the old techniques of food preservation include smoking, drying, salting/lye, freezing, fermentation, canning, spray drying etc. (Marshall & Mejia, 2011; Olurankinse, 2014). Fermentation is a Latin verb '*fervere*' meaning to boil (Bassey, 2013). This describes the warty appearance caused by

the action of yeasts on extracts of fruit or malted grain during anaerobic catabolism (Colombie *et al*., 2007; Bassey, 2013). The fermentation process is carried out by microorganisms which break down organic matter to obtain the energy required to remain viable and make organic compounds such as alcohol and organic acid (Mkondweni, 2002; Scott & Sullivan, 2008), as well as inorganic compounds such as CO_2 and H_2 (Weir & McSpadden, 2005; IP, 2007). The difference between fermentation and decomposition lies in the nature of the end product. The process is termed decomposition if substances such as H_2S and NH_3, that are harmful to humans are formed (IP, 2007; WSU, 2014) and is termed fermentation if beneficial substances are formed (IP, 2007). If a large amount of putrefactive bacteria are present, the process shifts to decomposition, and if there are many fermentative bacteria, it shifts to fermentation (GRNBA, 2012).

During storage, grains are metabolically inactive and have low water activity of less than 0.6 and moisture content of 9-12%. Microorganisms and enzymes are not active during storage due to this low water content (Achi & Ukwuru, 2015). If the grains are hydrated, the enzymes become active and microorganisms start to grow and multiply. The hydration process stimulates cereal fermentation to start and the activity of hydrolytic enzymes such as amylosis, lipolysis, proteolysis and physiological activities of microorganism carry out the fermentation (Achi & Ukwuru, 2015). During this phase organic acid is produced and lowers the pH of the medium. Different metabolites are produced which are beneficial to the consumers by improving the palatability and acceptability of the beverage by improving flavours and texture; and preservative by means of acidulants, alcohol and antimicrobial compounds. Cereal fermentation is influenced by different factors such as duration of fermentation, temperature and pH which are difficult to control in rural household families and require innovative technological methods. In addition, factors that influence fermentation include (i) amount of moisture content in the grain, (ii) extent of grain size reduction, (iii) type of cereal, (iv) growth requirements for microorganisms, (v) enzyme sources, (vi) materials added to fermenting substrate, (vii) pH, (viii) level of hygiene and sanitation and (ix) quality of starter culture (Achi & Ukwuru, 2015).

Fermentation can be divided into two, namely, aerobic and anaerobic. Anaerobic process takes place in the absence of oxygen which results in reduced pyridine nucleotides which needs to be re-oxidised. During this process the reduced pyridine nucleotide oxidation is followed by the reduction of organic compounds (Standburry *et al.,* 1995). Under aerobic process there is a supply of oxygen and re-oxidation of reduced pyridine nucleotides occurs by electron transfer through the cytochrome system with

oxygen acting as electron acceptor (Standburry *et al.,* 1995). The different types of fermentation named based on the end products are: (i) ethanol, (ii) propionate, (iii) mixed acid and butanediol, (iv) butyrate and acetone-butanol, (v) homoacetate and (vi) lactic acid fermentation.

2.8 Biochemical changes during fermentation

Cereals are grown on over 103 million ha with production quantities of 169 million tons in Africa as a source of dietary protein, carbohydrates, vitamins, minerals and fibre. However, these nutrients are sometimes lower in comparison to dairy products (Kohajdova & Karovicova, 2007; Katangole, 2008). Therefore, fermentation of cereal is used to: (i) enrich the diet through creation of flavours, aromas and texture modification, (ii) preserve food through the creation of organic acids (lactic acid), (iii) enrich the beverages with proteins, essential amino acids, essential fatty acids, and vitamins, (iv) detoxification during fermentation and (v) reduce the cooking times and energy usage (Steinkraus, 1996). The process of fermentation is carried out by microorganisms and their enzymes to achieve the desirable modification of cereals (Apena *et al*., 2015). Microorganisms which carry fermentation derive their food from their immediate environment (cereal). Water is not a nutrient but a basic need for biochemical reactions for synthesis of cell mass and energy. During cereal fermentation carbohydrates are broken down into monosaccharides by lactic acid bacteria (aerobic, anaerobic and facultative). These nutrient molecules are transported into the cell through the cell wall and cell membrane (Ray, 2004). In Gram positive lactic acid bacteria (LAB) the cytoplasmic membrane made up of two layers of lipids is a barrier to nutrient transport. Small molecules such as amino acids, small peptides, monosaccharides and disaccharides are easily transported into the cell. However, large carbohydrates such as starch need to be broken down using hydrolytic enzymes into small molecules before transportation into the cell. LAB breakdown the glucose through the Embden-Meyerhof-Parnas (EMP) and hexose monophosphate shunt (HMP) pathways to supply energy for survival. The EMP and HMP are carried out by homofermentative and heterofermentative lactic acid bacteria (Ray, 2004). All microorganisms are capable of utilizing carbohydrates but their ability to utilize it differs greatly among different microorganisms. The end-products (metabolic products) of fermentation are used to synthesize cellular components of microorganisms. Other end products such as organic acids are used to support the growth of other bacteria that tolerate acidic environment. The cereal grains have low buffering capacity and the pH therefore decreases quickly as acid is produced during

fermentation by LAB. As acid is produced pathogens are then inhibited (Simpson, 2012). In general, fermentation of cereals results in the reduction of carbohydrates and non-digestible polysaccharides and oligosaccharides (Katangole, 2008).

Proteins, conjugate proteins, peptides, non-protein nitrogenous compounds (amino acids, urea, ammonia, creatinine, trimethylalmine) are all major proteinaceous components utilized by microorganisms. The proteins which are amino acids polymers have different solubility which is the base to determine which microorganisms are capable of utilizing the particular protein (Ray, 2004). Most microorganisms are capable of using proteins which are soluble in water than the insoluble ones. Microorganisms such as *Lactococcus* transport amino acids and peptides into the cell and thereafter the peptides are hydrolyzed into amino acids within the cell (Ray, 2004). Sometimes proteinases and peptidase are produced by microorganisms to breakdown large proteins and peptides into small peptides and amino acids before transportation into the cell. Certain amino acids are synthesized and may improve the availability of B group vitamins (Katangole, 2008).

Lipids are less preferred by microorganisms for microbial survival. They are mostly found in food of animal origin rather than plant such as cereals. Fatty acids diffuse easily through the lipid bilayers in the cytoplasm (Ray, 2004). Their utilization in food by microorganisms is associated with spoilage of food. Microorganisms such as *Lactobacillus acidophilus* can breakdown cholesterol in the intestine and reduce serum cholesterol in humans (Ray, 2004).

Lactic acid bacteria like many microorganisms require small amount of minerals such as phosphorus, calcium, magnesium, iron, sulphur, manganese and potassium for survival. Organic acids produced during fermentation create an optimum pH environment for enzymatic degradation of phytate available in cereal grains in the form of polyvalent cations such as calcium, zinc, magnesium and proteins (Kohajdova, & Karovicova, 2007; Katangole, 2008). Consequently, antinutrients such as phytate and polyphenols are reduced during fermentation (Kohajdova, & Karovicova, 2007).

2.8.1 Growth of lactic acid bacteria during fermentation

Microorganisms in naturally fermented beverages are from internal and external sources such as water, air, raw materials (cereal grains, spices, malts etc.), equipment, and other sources. Proper sanitation during production is ideal to reduce the number of bacteria especially pathogenic organisms. Natural fermentation of traditional beverages involves mixed bacteria which can grow in mixed population, sequence, succession (diauxic), symbiotic, synergistic and/or antagonistically (Ray, 2004). During mixed population

growth, different species of bacteria, yeast, and/or molds are involved. Some species may be in large number and depending on the growth conditions others grow rapidly and overcome the others. In sequence growth different species grows predominant in sequence during storage. One or few species grow initially and create the environment which favours subsequent species. Succession microorganisms are separated by a short lag phase. Some bacteria utilizes one nutrient they prefer and once depleted uses the other nutrient for growth. The growth curve has a repetition of exponential and stationary phases. Synergistic growth refers to the symbiotic growing of bacteria independently producing metabolites at lower rates. When symbiotic bacteria are in a mixed culture they both produce high level of the end-product. Antagonistic growth happens when two or more microorganisms in the beverage affect the growth of each other, sometimes leading to the death of one bacteria. This can happen between bacteria and mould, mould and yeasts, bacteria and yeast. Typical example is the growth of Gram positive lactic acid bacteria which produces bacteriocins or proteins that kills other Gram positive bacteria (Ray, 2004).

2.9 Types of fermentation models used in the industrial production of fermented beverages

There are three main models of fermentation process in the industry namely, batch, continuous and fed-batch, depending on the feeding strategy of culture and the medium in the fermenter (Chisti, 1999).

2.9.1 Batch fermentation

Batch fermentation normally takes place in a closed system (Scott, 2004; Kumar, 2012). At the beginning, microorganisms are added to the sterile medium fermentor and incubated (Scott, 2004). During the course of fermentation only oxygen (in the case of aerobic microorganism), antifoaming agent and acid or base are added to the medium to control the pH (Chisti, 1999; Scott, 2004; Kumar, 2012; Diaz-Montano, 2014). The composition of the culture medium, the biomass concentration, and metabolite concentration generally change constantly as a result of the metabolism in the cells. After the inoculation of microorganisms and cultivation of sterile nutrient under physiological condition, four typical physiological phases of growth are observed, namely, lag, logarithmic/exponential, stationery and death phase (Kang, 2000; Diaz-Montano, 2014).

During the lag phase, microorganisms are introduced into the fresh culture medium; usually no immediate increase in cell number occurs (USDA, 2012). The

microorganisms have been injured and require time to recover. This is followed by exponential or logarithmic phase where microorganisms are rapidly growing and dividing at the maximal possible rate given their genetic potential, nature of the medium and environmental conditions (Todar, 2012; MHRD, 2014). In a closed system such as batch culture, population growth eventually ceases and the growth becomes horizontal. The stationary phase usually is attained when the bacterial population level is around 10^9 cells per ml but certain bacteria are unable to reach this level. Population growth is limited by three factors: (i) exhaustion of available nutrients; (ii) accumulation of inhibitory metabolites or end products; and (iii) lack of biological space (Todar, 2012). In the death phase the energy reserves of the cells are exhausted. It is assumed that detrimental changes in their environment such as nutrient deprivation and the build-up of toxic wastes causes irreparable harm and damage leading to loss of viability (Al-Qadiri *et al.*, 2008; MHRD, 2014). When bacteria are transferred to a fresh medium, no cellular growth is observed (Thiel, 1999). Due to loss of viability often accompanied by loss of total cell number; it is assumed that viable cells were dead but did not lyse. At the end of fermentation, contents are emptied preparing for next batch (Renge *et al*., 2012).

2.9.2 Continuous fermentation

Continuous fermentation takes place in an open system (Kumar, 2012). During fermentation substrates are continuously added to a bioreactor. At the same time, equal amounts of converted nutrient solution with microorganisms are removed from the system (Chisti, 1999; Renge *et al*., 2012; Diaz-Montano, 2014). Homogeneously mixed bioreactor is divided into two, chemostat or turbistat. In chemostat the growth of cells is controlled by adjustment of the substrate concentration (Scott, 2004). Turbistat is kept constant by using turbidity to monitor the biomass concentration. The rate of feed of nutrient solution is also adjusted.

2.9.3 Fed-Batch fermentation

Fed-batch fermentation is the type of system where nutrients are added only when their concentration falls (Chisti, 1999; Scott, 2004; Diaz-Montano, 2014). This mode of fermentation can be regarded as a combination of the batch and continuous operation (Caylak & Sukan, 1998; Standbury, 2006). The nutrients are added in several doses to ensure that there are no surplus nutrients in the fermenter at any time (Diaz-Montano, 2014).

2.10 Factors influencing fermentation process

There are numerous intrinsic and extrinsic factors which can influence the fermentation process, namely: nutrient availability, temperature, pH, heat and oxygen. Their variation may affect the rate of fermentation, product spectrum and yield, and organoleptic properties of the product (Chisti, 1999; Maukonen *et al*., 2003; Najafpour, 2006; Renge *et al*., 2012).

2.10.1 Nutrient availability

Microorganisms require nutrients (amount and type depend on the range of microorganisms) for growth and maintenance of metabolic functions (FDA, 2013). Microorganisms require a source of carbon, nitrogen and phosphorus; a respiratory substrate which is usually glucose; vitamins and minerals to act as co-enzymes and water for all metabolic reactions to occur (FDA, 2013). If a nutrient is depleted it will become a limiting factor and reduce the growth rate.

2.10.2 Temperature

Temperature influences the growth rate co-ordinated by enzymes (Enfors, 2008). Enzymes work most efficiently over a narrow range of temperatures (around optimum). If the temperature falls too low, the rate of enzymes-catalysed reactions becomes too low to sustain life of microorganisms (Thiel, 1999). If the temperature is high, the denaturation of enzymes causes cell death (Thiel, 1999). The optimum range of temperature for most microorganisms is between 25^0C and 45^0C (Thiel, 1999).

2.10.3 pH

Bacterial work efficiently within a narrow pH range between pH 6 and 7 (Lambert, 2011). However, microorganisms in general can tolerate a wider pH range. Some species grow in acidic and others in alkaline conditions (da Vinci, 2009).

2.10.4 Oxygen

Microorganisms have different oxygen requirements. Obligate aerobes are microorganisms that require oxygen all the times, without it they are unable to survive in the fermentation process (Fox, 2010). Obligate anaerobic are microorganisms that find oxygen toxic as it inhibits respiration and they cannot grow in its presence (Fox, 2010). Facultative anaerobic refers to populations of bacteria that grow in the presence of oxygen, but can survive without it, although their growth rate is slow (Clarke, 2013).

2.10.5 Heat production

Heat is produced during biological activity, thus the fermentation cycle should be carried out in a controlled temperature environment to obtain optimal yields (van Leeff *et al.,* 1993; Colombie *et al*., 2007). Energy is also created during stirring and aeration/gassing of the medium being fermented.

2.10.6 Gas exchange

The successful operation of aerobic fermentation requires adequate gas supply (Lee, 2001) in which oxygen is the most important gaseous substrate for microbial metabolism, and carbon dioxide is the most vital metabolic product (FDA, 2013). Limitation in oxygen supply may lead to an undesirable change in enzymatic make-up or death of the organisms which could lead to lower yield of desired end product (Lee, 2001). During fermentation by unicellular microorganisms, the rate of transfer is controlled by the resistance in the phase boundary between the gas bubble and the liquid phase (Colombie *et al*., 2007; BM, 2012; Rodriguez-Fernandez *et al*., 2012). Organisms near gas bubbles may absorb oxygen directly and the gas tranfer rate may be increased (BM, 2012).

2.11 Fermentation of non-alcoholic cereal beverages

Traditional beverages can be fermented in three ways depending on the source of desired microorganisms. The beverages can be fermented by either natural, back-slopping or controlled fermentation (Ray, 2004).

2.11.1. Natural fermentation

Many indigenous cereal beverages are fermented through natural fermentation. The raw materials used are not heat treated and contain a mixture of desired and unwanted microorganisms. The production process and incubation conditions favour the growth of desired microorganisms while slowing the undesired bacteria. These beverages have desirable aroma caused by the lactic acid bacteria metabolizing the available nutrients usually carbohydrates. The microflora in natural fermented beverages differs from batch to batch resulting in inconsistent characteristics of the final beverage. This type of fermentation is also risky since pathogens could be present in the final product.

2.11.2. Back slopping

Some cereal beverages are produced traditionally using clay pots in Africa. At the end of a successful fermentation the pots are not washed and a new batch is mixed inside the

pot. The remaining microorganisms on the surface of the pot are used as starter culture for the new batch of beverage. However, the quality of the beverages may differ over a long period because of the change in microbial types. Pathogens could also be found in the beverage at high level.

2.11.3 Controlled fermentation

During this type of fermentation, a starter culture is added to the beverage which had been heat treated. The starter culture used is a single or mixed strain of microorganism. The beverages are incubated at defined optimum temperature for starter cultures used. A consistent beverage with predictable qualities can be produced and the chances of failure are very low. However, the beverage may have different flavours in comparison to natural and back slope fermented beverages due to the use of selected bacteria.

2.12 Non-alcoholic cereal beverages in Africa

Recently health conscious consumers are looking for natural foods without chemical preservatives that will fit their healthy lifestyles (Judeikiene *et al*., 2012). The increasing consumption of pre-cooked and the import of raw foods from developing countries are among the main causes of people opting for healthy foods. Currently, fermented foods are increasing in popularity due to their nutritious properties. Fermented products are divided into porridges, beverages (alcoholic and non-alcoholic), breads and pancakes, fermented meat, fish, vegetables, dairy products and condiments that are produced from both edible and inedible raw materials in many countries (Marshall & Mejia, 2011). Fermented cereal beverages from Africa are of interest and are shown in Table 2.3. The classification is based on the raw material and beverage name, country of popularity and the microorganism that ferment the beverage (Solange *et al*., 2014). Non-alcoholic cereal beverages are popular in African countries (Terna & Ayo 2002). The cereals mainly used in their production are millets, sorghum and maize although composite cereal are sometimes used (Terna & Ayo 2002). The production of these African cereal beverages are still limited to household level where they are carried out by mixed microorganisms in succession. The end-product differs from each family in terms of quality, yield and safety and hinders commercialization of the beverages. To assure the homogeneity and large scale production, different processing and innovative approach needs to be applied to improve these non-alcoholic beverages (Judeikiene et al., 2012).

Table 2.3 Types of fermented non-alcoholic beverages from different raw materials and their names in selected African countries

Raw materials and beverage name	Country	Microorganisms	References
Sorghum			
Ogi	Nigeria	LAB, yeast, moulds	Chelule *et al.,* 2010; Mwale, 2014
Kunun-zaki	Nigeria	LAB, yeasts	Akoma *et al.,* 2014; Oranusia et al., 2003
Pito	Ghana, Nigeria	*Lactobacilli*,*Pediococcus*, yeasts	Kolawole & Kayode, 2013; Orji *et al*., 2003
Bushera	Uganda	LAB	Solane *et al*., 2014; Marsh *et al*., 2014; Mwale, 2014
Gowe	Benin	*Lactobacillus* species	Solane *et al.,* 2014; Adinsi, 2014
Motepa	South Africa	Unknown	
Motoho	Lesotho/South Africa	Unknown	Gadaga et al., 2013
Togwa	Tanzania	LAB	Chelule *et al.,* 2010
Kisra	Sudan	LAB	Chelule *et al.,* 2010
Munkoyo	Zambia	LAB	Chelule *et al.,* 2010
Uji	Kenya, Uganda, Tanzania	*Lactobacillus species, Pediococcus*	Mwale, 2014
Maize			
Ogi	Nigeria	Bacteria, yeasts, moulds	Evans *et al*., 2013; Chelule *et al.,* 2010
Kunun-zaki	Nigeria	LAB, yeasts	Akoma *et al*., 2014
Gowe	Benin	*Lactobacillus* species	Solange *et al.,* 2014
Kunun-zaki	Nigeria	LAB, yeasts	Solange *et al*., 2014
Mahewu	South Africa, Zimbabwe	*Streptococcus lactis, Lactococcus lactis* subspp *lactis*	Gadaga *et al*., 1999; Awobusuyi, 2015; Chelule *et al*., 2010; Mwale, 2014
Incwancwa	South Africa	LAB	Chelule *et al*., 2010
Togwa	Tanzania	*Lactobacillus* species, *Issatchenkia orientalis*	Mwale, 2014
Kwete	Uganda	LAB, yeasts, coliforms	Namagumya. & Muyanja, 2009
Uji	Kenya, Uganda, Tanzania	*Lactobacillus species, Pediococcus*	Mwale, 2014
Millet			
Ogi	Nigeria, West Africa	Bacteria, yeasts, moulds	Evans *et al*., 2013; Chelule *et al*., 2010

Table 2.3 Types of fermented non-alcoholic beverages from different raw materials and their names in selected African countries (continued)

Raw materials and beverage name	Country	Microorganisms	References
Millet			
Kunun-zaki	Nigeria	LAB, yeasts	Akoma *et al*., 2014; da Vinci, 2009
Bushera	Uganda	Unknown	Solange *et al*., 2014; Marsh *et al*., 2014
Gowe	Benin	*Lactobacillus* species	Solange *et al*., 2014
Mangisi	Zimbabwe	Unknown	Solange *et al*., 2014
Masvusvu	Zimbabwe	Bacteria, LAB, yeasts, moulds	Solange *et al*., 2014; Zvauya *et al*., 1997
Togwa	Tanzania	LAB	Gadaga *et al*., 2013
Uji	Kenya, Uganda, Tanzania	*Lactobacillus species, Pediococcus*	Mwale, 2014
Maize and Millet			
Kwete	Uganda	LAB, yeasts, coliforms	Namagumya & Muyanja, 2009
Maize, sorghum, wheat, millet, tef, barley			
Borde	*Ethiopia*		Solange *et al*., 2014

2.12.1 Fermenting organisms in non-alcoholic beverages

Many indigenous non-alcoholic cereal beverages (NACB) are carried out by natural fermentation involving mixed cultures of bacteria (mainly lactic acid bacteria) and yeast (Table 2.4) [Franz *et al*.,2014]. Lactic acid bacteria (LAB) are a large group of beneficial microorganisms that usually carry out fermentation of traditional cereal beverages. There are numerous LAB genera found within the phylum Firmicutes. The genera of LAB are: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococccus* and *Weissella* (Beasly, 2004; Rattanachaikunsopon & Phumkhachorn, 2010; Halasz, 2011; Ongol, 2012). The organisms are Gram-positive, anaerobic microorganisms with the ability to grow in the presence of oxygen and produce lactic acid from the breakdown of carbohydrate (Beasly, 2004; Campos *et al*., 2009; Rattanachaikunsopon & Phumkhachorn, 2010; Halasz, 2011). They do not form spores, are cocci, coccobacilli or rods in shape with less than 53 mol% guanine-cytosine content of the DNA base composition. They are usually non-respiratory and catalase negative (Gaffa *et al.,* 2002). The organisms require a complex nutrition for growth made up of

Table 2.4 Lactic acid bacteria associated with the fermentation of non-alcoholic cereal beverages

Genus	Morphology	Homo-fermenter	Hetero-fermenter	References
Lactobacillus	Rods-single or chains	+	+	Guizani & Mothershaw, 2005; Konig & Frohlich, 2009, Wassie & Wissie, 2016;
Lactococcus	Oval cocci- pairs or chains	+	+	Guizani & Mothershaw, 2005; Wassie & Wissie, 2016
Leuconostoc	Oval- pairs or chains	-	+	Guizani & Mothershaw, 2005; Konig & Frohlich, 2009, Wassie & Wissie, 2016
Pediococcus	Cocci- pairs and tetrads	+		Guizani & Mothershaw, 2005, Konig & Frohlich, 2009
Streptococcus	Cocci- pairs and chains	-	-	Guizani & Mothershaw, 2005; Wassie & Wissie, 2016
Wissella	Coccoid/short rods- singlfe, pairs or short chains	-	+	Guizani & Mothershaw, 2005, Konig & Frohlich, 2009
Enterococcus	Cocci- single, pairs or short chains	+	-	Guizani & Mothershaw, 2005; Wassie & Wissie, 2016

carbohydrates, amino acids, peptides, nucleic acids and vitamins (Rattanachaikunsopon & Phumkhachorn, 2010; Ongol, 2012).

A large number of different bacteriocins are produced by LAB with antimicrobial activities such as acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins that prolong the shelf life and safety of beverages by preventing the growth of pathogens and spoilage microorganisms (Silva *et al*., 2002). Bacteriocins are antimicrobial peptides produced by bacteria (Grazina *et al.,* 2012), including some of the LAB. Bacteriocins produced by LAB are used as natural bio-preservatives since they are degraded by proteases of gastrointestinal (GI) tract and are generally recognized as safe microorganisms (GRAS) [van Geel-Schutten *et al*., 1998; Morelli, 2001; Chauhan, 2012; Ongol, 2012]. The peptides are able to prolong the shelf life of many foods due to their bio-preservative properties (Morelli, 2002).

2.12.2 Technologies to improve the quality, shelf life and acceptability of cereal beverages

Climate change which is linked to the rise in temperatures has threatened food security but its impact can still be minimized. This increase in temperatures will have severe implications on the physical and biological systems and human livelihoods. Africa is among the most vulnerable continent to the negative effects of climate change due to its limited financial resources, skills, technology and dependence on climate-sensitive primary sector. Pearl millet is a better suitable climate change compliant crop to use than other cereals since it can survive adverse environment (low rainfall and high temperatures) [Andreas, 2013].

Traditionally prepared non-alcoholic beverages (NAB) last for one to few days (Oranusia, *et al*., 2003; Amusa, & Odunbaku, 2009; Ratau, 2011) due to the unhygienic environment where the beverages are produced, pitching of different microorganisms from fresh milled mixture; differences in the production process from area to area, tap water used to increase volume and profit margin not portable etc. These factors also result in variations in taste and flavour from different producers and are a limitation to large scale production (Oranusia, *et al*., 2003). Various researchers have reported on technological approaches to improving fermented beverages. These processing methods include: (i) heat treatment, (ii) freeze-drying, (iii) steeping of grains in sodium metabisulphite, (iv) hurdle effect of sodium metabisulphite and refrigeration, (v) enrichment of the beverage with soy milk, (vi) effect of preservation, (vii) enrichment with ProVitamin A, (viii) enrichment with Moringa flour, and (vx) production of quality cereal malt. Most of these techniques are practised in the food industry and could be used to commercialize traditional cereal beverages. In order to standardize the beverages in terms of quality and safety for commercialization these different technologies need to be improved and/or implemented.

2.12.2.1 Thermal processing (pasteurization) of the beverage

Thermal processing has been used in modern day to enhance the shelf-life of food without altering with their organoleptic qualities. Studies on thermal processing as undertaken by Oranusia *et al*. (2003) showed that heat reduces the number of bacteria, yeasts and molds that causes food spoilage and enhance the taste, smell, appearance and digestibility of foods. *Sekete*, a fermented maize beverage, was pasteurised at three temperatures (65, 70 and 75°C) for 30 min and the microbial load and sensory properties of the beverage evaluated (Onaolapo & Busari, 2014). Pasteurization at 75°C for 30 min

eliminated all bacteria and increased the shelf-life of *Sekete* by four weeks in comparison to 65°C and 70°C for 30 min (Onaolapo & Busari, 2014). The sensory qualities between the beverages did not differ significantly but the taste of the sample pasteurised at 65°C for 30 min was preferred (Onaolapo & Busari, 2014). These results were in agreement with Egbere, (2009) during the pasteurization of sorghum cereal beverage at 70°C for 30 min (Onaolapo & Busari, 2014). The results showed that after 20 min 80% of the microbial load was destroyed except *Bacillus subtilis* (D_{value} = 6.5 min) and *Saccharomyces cerevisiae.* This indicated that ultra-high temperature or combinational of pasteurization and chemical preservatives could be used to eliminate *B. subtilis*. However, Maji *et al*. (2011) reported chemical deterioration of cereal beverage in one week when pasteurised at 60°C for 1 h in comparison to samples treated with 0.1% sodium benzoate or sodium metabisulphite which deteriorated in 2 weeks. Although the beverage shelf life can be extended through pasteurization, the heat destroys labile probiotics (Amusa & Odunbaku, 2009) and could results in the reduction of desired chemical properties which may lead to reduced acceptability of the beverage by potential consumers. Nutritious foods such as non-alcoholic cereal beverages with long shelf life are in demands from consumers. Heat treatment extends the shelf life but may have detrimental effects on the nutrition and fresh like flavors of beverages (Aneja *et al*., 2014). New technologies such as high hydrostatic pressure, high pressure processing, pulsed electric field, ultrasound, irradiations etc. developed in the food industry needs to be developed for cereal beverages (Aneja *et al*., 2014).

2.12.2.2 Freeze-dried cereal beverage

Freeze drying (lypholisation) of fermented *Kunun-zaki* prepared using starter culture was investigated (Nkama *et al*., 2010). The difference in nutritional and sensory quality attributes between the reconstituted dried beverage and freshly prepared beverage were investigated. There were no significant differences in overall acceptability and mineral contents of the beverages. However, there was a marginal difference in titratable acidity (as % lactic acid), pH, proximate and amino acids content between freeze-dried and freshly prepared beverage. Freeze drying techniques however, prolonged the shelf life of the beverage by 6 months and offered consumers the option to decide when to consume the beverage (Nkama *et al*., 2010). However, lyophilisation technology has disadvantages such as high cost, increased handling and processing time. Other factors to contribute the cost could be cryogenic protector used during freeze drying. Chen *et al.* (2006) reported loss of microbial viability after freeze drying without lyoprotectants but

there was a significant growth of surviving microorganism when 10% galactose or sucrose was used as lyoprotectants. An instant *kunun-zaki* was also produced by Uvere and Amazikwu (2011) with an addition of cowpea and soybean. The instant beverage had high crude protein and sugar content compared to traditional beverage. However, the moisture content and viscosity of the beverage was reduced when compared to freshly prepared beverage. A proper package such as vacuum packaging should also be used to avoid the loss of volatile compounds, control the growth of mold; and sterile diluents needs to be used when reconstituting (Uvere and Amazikwu, 2011; Nireesha *et al*., 2013). Ndulaka *et al.* (2014) also reported no-significant differences in the proximate composition between (protein, ash, fibre and carbohydrates) reconstituted *kunun-zaki* and freshly prepared beverage. However, there was a significant difference in terms of the taste and pH of the beverages. The pH of the reconstituted beverage was 3.61 while the freshly prepared sample had a pH of 2.03 which is in agreement with Nkama *et al*. (2010). The freshly prepared beverage was scored high in terms of taste compared to reconstituted beverage. The unacceptable taste could be due to the drying method (oven).

2.12.2.3 Steeping of grains using sodium metabisulphite

Terna & Ayo (2002) investigated the use of sodium metabisulphite at 5% in warm steeping water (60 - 70ºC). This helped soften the grains during the steeping process. The main aim was to improve the traditional production process while maintaining nutrient and improving microbiological quality. The usual production process was shortened to 12 h instead of the traditional 24 h. The shorter liquefication time resulted in less chances of contamination and food borne infections. As a result of shortened saccharification process, the protein content of the beverage with shortened steeping process increased from 4.1 to 5.4%. Yang and Seib (1996) reported an optimum recovery (51%) of starch in sorghum grains steeped for 4 h at 58°C with initial SO_2 concentration at 0.3%. However, the use of sodium metabisulphite may cause allerrgic reaction to people sensitive to sulfite. Sulfite is known to results in asthma, rhinoconjunctivitis, urticaria and anaphylactic shock (Oliphant, 2012). The use of NaOH as a steeping agent also resulted in high starch purity (Nyakabau *et al.,* 2013). Deepe and Vilayakuwa (2013) reported a reduction in steeping time to 10 h using 0.03N NaOH resulting in optimum isolation, yield, hydration index, swelling index and micrometric properties of starch.

2.12.2.4 Hurdle effect of sodium metabisulphite and refrigeration or pasteurisation on the beverage

Microbiological, physicochemical and sensory qualities of cereal beverage made with millet and stored at refrigerated condition were investigated by Abimbola *et al.* (2013). The study was aimed at producing the beverage under hygienic conditions, improving the traditional production process thus reducing microbial count and improving storage stability. The improved method included the use of 0.5% sodium metabisulphite during the 3 h steeping process, addition of 0.1% sodium metabisuphite after saccharisation at 60 - 70ºC, filtration step and lastly storing the beverage at refrigerated conditions (4 - 8ºC). The new improved method in comparison to traditional method showed that the old method affected the quality and acceptability. There was a significant difference in taste, consistency and colour but no significant difference in overall acceptability. However, to maintain the colour, odour, taste and consistency using the improved method there should be a constant supply of electricity. There was no growth of pathogens in refrigerated samples as compared to ambient stored samples. Maji *et al*. (2011) also investigated the hurdle effect of 0.1% sodium benzoate and sodium metabisulphite on the shelf life of cereal beverage. The shelf-life was stable for 3 weeks and the beverage was accepted by potential consumers. This was also supported by Ojimelukwe *et al*. (2013) who reported an increase in shelf life by five days and acceptability of non-alcoholic cereal beverage from sorghum treated with 0.2 g/L sodium metabisulphite followed by pasteurisation at 65°C for 30 min.. The use of sodium metabisulphite may cause allerrgic reactions to people sensitive to sulfite. Sulfite is know to results in asthma, rhinoconjunctivitis, urticaria and anaphylactic shock (Oliphant, 2012). Refrigeration is cost intensive and some villages do not have electricity.

2.12.2.5 Enrichment (fortification) and supplementation of the cereal beverage

The nutrition and safety of improved non-alcoholic beverage produced from malted cereal enriched with malted soymilk at different substitution levels (0 - 30%) was investigated by Adelakan *et al*. (2013). The aim was to improve the nutritional and acceptability of the beverage to suite the daily dietary requirements of the consumers. The use of soymilk and malting increased the protein, amino acid, ash and moisture content of the beverage. Malting resulted in the reduction of carbohydrates and fat content. The protein content in malted samples ranged from 2.79 - 3.82% while proteins in un-malted samples was 2.36%. The concentration of phytic acid and trypsin inhibitors decreased after malting but the concentration increased when more soymilk was added. In addition the amino acid

content increased when more soymilk was added. Therefore, the beverage with soymilk was more nutritious. Haard *et al*. (1999) also reported that the use of 10% soya flour in *ogi* enhanced the nutritive value, shelf life and theurapeutic properties of the beverage. Meanwhile, Oluwole *et al*. (2012) reported an increase in acceptability of beverage made from 72 h maize malt and soymilk. The beverage was nutritious with high levels of energy and acceptable fat, proteins and crude fibre. However, the nutritious soy milk enriched beverage was not accepted as the soy milk content increased. This is due to the beany taste caused by soy milk. It is advisable to use flavours to reduce the beany taste and preservatives used to improve the shelf-life and the beverage stored at refrigerated conditions (Adelakan *et al*.,2013). However, consumers need less additives in food and refrigeration is not ideal for rural villages with no electricity. The fortification may produce nutritious beverage but may have a low acceptability by beverage consumers (Sowonola *et al*., 2005). Soy contains phytoestrogens which can have negative health effects and needs to be regulated. Some of the soy is produced using genetic modified organisms. Individuals allergic to soy will not be able to consume the beverage. Beside using malt, Bede *et al*. (2015) used date fruit which is knowwn to be nutrtious containing high carbohydrates, sugars (44 -88%), 1 mg salt bettwer than 55 mg in sweet potatoes and essential vitamins and minerals (such as potassium – 696 mg/100 mg) to boost the nutrtive and sweetness on non-alcoholic cereal beverage. The beverage made with millet and date fruit was preferred than the sample made with millet and sweet potatoes.

Awobusuyi (2015), investigated the optimal processing parameters of M*ahewu* produced using Pro-Vitamin A-biofortified maize. *Mahewu* was prepared traditionally with slight modification. Maize meal was mixed with water (1:7 w/v) at 90ºC while stirring for 15 min. The porridge was cooled to 40ºC then inoculated with wheat bran, maize malt and *Lactobacillus* starter culture. The concentrations used for inoculums were 0.5, 1 and 2% w/w. The different varities of Pro-Vitamin A maize used were PVAH 62 and PVAH 19 (Awobusuyi, 2015). A white *Mahewu* processed beverage was used as control. Pro-Vitamin A was retained in *Mahewu* after fermentation, the starter cultures improved the taste, aroma and overall acceptability and the beverage was shelf stable for 3 days at room temperature (Awobusuyi, 2015). However, the beverge was accepted mostly by females than male who were undecisive. The enrichment may lead to segmentation of the consumers.

Other minerals such as zinc and iron content which help with physical growth, cognitive developemnt and reproduction could be made available through the addition of ascorbic acid and/or NaFeEDTA to encourage their release during fermentation. The

naturally available bacteriocins could eliminate spoilage microorganisms in the beverage. Like milk based beverages, prebiotics such as fructooligosaccharides, inulin, and galacooligosaccharides could also be added to the beverage to support the growth of LAB bacteria (Adelakan, 2013). Different vitamins and minerals such as vitamin D, vitamin E, vitamin C, calcium and magnesium could also be added to supplement and/or fortify the beverage to improve the growth of children. Other technologies such as flavour enhancer could be used to mask any undesirable taste and flavours that arise during fortification (Sowonola, 2005).

Olosunde *et al*. (2014) investigated the use of *Moringa* (*Moringa oleifera*) flour to determine the nutritional quality of cereal beverage. The beverage was prepared traditionally with slight modification. Three varied levels (5, 10 & 15%) of *Moringa* seed flour was added to the slurry during production. The protein, mineral, physicochemical, anti-nutritional and sensory qualities of the beverage were determined. There was a decrease in moisture and carbohydrate as the *Moringa* seed flour increased while the protein, fat, ash and crude fibre contents increased. Enriched beverage with 15% *Moringa* seed flour had higher mineral and anti-nutritional contents. The pH and soluble solid also increased as *Moringa* flour increased. There was no significant differences in taste, appearance and overall acceptability of 5 and 10% enriched beverage samples and the control. It is recommended that up to 10% *Moringa* seed flour is desirable, as higher concentrations (15%) impact undesirable effect on the taste and appearance. Therefore, the level of *Moringa* to use in supplementing the beverages need to be monitored not only for increasing nutritional benefit but also for taste, aroma and appearance. Abidoye (2017) investigated the effect of cocoa powder on the nutritional properties of kunun-zaki made from sorghum due to its antioxidants activities. The most prefered beverage was made-up of sorghum as base and cocoa powder at 80 and 20%, respectively, scoring 3.4 compared to traditional beverages at 1.8 on a 9 point Hedonic scape. The antioxidant of the beverage increased from 40 to 50%. Folasade and Oyenike (2012) also reported that sesame seeds (20%) added to sorghum non-alcoholic beverage produced an acceptabe beverage. The beverage was high in protein, ash, fat and minerals (calcium, phosphorus, potassium, magnesium and iron) in comparions to indegenous beverage. The effect of *Aframomum danelli* and black pepper on the physicochemical, sensory and shelf life of cereal beverage were investigated (Adedokum *et al*., 2012). The hurdle effect of local spices, refrigeration and/or freezing increased the shelf life, physicochemical and sensory (flavor) of the beverage.

2.12.2.6 Effect of preservatives on cereal beverage

The effect of preservatives only and in combination with pasteurisation on microbiological qualities of *Kunun zaki* were investigated by Ayo *et al*. (2013) using four varied levels (0.01 - 0.05%) of sodium benzoate and metabisulphite as preservatives on the physicochemical, sensory and microbial quality. The unpasteurised beverage had high total acidity, while the pasteurised beverage with 0.05% sodium metabisulphite had low total acidity. The decrease was due to the destruction of *Lactobacillus spp.* by heat and chemical preservatives. The total soluble solids decreased in all samples over storage time due to breakdown of sugars by surviving organisms. The protein content was high in unpasteurised samples (4.25 - 4.31%) whereas pasteurised samples had a 10% denatured protein. The increase in protein of unpasteurised (UP) samples was due to protein hydrolysis during fermentation. There was an increase in total solids and moisture content while a decrease in ash content over storage time was observed. The latter was due to the increased metabolism of nutrients. The microbial quality of the beverage improved in pasteurised and preserved samples. There was high microbial count in unpasteurised samples which increased with storage period. It was noted that the microbial count decreased with a reduction in the level of chemical preservatives. This was due to the inhibitory and destructive effect of chemical preservatives against yeast, mould and bacteria (Ayo *et al*., 2013). Unpasteurised beverage had high count of microorganims than pasteurised beverage which increased on storage. The pasteurised samples had high count of 4×10^3 cfu/ml which indicated that pasteurisation was not effective or recontamination after processing and/or package leak. There was no microbial growth in 0.0.3 - 0.05% sodium benzoate samples. Sodium metabisulphite (0 - 0.05%) samples had no growth at the beginning of storage, however, there was growth in subsequent storage which could be due to recontamination as a result of leak in packaging. The sensory qualities showed that 0.03% sodium benzoate was most preferred (Ayo *et al*., 2013).

Hussain *et al.* (2014) investigated the effect of niacin (400 RU/ml) at 1% and potassium sorbate (0.15%) on the shelf life of sorghum based fermented milk beverage and combination of preservatives and thermal treatment (65°C for 5 min). The use of potassium sorbate only was the best preservation enhancing the shelf life of the beverage at refrigeration conditions. The use of many food additives including preservatives is still a subject of debate among academics. The additives are believed to cause a lot of reactions in humans, estimated at a rate of 1% in adults and 2% in children (Abdulmumeen, 2012). Chemical preservatives such as sodium benzoate and potassium

sorbate are used in the preservatives of cereal beverages to extend their shelf life. However, consumers of cereal beverages demand fresh and safe foods with no added chemically synthesized preservatives which opens doors for research into naturally synthesized preservatives such as bacteriocins, organic acids, essental oils and phenolic compounds (Aneja *et al*., 2014). Sulphites causes a variety of symptoms while benzoates are responsible for asthma, allergic rhinitis, chronic urticaria (Oliphant, 2012). Thus, most heath conscious consumers prefer clean label on food products.

2.12.2.7 Production of quality cereal malt

Cereal malts are used in various non-alcoholic beverages where they act as carriers for fermenting microorganisms (inoculum). Malting of cereal grains under favourable conditions of heat and humidity leads to a product rich in enzymes, vitamins and other soluble compounds. The process is carried out in three phases, namely, soaking, germinating and drying. Traditionally, malting is done by women at home under uncontrolled conditions which pose a health risk due to the cyanogenic compounds, enterobacteria or moulds which can grow. This uncontrolled process conditions could also affect the enzymatic activity including amylase and affect the organoleptic qualities. Traditional malt production could also generate aflotoxins in excess of 8 µg/kg recommended as a limit by Codex Alimentarius. In view of this, soaking of grains in alkaline solution could be implemented as an improvement to increase the grains diastatic properties and control bacterial population. As suggested by Hounhouigan (2010), the production of quality malts can create an inoculum for production and marketing for high-quality malts to use in beverages. The effects of roasted malt on the physicochemical characteristics of non-alcoholic maize beverage were investigated by Akonor *et al*. (2014). Maillard and caramelisation which took place in roated beverages caused the beverage to be darker in comparison to traditional beverage. The was no significant differences between the beverage made with roasted malt and traditional beverage. This idicated that roasted malt could be used to produce non-alcoholic beverages during commercialisation instead of using commercial caramel.

2.13 Importance of fermented beverages in Africa

A number of methods such as genetic improvement and amino acid supplementation with protein rich concentrates or other protein rich sources have been used to improve nutritional qualities of cereals (Coulibaly *et al.*, 2011). In addition, processing technologies such as cooking, sprouting, milling, and fermentation have been put into practice to improve the nutritional properties of cereals. Fermentation is the most simple and

economical way of improving the nutritional value, sensory and functional qualities of cereals (Hui, 2012). Several changes occur during fermentation of cereals such as the increase of biological availability of essential amino acid such as lysine and starch and the decreases in fibre while the vitamins vary depending of raw materials used (Hui, 2012).

The major objective of fermentation is to extend shelf life of beverage products (Marshall & Mejia, 2011). In addition, fermentation makes the beverage to be wholesome, acceptable and of improved quality (Marshall & Mejia, 2011). Fermented beverages have contributed to cultural evolution and preservation since 800 BC (Marshall & Mejia, 2011). During the production of cereal beverages, various sectors within the economy will supply water, sugar, cereals (farms), packaging, fuel and power. The purchase of these goods and/or services will have an economic benefit. Commercialisation of non-alcoholic cereal beverages (NACB) would employ people both in the production of the beverages and indirectly at the farms, spend on capital expenditure and tax contribution. There will be an indirect boost on direct suppliers in terms of production, employment and tax revenue. The economy will also benefit indirectly when the farmers, their employees and their families re-spend in the country's economy further boosting economic activities. The gross domestic product (GDP) of the continent which recently grew to an estimate of 5% (2001 – 2014) from 2% (1980 – 1990) will be boosted through the production and sales of NACB locally and abroad resulting in foreign earnings (Chelule *et al*., 2010). Other sectors which will benefit on the long run include insurance companies, finance, wholesale, transport, catering and accommodation and storage among others.

Food fermentation has many advantages and disadvantages. Some advantages include the conversion of sugars and other carbohydrates such as juice to wine, grain to alcohol, sugars in to organic acids. Other effects of food fermentation are the controlled action of microorganisms that alter the texture of food, to preserve and produce characteristic flavours and aromas. Additional benefits of fermented beverages as reported by Marshall & Mejia (2011) include (i) food security and cultural importance, (ii) nutritional and health benefit, (iii) benefits to small scale farmers, (iv) value-added products, (v) employment benefits and (vi) gender development.

2.13.1 Food security and cultural identity

Fermented non-alcoholic cereal beverages serve as food security items for millions of marginalised and vulnerable people around the globe. Fermentation is a food processing way of preserving perishable food, thus bringing benefits to people. It offers the opportunity for a range of raw materials that can be used and remove anti-nutritional

factors making food safe to eat. The process is cheaper to set up and energy efficient, which is accessible to the marginalised, landless, physically incapacitated rural, semi-urban and urban poor people. It makes use of less sophisticated equipment to undertake or less subsequent storage for fermented product. Fermentation is still seen as a substitute for refrigeration, safe keeping of food and utilised to make edible left overs. Food fermentations are culturally important; they have been passed from one generation to another. The variety of fermented foods and beverages reflects cultural diversity and cuisine. People carry their type of fermented foods and beverages as they migrate among countries.

2.13.2 Nutritional and health benefit

The nutrition and health of a human depend on a balanced supply of food and water. The most susceptible group to malnutrition are women, children and weaning infants. It is predicted that approximately 30% of women consume less than recommended daily energy and at least 40% of them suffer from iron-deficiency (Marshall & Mejia, 2011). Fermented beverages provide one third of worldwide diet and cereal are important raw materials in fermented beverages. Fermentation makes beverages safe, nutritious, palatable, improve digestible proteins and carbohydrates and remove toxins. Cereals used as substrates contain anti-nutritive compounds and reduced availability of minerals, calcium, iron, magnesium and zinc; and deficiencies in essential amino acid, which are building blocks for proteins (Marshall & Mejia, 2011). However, fermentation of cereals improves the nutritional value of protein quality. Beverages are made up of a lot of water and this help prevent dehydration for millions of people in sunny Africa. The mechanisms of probiotics are still not well understood, but are commonly suggested to relate to (i) pathogen interference, (ii) exclusion or antagonism, (iii) immune modulation, (iv) anticarcinogenic and antimutagenic activities, (v) alleviation of lactose intolerance symptoms, (vi) reduction in serum cholesterol levels, (vii) reduction in blood pressure, (viii) prevention and decreasing incidence and duration of diarrhoea, (ix) prevention of bacterial vaginisos and (x) urinary tract infection, (xi) maintenance of mucosal integrity , and (xii) improved odontal health (Franz *et al*., 2014).

2.13.3 Farmer benefit

A fermentation business requires minimal entry costs to set up and run, because it uses produce from the farm and has no major impact on farm production and labour as most of the job is carried out by fermenting microorganisms. Produces not sold to commercial

producers can be used for small scale fermentation. The fermentation enterprise can use raw materials from other farm by-products. Farm by-products can also be made from fermented products to enhance livestock nutrition and health. Fermented livestock wastes are excellent source of energy (Marshall & Mejia, 2011).

2.13.4 Range of fermented products

Fermentation enterprises involve adding value to produce on the farm and its by-products. This can increase income to the farmer and extend the shelf life of products. Fermentation results in the production of nutritionally enriched, staple food products from substrates with low value carbohydrates and protein. It also improves the flavour, aroma, texture and appearance of food. It increases the range of products to be sold thus adding value to farmer produce.

2.13.5 Employment benefits

Large scale fermentation business involves setting-up process, preparation, packaging, marketing and all of these involve employing people. However, most of employment comes from small scale fermentation enterprise that employs few people commonly related members. Traditional and small scale fermentation enterprises are popular in remote areas with limited resources. Popularity of fermented products will have the potential to increase employment options due to increased demands leading to more sales. This business when set up on farms will provide immediate family members with employment and advance their knowledge through training in fermentation, skills such as process management, quality control, business management and/or transferable skills will be gained. These enterprises may also create indirect jobs by requiring other inputs not available on farms such as sugar, transport, packaging or marketing.

2.13.6 Empowerment

Fermentation is normally done on farms by women. This represents an economic opportunity for women in farms since the start-up capital is low and no particular assets are required and not physically challenging. As fermentation skills are passed down among generations from mother to daughter, women are the most traditionally knowledgeable people on fermentation processes. The fermentation process is not labour intensive and can be combined with other household responsibilities such as child-care and allow flexible hours. However, due to marketing, women are required to leave their homes to earn a living elsewhere. This in turn empowers them through social and

business interaction. They develop literacy and numeracy skills to improve the status of their community. The earning generated provide for their family needs, and security in case of abandonment and widowhood.

2.14 Trends in the near future of commercialization and production of non-alcoholic cereal beverages

Food supply and demands globally are projected to substantially undergo transformations in consumption pattern, technologies, policies and international trade. The change in food consumption is mainly due to population and income growth and changes in food habits. Although most of the population in Africa still leaves in rural areas this is likely to improve as the income is expected to rise in many developing countries. Many people in developing countries are likely to demand foods that are healthy, nutritious and have theurapic value as they become conscious of what they eat. As such the demand for traditional foods such as the non-alcoholic cereal beverages (NACB) is likely to grow. Due to factors such as urbanization, the growing number of females in the workplace and single person household the demand for convenient food is likely to increase. Indigenous food such as NACB may considerable benefit the consumers' need of greater variety in food of which they are familiar with (Henderson, 1998). The beverages are cheap and the cereal used is widely grown. Although there has been a growing scientific interest in these beverages, the age-long techniques used during their production still need to be improved for industrialization (Brian, 1998). The fermentation of cereal for beverages production with health-promoting properties (functional) is well known in Africa. The global production and demand of functional beverages is growing, it was said to have increased by 1.5 fold between 2003 and 2010, by 22.8% between 2010 and 2014 with the value of €29.8 billion and have been around €65 billion in 2016 (Mash *et al*., 2014). The availability of technology and product development processes is paving the path to get these beverages to the store shelves (Gaffa *et al.*, 2002). In addition, beverage producers have a chance to improve the acceptability of the beverages due to advancement in technologies. This opens opportunities for manufacturers to respond quickly to consumer demands. There is a need to move this household fermentation technology to an industrial scale to meet the increasing consumer demand.

2.15 Chemometrics

Chemometrics is the branch of chemistry which deals with the evaluation of chemical data and ensures that data collected from experiments contain maximum information (Otto,

2007). The notation chemometric was developed in 1972 by Swede, Svante Wold, and the American, Bruce Kowani (Otto, 2007). It originates in chemistry and applied in the development of qualitative structural activity relationships and/or in the evaluations of analytical chemical data. Chemical systems or process are too complicated to understand fully by theory hence, chemometrics is necessary to achieve information (Wold, 1995).

2.16 Conclusion

Pearl millet is a principal source of energy, protein, vitamins and mineral and is able to grow in a broad range of ecological environment. However, this nutritional quality is considered lower due to the presence of anti-nutritional factors which results in poor digestibility of proteins and carbohydrates. Fermentation of pearl millet removes anti-nutrients such as phytic acid and tannin and improves the digestibility of complex proteins. Due to the nutritional richness and growth conditions of pearl millet, it has the potential to be used in fermented beverages.

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CHAPTER THREE

ISOLATION, IDENTIFICATION AND PURIFICATION OF LACTIC ACID BACTERIA FROM PEARL MILLET SLURRY DURING FERMENTATION FOR NON-ALCOHOLIC CEREAL BEVERAGE

Abstract

The aim of this investigation was to isolate, identify and purify bioburden lactic acid bacteria from naturally fermented pearl millet slurry during the production of non-alcoholic cereal beverage (NAPMB). NAPMB was produced through natural fermentation of pearl millet slurry at 37°C for 36 h. During the fermentation the pH, total titratable acidity (TTA), correlated optical density (OD_{corr}), microbial growth (lactic acid bacteria and total viable count) and the soluble sugar of the beverage were determined at 3 h interval. The total viable cells were enumerated on total plate count agar and the lactic acid bacteria on deMan Rogosa and Sharpe agar. The presumptive lactic acid bacteria were characterized using scanning electron microscope and identified using Vitek 2 system. There was a significant ($p \leq 0.05$) difference in the total viable counts over the 36 h fermentation. The total viable count increased from 6.98 to 7.82 log cfu/ml after 27 h. There was a significant ($p \leq 0.05$) difference in the lactic acid bacteria (LAB) at 3, 15, 21, 24, 27 and 30 h fermentation periods. The initial numbers of LAB were 7.04 log cfu/ml and increased to 8.00 log cfu/ml after 21 h. The beverage was dominated by LAB from the genera *Leuconostoc, Pediococcus, Streptococcus* and *Enterococcus*. There was a significant ($p \leq 0.05$) change in the pH and TTA of the beverage during fermentation. The pH of the beverage at the start fermentation was pH 6.37 and decreased to pH 4.06 in 18 h. Thereafter, the pH did not significantly change over time. The TTA was inversely proportional to the pH and ranged from 0.12 to 0.53% in 36 h. There was a moderate, negative linear correlation between the LAB and pH (r = -0.535, $p < 0.05$). The TTA had a very strong, negative linear correlation with the pH (r = -0.975, $p < 0.05$) while the yeast and mould (YM) had a strong, negative correlation with the OD_{corr} (r = -0.713, $p < 0.05$). Principal component analysis (PCA) showed that LAB, TTA, YM and OD_{corr} were important parameters at 30 – 36 h of fermentation of pearl millet slurry during the production of NAPMB. The optimum fermentation time for the beverage was 18 h at 37°C with pH of 4.06.

3.1 Introduction

Indigenous non-alcoholic cereal beverages play a vital role in the everyday lives of people in developing countries. They are considered as after-meal drinks or refreshing drinks during dry season in rural and urban areas. These beverages are made up of about 90% water, cereal powder, sugar, flavours and sometimes preservatives (Osuntogun & Aboaba, 2004; Ikpoh *et al*., 2013). Different organisms are present in these traditional beverages with raw materials being the main sources. The microbiological composition of these products is complex and unexploited. It involves mixed cultures which may work in parallel, while others act in a successive manner with changing principal microflora during fermentation (Katongole, 2008).

Non-alcoholic cereal beverages (NACB) are popular in many African countries (Terna & Ayo, 2002). The cereals mainly used in their production are millets; sorghum and maize although composite cereals are sometimes used (Terna & Ayo, 2002). The choice of cereal to use depend on availability and affordability. Sprouted cereals are also used in the production of NACB. Sprouting modifies the cereal grains physically, chemically and biologically. During sprouting the starch and proteins are hydrolysed into sugars and amino acids, respectively. The sprouted cereal grains are a major source of hydrolytic enzymes particularly the alpha-amylase (Akonor *et al*., 2014) and are used in weaning food where they breakdown starch resulting in decreased viscosity and increase in the nutritional value of the beverage (Grossmann *et al*., 1998). Rice is sometimes used in the production of sprout because it is belived to produce a tasty beverage.

The increasing consumer awareness towards healthy diets and changing eating habits due to urbanization has created a huge market demand for new functional foods with beneficial effects on health (Rathore *et al.,* 2012). Hence, the indigenous fermented beverages are becoming popular due to their nutritional and therapeutic value. Hence, there is a need to commercialise these beverage and make them available on the supermarket shelves. The beverages made from cereal grains are sources of dietary proteins, energy, vitamins and minerals (Blandino *et al.,* 2003). Thus, starter cultures need to be developed to produce beverages with similar and consistent quality with the traditionally prepared beverage.

Lactic acid bacteria (LAB) are made up of the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococccus* and *Weissella.* These bacteria are probiotics producing different antimicrobials such as acetic acid, carbon dioxide and bacteriocins which are of interest. The preparation of NACB is

carried out by LAB through chance fermentation. LAB have been isolated from different traditional foods but the organisms differ from region to region and house to house as a result of raw materials used and other factors. This leads to differences in terms of sensory, yield, quality, shelf-life and safety among the products.

The aim of this study was to isolate and identify the LAB involved in the natural fermentation of pearl millet beverage with a view to obtain pure cultures of bioburden LAB. Bioburden refers to viable microorganisms present on or in a product that has not been sterilised.

3.2 Materials and Methods

3.2.1 Sources of materials and equipment

Pearl millet and rice grains were purchased from Agricol in Brackenfell, Cape Town. Ground ginger was purchased from Deli Spices in Cape Town.

Perten 3100 laboratory mill, Bauermeister Incorporation Vernon hammer mill, water-bath, cabinet oven, Hanna Edge pH meter (HI - 11310), Incubator, Geiger & Klotzbucher cabinet oven (Model: 1069616) and compound microscope were provided by the Department of Food Science and Technology, Cape Peninsula University of Technology. The Agilent 1100 HPLC-RID, Biomerieux Vitek 2, BenchTop Pro with omnitronics (VirTis-SP Scientific) freeze-dryer, Ultra-freezer (Glacier, -86ºC ultralow temperature freezer) and UV 1700 Pharmaspec spectrophotometer system were provided by Agrifood Technology Station, Cape Peninsula University of Technology. Scanning electron microscope was provided by the Department of Geology, Stellenbosch University (Zeiss MERLIN FE-SEM).

3.2.2 Production of pearl millet flour (PMF)

The method of Ratau (2011) was used to produce pearl millet flour. Dry pearl millet grains were manually cleaned to remove physical foreign objects (seeds, broken grains, sands etc.) and washed. Excess water was drained off by spreading the pearl millet grains on a stainless sieve and then dried for 3 days (72 h) at 50°C in a Geiger & Klotzbuche cabinet oven. The grains were dry milled into flour using a 0.8 mm Bauermeister Incorporation Vernon hammer mill. The resulting flour was kept at 5°C until further use.

3.2.3 Production of sprouted rice flour (SRF)

Dry rice grains were manually cleaned to remove physical foreign objects (by removing seeds, broken grains, sands etc.) and hydrated with cold water for 24 h. Excess water

was drained by spreading the rice grains in a plastic colander. The soaked grains were left to sprout in the colander in an incubator at 25^{o}C for 96 h with a 12 h rinse interval. The sprouted grains were spread on an oven tray and dried at 50^{o}C for 96 h in a Geiger & Klotzbucher cabinet oven. The dried seeds were milled into flour through a 0.8 mm sieve using Perten 3100 hammer mill. The resulting flour was kept in a clear plastic bag and stored at 5ºC until further use.

3.2.4 Determination of alpha amylase activity (falling number) in sprouted rice flour (SRF)

The alpha-amylase (α-amylase) activity of sprouted rice flour (SRF) was measured using a Perten Falling Number instrument. Prior to falling number (FN) measurement, the moisture content of the flour was measured using oven method. The FN of flour was determined by weighing separately 6.40 g of SRF and 6.65 g of unsprouted rice flour (uSRF) into a 75 ml viscometer tube and thoroughly mixed with 25 ml of distilled water by means of a stirrer. The mixture was placed in a boiling water bath fitted within the instrument and allowed to run. The boiling water gelatinised the starch and the slurry became viscous. The stirrer inserted mixed the gelatinised slurry into more homogenous slurry. When the stirrer was dropped during the test, the time taken for the stirrer to reach the bottom of the viscometer tube was calculated as the FN. The readings were automatically measured after 5 seconds. The lower FN indicates that the α-amylase activity was higher (Perten, 2016; Perten, 2017). The samples were run in triplicates.

3.2.5 Production of non-alcoholic pearl millet beverage (NAPMB)

The pearl millet flour (200 g) was hand mixed with 250 g water and left to hydrate for 3 h at ambient temperature (approximately 25 ^{o}C). After hydration, the paste was divided into two unequal portions (¼ and ¾). The ¾ paste was gelatinised with 1000 ml boiling water and cooled to 40^{o}C. The ¼ slurry was hand mixed with 10 g ground ginger, 30 g sprouted rice flour and 50 ml cold water. The two portions (¼ and ¾) were mixed together. Aliquots (45 ml) of the slurry were distributed into sterilized 100 ml Schott bottles and left to ferment at 37^{o}C for 36 h in a water bath with a shaker set at 32 rpm. The production process of non-alcoholic pearl millet beverage is shown in Figure 3.1. Samples were drawn at 3 h interval during the 36 h fermentation and analysed for pH, total titratable acidity, total soluble sugar, optical density and microbiological analysis following the methods described in Chapter 3, Sections 3.2.6 – 3.2.13.

3.2.6 Physicochemical analysis of pearl millet slurry (PMS) during fermentation for the production of non-alcoholic pearl millet beverage (NAPMB)

The pH of the pearl millet slurry (PMS) [10 ml] was measured at 3 h interval in triplicates using Hanna Edge glass electrode pH meter standardised with pH buffer solution of 4, 7 and 10.

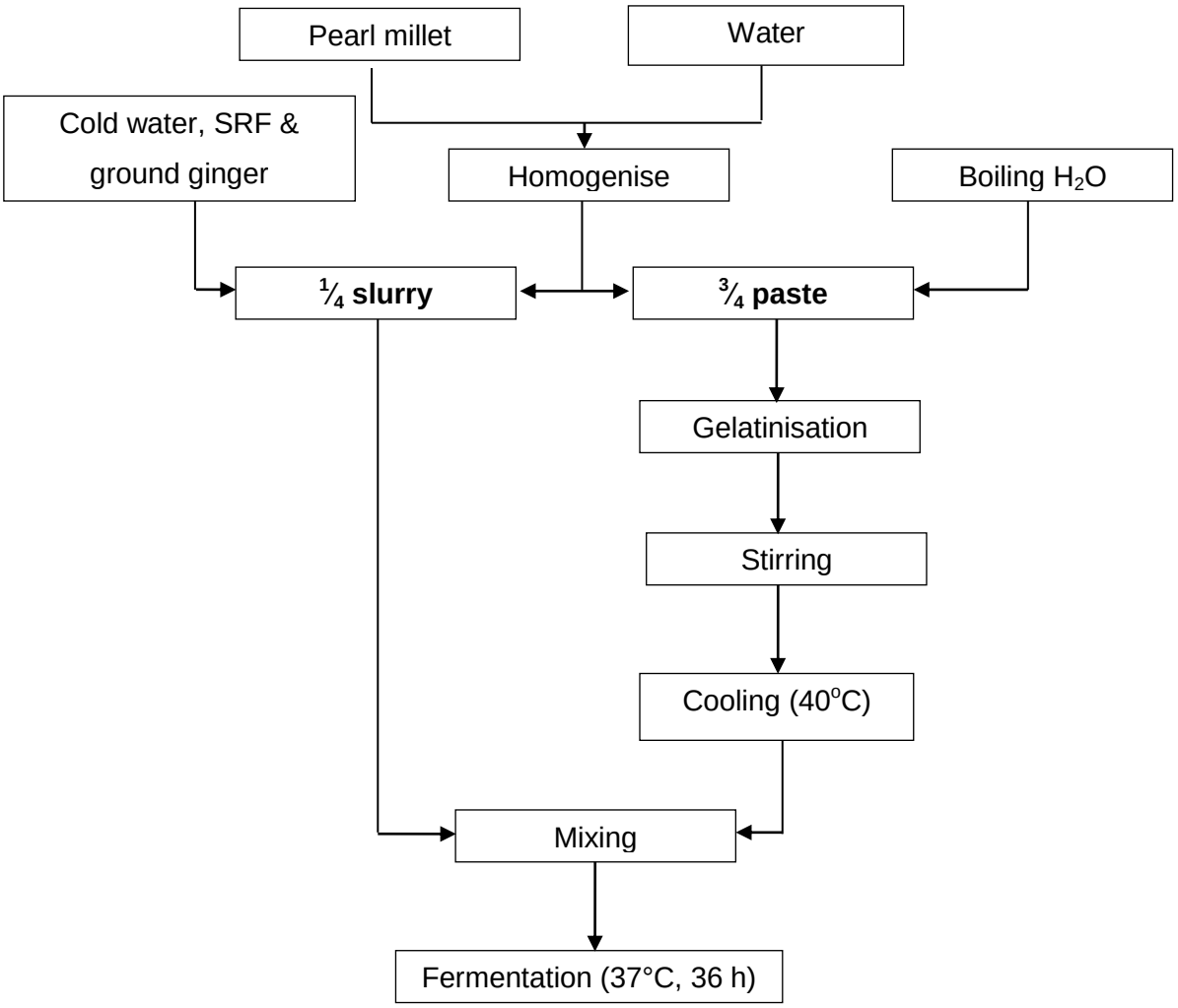

Figure 3.1 Flow diagram for the production process of non-alcoholic pearl millet beverage (NAPMB). SRF – sprouted rice flour

Total titratable acidity (TTA) was assessed at 3 h interval during fermentation. The TTA of the pearl millet slurry during fermentation was determined in triplicates by titrating 10 ml of the sample with 0.1N NaOH using phenolphthalein as indicator until a light pink

colour appears. The TTA was expressed as percentage lactic acid (AOAC, 1980). Equation 3.1 was used to calculate the percentage acidity, with each 0.1M NaOH equivalent to 90.08 mg lactic acid.

$$\text{TTA (\% lactic acid)} = \frac{\text{ml NaOH x N NaOH x M.E}}{\text{volume of sample used x 1000}} \times 100 \qquad 3.1$$

Where, ml NaOH = volume of NaOH (ml), N NaOH = molarity of NaOH, M.E = the equivalent factor of lactic acid being 90.08 mg, 1000 = factor used to convert the M.E which is normally in mg to grams, and 100 used to express the lactic acid concentration in percentage.

3.2.7 Determination of total soluble sugars in pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage (NAPMB)

The method of AOAC 982.14 as described by Li *et al*. (2002) was used to determine the total soluble sugars in pearl millet slurry (PMS) during fermentation for the production of non-alcoholic cereal beverage (NACB). Sugar extraction was done by mixing 5 g (W_1) of the pearl millet slurry (PMS) with 100 ml (W_2) of 50% ethanol. The mixture was heated in a water bath for 25 min at 85ºC with a shaker set at 25 rpm to break-up and dissolve the sample. The mixture was cooled to room temperature and ethanol (95%) was used to bring the sample weight to original weight (W_2). The sample was filtered through a 0.45 µm nylon syringe into 1.5 ml screw neck high performance liquid chromatography (HPLC) sample vials (11.6 mm outer diameter × 32 mm height). The total sugar content of the extracts was determined in triplicates using HPLC (Agilent 1100 HPLC – RID system) equipped with Zorbax carbohydrates column (4.6 × 150 mm, 5 µm) and Zorbax NH_2 guard column (4.6 × 12.5 mm, 5 µm). The mobile phase used was acetonitrile mixed and de-gassed with millipore distilled water at 75:25 (acetonitrile:water) ratio. The sugar standards were prepared by mixing sucrose (6 mg/ml), fructose (6 mg/ml), glucose (6 mg/ml), maltose (6 mg/ml), lactose (6 mg/ml) and sucrose (30 mg/ml) in a water/ethanol (50:50) solution. The resulting stock solution was then used to prepare concentration solutions used for the calibration curve. The concentration used to draw a standard curve were 0.375 (1.875) mg/ml, 0.75 (3.75) mg/ml, 1.5 (7.5) mg/ml and 3.0 (15.0) mg/ml. The value in parenthesis shows the sucrose concentration in each solution.

3.2.8 Measurement of cell concentration in pearl millet slurry during fermentation by optical density

The growth of lactic acid bacteria was determined using optical density (OD). Pearl millet slurry (PMS) in 100 ml Schott bottles was thoroughly mixed for 1 min. PMS (1 ml) was aseptically transferred into deMan Rogosa and Sharpe (MRS) broth [HG000C87.500] and incubated at 30°C for 48 h. After incubation, 0.2 ml of the broth was transferred into sterile 3 ml de-ionised water (*d*). The dilution was done where necessary since the relationship between microorganisms and OD is non-linear if the OD is above 1.0 (Champagne *et al*., 2007). The sample was mixed by vortexing for 30 sec prior to the OD measurement using UV 1700 Pharmaspec visible spectrophotometer set at 20ºC and 600 nm (OD_{600}) wavelengths (Widdel, 2010). The reference sample used was sterile MRS broth (0.2 ml) mixed with 3 ml sterile de-ionised water (*d*) where necessary. The absorbance was divided by the defined dilution factor (*d*) to get correlated or calculated optical density (OD_{corr}).

3.2.9 Enumeration of bacteria in pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage (NAPMB)

Pearl millet slurry (PMS) [45 ml] was added into 100 ml Schott bottles and thoroughly mixed by shaking for 1 min. Dilutions of PMS were carried out by transferring 10 ml to a bottle containing 90 ml sterile ¼ strength of Ringer solution (Abegaz, 2007, Kivanc *et al*., 2011) to give 10:100 dilutions followed by a 10 fold serial dilution from 10^{-1} to 10^{-10}. Each dilution was sub-cultured in triplicate. A portion of the sample dilution (1 ml) was added into a 15 x 100 mm plastic Petri plates containing cooled molten agar by means of a pipette, mixed and left to solidify (Omemu, 2011). Lactic acid bacteria (LAB) were plated on deMan Rogosa and Sharpe (MRS) agar [Merck HG00C107.500] (Nwachukwu *et al*., 2010; Temitope & Taiyese, 2012) under anaerobic condition using Anaerobic Gas-Pack system and anaerobic indicator strips at 30°C for 48 h (Osuntogun & Aboaba, 2004; Nwachukwu *et al*., 2010). The total viable count (TVC) was enumerated on plate count agar (PCA) [Merck HG 0000C6.500] and incubated aerobically at 37ºC for 48 h. After incubation, Petri plates with colonies between 30 and 300 were counted. All microbiological data were expressed in logarithms of numbers of colony forming unit per ml (log cfu/ml).

3.2.10 Isolation and identification of lactic acid bacteria in pearl millet slurry during fermentation

Pearl millet slurry (PMS) [45 ml] was homogenized in centrifuge tubes using vortex at 5 speeds for 30 sec. After homogenisation, 1 ml of PMS was transferred aseptically into a 9 ml of ¼ strength Ringer solution and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-4}) were carried-out and 0.1 ml portion of the appropriate dilutions spread onto deMan Rogosa and Sharpe (MRS) agar plates. In addition, 1 ml of the serial dilution (10^{-1} to 10^{-4}) was pour-plated onto MRS agar. Each dilution was cultured in triplicate (Mavhungu, 2005; Omemu, 2011). The plates were incubated anaerobically for 48 h at 30ºC. Distinct colonies grown on and/or in MRS plates with 30 - 300 colonies were harvested and sub-cultured on fresh MRS agar and incubated for 48 h at 30ºC. Presumptive lactic acid bacteria (LAB) colonies were further sub-cultured in triplicates on MRS agar plates and anaerobically incubated for 48 h at 37ºC.

Presumptive LAB isolates on MRS agar were examined for Gram reaction, catalase reaction, production of CO_2 from glucose using hot loop test and gas production using 3% H_2O_2 (Schillinger and Lucke, 1987). Cell morphology was examined by compound microscope and scanning electron microscope (SEM). The growth of isolates at 4, 10, 45ºC and 6.5% in NaCl concentration in MRS agar were evaluated after 48 h. The colonies were identified using Vitek 2 compact system. The gram-positive (GP) cards for Vitek 2 compact system were used to identify isolates (*Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus and Vagococcus*) to species while anaerobic cards (ANC) were used to identify *Lactobacillus* to species. Colonies were identified according to the instructions provided by the manufacturer.

3.2.11 Determination of the generation time of bacteria in pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage (NAPMB)

The generation time or doubling time of microorganisms refers to the time it takes for a cell (or population) division to take place. The equation used to calculate the generation time was derived from the binary fission growth and is shown in Equation 3.2 where, G = generation time given in minutes; t = time interval in hours or minutes; B = the initial bacterial count of the given time interval and b = final bacterial count of the given time interval (Todar, 2012). The data collected in Chapter 3, Section 3.2.9 was used to calculate the generation time.

$$G = {}^{t}\!/_{3.3 \log \frac{b}{B}} \qquad 3.2$$

3.2.12 Lactic acid bacteria preparation for scanning electron microscope (SEM) images

The methods of Mavhungu (2005), Katongole (2008) and JCU (2016) were used to get images of lactic acid bacteria (LAB) under scanning electron microscope (SEM). LAB colonies were grown in MRS broths at 30ºC for 36 h. The broth was mixed thoroughly for 1 min and few drops (4 - 5) placed on a 0.45 µm filters and then left to air dry at room temperature for 30 min. The specimens were then fixed using 2.5% glutaraldehyde in phosphate buffered saline (PBS) with pH of 7.2 for 30 min at 4ºC. The specimens were fixed using osmium tetroxide (OsO_4) for 1 to 2 h prior to dehydration in a series of ascending different ethanol concentration (30, 50, 70, 80 and 100%) for 15 min at each concentration. The final stage in 100% ethanol was repeated twice. The specimens were then critically-point dried at 1072 psi and 31ºC, coated with gold before viewing under Zeiss MERLIN FE-SEM. Beam conditions during imaging were 5 kV accelerating voltage, 250 pA probe current, with a working distance of approximately 4 mm.

3.2.13 Storage of purified cultures of lactic acid bacteria isolated from pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage (NAPMB)

The isolates were grown in 500 ml deMan Rogosa and Sharpe broth at 30ºC for 60 h. The broths were hand mixed thoroughly and 2 ml of the broth mixed with 1 ml of 10% skim milk (Kandil & Soda, 2015) in 5 ml bench-top freeze-dryer vials. The samples were frozen in an ultra-freezer (Glacier, -86ºC ultralow temperature freezer) at -76°C for 12 h then freeze dried using BenchTop-Pro with omnitronics (VirTis SP Scientific) freeze dryer. The dried samples were sealed under vacuum and stored in the freezer at -18ºC.

3.3 Data analysis

The results reported are mean of three independent trials. Multivariate analysis of variance (MANOVA) was used to determine mean difference between treatments at $p = 0.05$. Duncan's multiple range tests was used to separate means where differences exist using IBM SPSS ver 23 (IBM, 2015). Principal component analysis (PCA) was used to summarise and uncover any patterns in the fermentation data set by reducing the complexity of the data. The statistical relationships between the dependent variables [pH,

total titratable acidity (TTA), total viable count (TVC), yeast and mould (YM), and correlated optical density (OD_{corr})] during the fermentation of pearl millet slurry (PMS) for the production of non-alcoholic pearl millet beverage (NAPMB) were determined using Pearson correlation (*r*). Pearson correlation is scaled between -1 and +1 ($-1 \leq r \leq +1$). When the correlation coefficient is -1 or +1 the linear relationship is said to be the strongest. The weakest linear relationship is observed when the correlation is 0. Any correlation that is positive indicates that if one variable increase the other variable tends to increase. A negative correlation means that when one variable decreases then other variable tends to decrease (Beldjazia & Alatou, 2016). This type of correlation only focuses on linear relationships, thus, a 0 relationship between variables does not indicate zero relationship but rather a zero linear relationship. Evans (1996) suggested the strength of the absolute correlation value as: 0.00 - 0.19 = very weak, 0.20 - 0.39 = weak, 0.40 - 0.59 = moderate, 0.60 - 0.79 = strong and 0.80 - 1.0 = very strong (Beldjazia & Alatou, 2016). Furthermore, a significant test was performed to determine if there is any evidence that a linear correlation exists between variables at 0.01 and 0.05 levels.

3.4 Results and Discussion

3.4.1 Physical, chemical and biological changes in rice grains during sprouting

The amylolytic enzyme was low in unsprouted rice grains with a falling number of (FN) of 410.33 and increased in sprouted grains with a FN of 194.67. The activity of α-amylase in the grains differed significantly between the sprouted and unsprouted rice flour ($p < 0.05$). The rice grains showed the radicle on day 3 (after 72 h) which grew larger after 96 h. Figure 3.2 shows the sprouted rice grains over 96 h. Seeds or grains contain all the necessary nutrients required for germination to take place. The main parts within the seed are, the embryo – found within the seed coat and develop into a plant. It is made-up of a plumule, radicle and one or two cotyledons; endosperm - which serves as the source of food stored in the seed; and the seed coat - which is a protective layer of the seed. During germination, there are three main stages, namely, inhibition of water, increased metabolic activity and the swelling of the cells. The main factors which play a role in the sprouting of seeds are water, oxygen and temperature. Water triggers the chemical process; oxygen is required for cellular respiration and temperature is required to influence the rate of metabolic activities. Prior to sprouting the grains were soaked in water at 1:2 ratio (grains:water) and the kernels absorbed enough water. The dormant seeds contain about 5 – 10% moisture and this increased during soaking. During this stage, the water update was through the micropyle. As the water was absorbed into the

Figure 3.2 Paddy rice seeds during sprouting (a) Unsprouted, (b) 24 h of sprouting, (c) 48 h of sprouting, (d) 72 h of sprouting, (e) 96 h of sprouting and (e) sprouted rice flour (SRF)

seed, the seed increased in size nd became turgid. The process of water absorption stimulated the increase in cellular respiration which resulted in the breakdown of starches to energy in the presence of oxygen. The absorption of water also activated the enzymes within the grains. These enzymes during sprouting broke down the polysaccharides (mainly starch) into oligosaccharides and monosaccharides, the fats into free fatty acids, and proteins into oligopeptides and free amino acids. The breakdown of starch resulted in the reduction of viscosity. Pressure was created during the swelling of cells within the seeds which caused the seed coat to lyse. The radicle emerged through the seed coat with the root facing downward and the stem facing upwards. The aim of sprouting rice grains was to stimulate the development and activity of hydrolytic enzymes (amylases, proteases and other endogenous hydrolytic enzymes) which are not active in non-germinated grains. As reported by Mella (2011) the germination usually lasts for 4-6 days and takes place between 20 and 30°C with the optimum temperature of 25 - 28°C. Ground sprouted rice grains added to either millet, sorghum or maize is believed to increase the crude protein, fats and carbohydrates by 33, 44 and 63%, respectively (Adelekan et al., 2013). Sprouted rice flour with high alpha amylase activity was produced after 96 h at 25°C.

3.4.2 Effect of fermentation time on the pH and total titratable acidity (TTA) of pearl millet slurry during fermentation

The changes in pH and total titratable acidity of pearl millet slurry (PMS) over the 36 h fermentation for the production of non-alcoholic pearl millet beverage (NAPMB) are shown in Figure 3.3. There was a significant ($p < 0.05$) change in pH during the fermentation cycle ranging from 6.37 ± 0.15 to 3.77 ± 0.01 in 36 h. The pH decreased as fermentation time elapsed due to the increase in population of lactic acid bacteria (LAB) which fermented glucose to lactic acid and CO_2. At the beginning of fermentation the lactic acid organisms were in lag phase (0 - 3 h) and as the fermentation time increased the organisms exponentially produced significant acid until 21 h followed by stationary phase where the amount of acid did not increase significantly (24 – 30 h). The decrease in pH could be due to the built up of hydrogen ions content when microorganisms breakdown starch. Meanwhile, the stationary phase could have been caused by exhaustion of nutrient and build-up of waste by LAB. Thereafter, the pH did not significantly change until 36 h. This could be caused by dying of parent cells as death phase set in. These results were in agreement with a report by Obadina *et al*. (2008) who reported a decrease

in pH during the fermentation of *Kunun-zaki* caused by the formation of organic acid from carbohydrates and other food nutrients.

The total titratable acidity (TTA) [expressed as % lactic acid] was 0.12 ± 0.01% at the start of fermentation and increased to 0.53 ± 0.03% at the end of fermentation (36 h). The TTA did not significant change between 0 and 3 h of fermentation. After 3 h, the TTA rapidly increased from start to 0.42 ± 0.01% in 18 h. Thereafter, there was no significant change for 6 h (18 – 24 h) then significantly increased ($p < 0.05$) for 3 h (24 - 27 h).

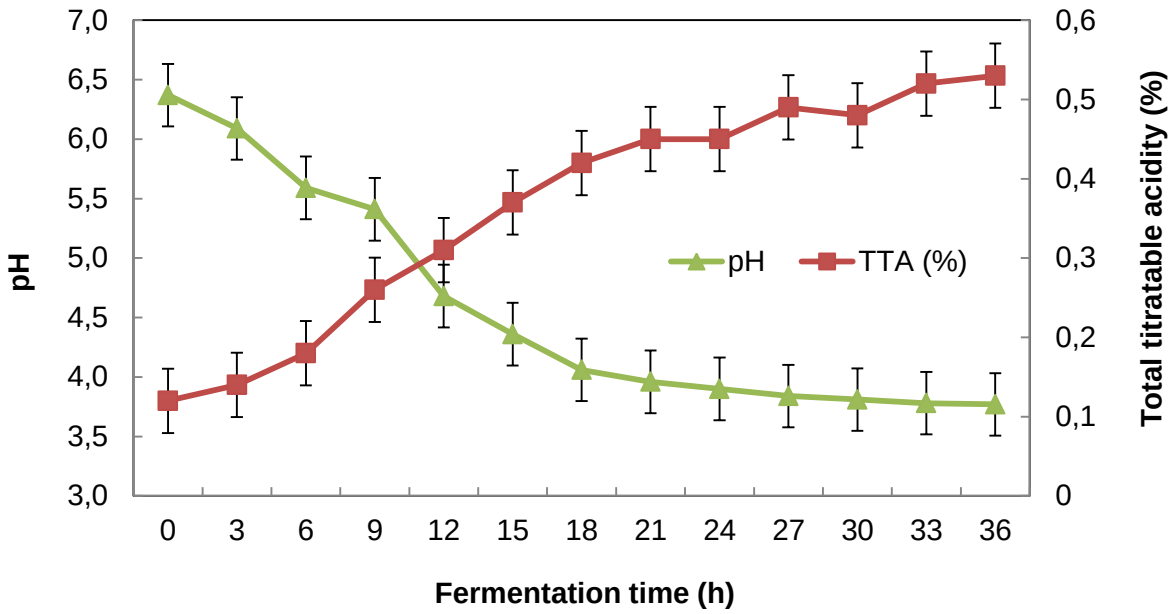

Figure 3.3 Changes in the pH and total titratable acidity of pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage. TTA – total titratable acidity

It remained significantly unchanged for another 3 h (27 – 30 h) followed by the gradual significant increase of TTA between 30 and 36 h. Overall, there was a significant ($p < 0.05$) change in TTA over the 36 h fermentation time. This could be attributed to the decrease in pH as the concentration of acid increased. The increase in LAB produced more lactic acid from fermentation of sugars. The increase in acidity could be the cause of a sweet-sour taste of non-alcoholic pearl millet beverage (NAPMB). This is in agreement with Zvauya *et al*. (1997) during the fermentation of *Masvusvu* and *Mangisi*.

In addition, after 18 h of fermentation there was no significant change in the pH and TTA of PMS. This is in agreement with the growth curve of LAB which reached the highest count after 18 h. Thus, the optimum fermentation time for the beverage could be

18 h at 37°C with the pH expected to be 4.06 ± 0.06. These results showed that the pH and TTA of cereal beverages decreases and increases, respectively during fermentation.

3.4.3 Effect of fermentation time on the soluble sugar content of pearl millet slurry during fermentation

The main soluble sugar identified in pearl millet slurry (PMS) was glucose which ranged from 0.54 ± 0.10 to 2.05 ± 0.03% during the 36 h fermentation period. Figure 3.4 shows a significant ($p < 0.05$) increase in glucose content during the 36 h of fermentation. Glucose could be from the starch found in pearl millet grains. Cereal grains are made up of 66 - 76% carbohydrates which consist of 55 - 70% starch, 1.5 - 8% arabinoxylans, 0.5 - 7%

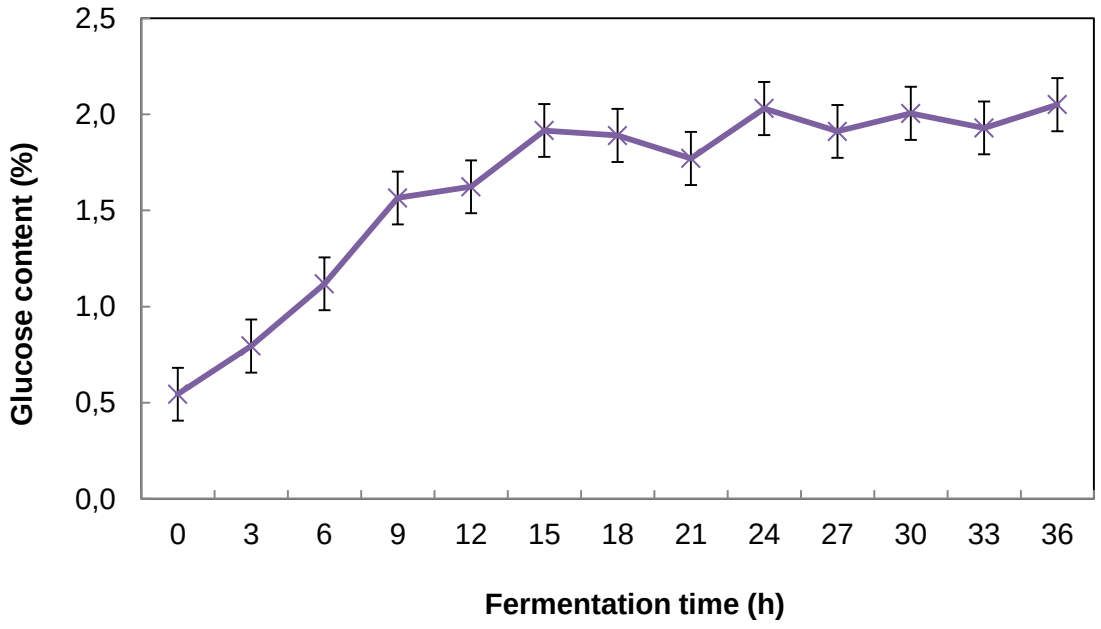

Figure 3.4 Effect of fermentation time on the glucose content in pearl millet slurry during the preparation of non-alcoholic pearl millet beverage (NAPMB)

β -glucans, 3% sugars, 2.5% cellulose and 1% glucofructans. Starch is the main constituent of the cereal grains and is found in the endosperm. It is made up of two water insoluble homoglucans called amylose (25 - 28%) and amylopectin (72 - 75%). Amylose is linear in structure with 500 - 6000 glucose units during polymerization while amylopectin is highly branched granular polysaccharides with 3×10^4 - 3×10^6 glucose units (Koehler & Wieser, 2013). The increase in glucose during PMS fermentation could be attributed to the decrease in starch caused by the action of alpha amylase (α-amylase) and beta-

amylase (β-amylase) acitivity. During fermentation enzymes hydrolyse starch to produce monomeric sugar glucose. Although there was an increase in glucose content from the onset of fermentation, the glucose did not significantly increase between 9 and 12 h; 15, 18 and 27 h; and 24, 30, 33 and 36 h. This could be due to the acidfication (low pH) of the beverage which terminates the activity of alpha-amylase and/or the termination of amylolytic enzymes inhibited by the build-up of tannins (Osman, 2011). The lower pH could have been caused by the lactic acid bacteria (LAB) converting the sugars from starch into mainly lactic acid and some traces of alcohol/acetic acid and/or CO_2. Tannins are natural polyphenols found in most cereal grains. They can act as antioxidants together with phytic acid and phenols (Pushparaj & Urooj, 2014). Similarly, Osman (2011) reported glucose as the main soluble sugar which gradually increased in the first 20 h during the fermentation of pearl millet flour during the production of *Lohoh* bread. Ibegbulem & Chikezie (2013) also identified 0.5% glucose during the fermentation of *Kunun-zaki*. Therefore, fermentation time has an effect on the glucose content of pearl millet slurry.

3.4.4 Effect of fermentation time on the viability of lactic acid bacteria and total microbes in pearl millet slurry during fermentation

The growth pattern of lactic acid bacteria (LAB) and total viable counts (TVC) in pearl millet slurry during fermentation for the production of non-alcoholic cereal beverage (NACB) are shown in Figure 3.5. The growth of LAB in pearl millet slurry significantly increased ($p < 0.05$) at 3, 15, 24, and 30 h and significantly decreased ($p < 0.05$) at 21 and 27 h during fermentation. The LAB counts were 7.04 ± 0.95 log cfu/ml at the onset of fermentation and decreased to 6.73 ± 0.46 log cfu/ml after 3 h. This period can be regarded as the apparent lag phase. During this phase a certain fraction of microorganisms are dividing (duplication) while a certain fraction is dying or not dividing (non-duplicating) due to the new environment they are introduced to. As a result of the non-duplicating bacteria exceeding the duplicating bacteria, these have led to the reduction in total cell count prior to exponential phase. The results are in agreement with those reported by Mwale (2014) during preparation of *Chibwantu*. This similar concept was explained by Yates & Smotzer (2007). The growth of LAB cells increased from 6.73 ± 0.46 log cfu/ml (3 h) to 7.74 ± 0.47 log cfu/ml (6 h) then decreased to 6.76 ± 0.02 log cfu/ml (9 h). Although most LAB tolerate low pH (acidophilic), certain strains of LAB may have been retarded (Menconi *et al*., 2014). A typical example as reported by Steinkraus

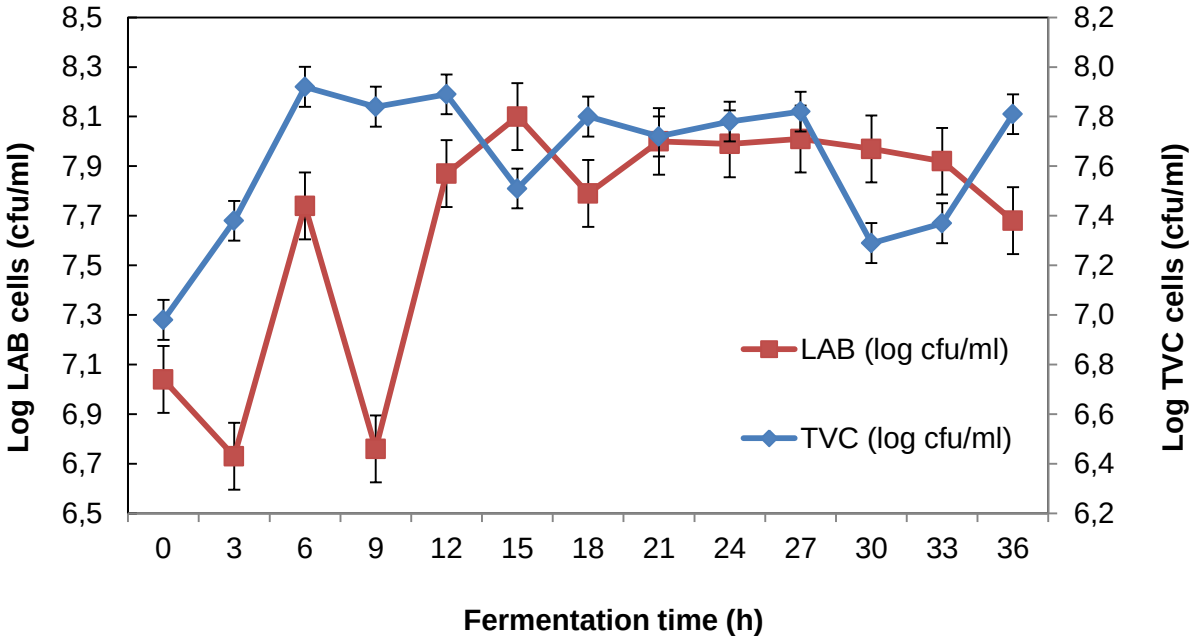

Figure 3.5 Bacterial growth curve in fermented pearl millet slurry during the preparation of non-alcoholic pearl millet beverage. LAB – lactic acid bacteria. TVC – total viable count

(1992) is the growth of *Leuconostoc* and lactic Streptococci which rapidly drops the pH of the beverage during fermentation to 4.0 – 4.5 and then retard their growth thus giving way to subsequent bacteria. Lactobacilli and Pediococci succeed *Leuconostoc* bacteria during fermentation and result in their growth retardation when the pH reaches 3.5 (Steinkraus, 1992). These results are similar to those reported for spontaneous fermentation of millet by Nwachukwu *et al*. (2010). This was followed by the exponential increase of LAB from 6.76 ± 0.02 (9 h) to 7.87 ± 0.34 log cfu/ml (12 h) in 3 h with a generation time of 16 min, and then accelerated to the highest count of 8.10 ± 1.01 log cfu/ml after 15 h. During this growth phase the cells surviving acidic environment could be growing and dividing at the maximum rate. There was a slight decrease in LAB to 7.79 ± 0.25 log cfu/ml (18 h) then the organisms remained stationary for 9 h (18 – 27 h) with an average of 7.95 ± 0.37 log cfu/ml. Since this is a batch type fermentation system the growth of organisms could have been limited by depletion of nutrients, build-up of inhibitory metabolites or end-product (lactic acid) and/or shortage of biological space. Thereafter, there was a death phase as the cells started to decrease from 7.97 ± 0.43 (30 h) to 7.68 ± 0.60 log cfu/ml (36 h). A similar trend of LAB growth was reported by Katongole (2008) during the fermentation of *Umqombothi*. The strength of the observed power of the test was 0.721 and can conclude

that the growth of LAB was between 7.04 ± 0.95 and 7.68 ± 0.60 log cfu/ml with the highest count of 8.10 ± 1.01 log cfu/ml during fermentation for the production of NACB.

There was a significant ($p < 0.05$) growth in total viable count (TVC) over 36 h fermentation period (Figure 3.5). The TVC cells accelerated from 6.98 ± 0.05 log cfu/ml at the onset to 7.38 ± 0.40 log cfu/ml in 3 h. This was followed by a significant ($p < 0.05$) exponential increase to 7.92 ± 0.14 log cfu/ml (6 h) with a generation time of 97 min. The lag phase was not visible during the growth of TVC. This may be caused by the rapid growth of mixed microbes which dominated the spontaneous fermentation of the pearl millet slurry. At this stage certain bacteria other than LAB could be growing at a faster rate. This could also have been caused by mixed microbes not taking long to adapt to the new environment. The growth went into stationary phase which lasted for 6 h (6 – 12 h) followed by the death phase. The numbers of cells during the death phase were reduced from 7.88 ± 0.19 to 7.51 ± 0.04 log cfu/ml (12 – 15 h). The decrease in cells may be due to the build-up of lactic acid caused by mostly LAB and other bacteria which may have unfavoured certain types of bacteria. This was followed by exponential increase in cells for 3 h (15 – 18 h) then a stationary phase followed for 9 h (18 – 27 h). There was a significant ($p < 0.05$) reduction in cells (death phase) after 27 h for 3 h followed by an acceleration phase for 3 h (30 h). Bacteria not tolerating low pH could have caused the decrease in TVC. The cells exponentially increased significantly ($p < 0.05$) again for 3 h until a cell count of 7.81 ± 0.17 log cfu/ml was noticed. The decrease and increase of cells could be mainly due to the unbalanced growth. During unbalanced growth the synthesis of cell components vary in relation to the other cells until a new balanced growth is observed. These usually happen when the environmental factors such as the lactic acid, anaerobic environment and depletion of nutrients and built-up of waste accumulating making the condition unfavourable and favourable for different organisms. The beverage was carried-out by spontaneous fermentation as a result different cells compete for survival, hence the rapid growth and decrease dominating the process (Achi & Ukwuru, 2015). The shift-up and shift-down could also be caused by the environmental conditions resulting in competition for survival among different species of LAB. In particular, the extended stationary phase could have led to cell reduction (death) with no new nutrients fed into the system. These results are similar to those reported by Zvauya *et al*. (1997) during the fermentation of *Masvusvu* and *Mangisi.*

There was a significant change in turbidity at 0, 21 and 33 h of the fermentation time. Table 3.1 shows the correlated optical density of total cells in pearl millet slurry during fermentation for the production of NAPMB. The correlated optical density (OD_{corr})

of LAB increased slowly over the 36 h fermentation period. Initial OD_{corr} was 6.71 ± 0.23 and after 36 h was 8.08 ± 1.25. This shows that the bacterial growth of LAB was slow. The lag phase was not visible which may be due to the higher number of cells in the beginning. Similarly, Jooyandeh (2013) reported a short lag phase (6 h) of viable cell count, optical density and cell biomass during the production of non-alcoholic plum beverage. The population went to exponential phase in 3 h from the onset of fermentation until 7.68 ± 1.69 OD_{corr} was reached. Thereafter, the number of cells decreased between 9 and 12 h followed by a slight increase in turbidity between 15 and 18 h. The sudden decrease may be attributed to the

Table 3.1 Optical density of total cells in pearl millet slurry during fermentation for the production of NAPMB

Fermentation time	OD_{corr} (600 nm)*
0	6.71 ± 0.23^{a}
3	$7.68 \pm 1.69^{a,b}$
6	$7.83 \pm 0.86^{a,b}$
9	$8.52 \pm 1.26^{a,b}$
12	$7.73 \pm 2.32^{a,b}$
15	$7.75 \pm 1.38^{a,b}$
18	$7.81 \pm 1.87^{a,b}$
21	7.03 ± 1.76^{a}
24	$8.34 \pm 1.87^{a,b}$
27	$8.58 \pm 0.83^{a,b}$
30	$9.27 \pm 1.27^{a,b}$
33	9.91 ± 0.87^{b}
36	$8.08 \pm 1.25^{a,b}$

*Values are mean ± standard deviation. Values with different superscripts in each row are significantly different ($p < 0.05$) from one another. NAPMB – non-alcoholic pearl millet beverage

acidic environment halting the growth of other bacteria which does not tolerate acidic condition. The slight increase in turbidity could be during the adaptation, recovery or size growth of acid tolerant organisms. The cell population increased after 24 h from 8.34 ± 1.87 to 9.27 ± 1.27 (30 h) followed by a significant increase to 9.91 ± 0.87 (33 h) then the

OD_{corr} decreased to 8.08 ± 1.25 (36 h). The increase could be due to the emergence of bacterial cells that tolerate acidic environment. The decrease of OD_{corr} could be caused by the death phase of bacteria, which occurs usually when conditions are unfavourable for most organisms. There was a possible built-up of toxins and/or waste which reached a threshold level. At this point, the growth of bacteria was not balanced due to the lack of growth requirements. The viable cells were lower than those dying. Therefore, pearl millet supports the growth of LAB during fermentation.

3.4.5 Lactic acid bacteria associated with pearl millet slurry

The isolates identified from pearl millet slurry (PMS) during fermentation over 36 h are shown in Table 3.2. Lactic acid bacteria (LAB) from the genera *Leuconostoc, Pediococcus* and *Enterococcus* were the main species involved in the fermentation of PMS. The *Leuconostoc mesenteroides ssp. Dextraicum* (Figure 3.6a).and *Leuconostoc pseudomesenteroides* were identified at the beginning of fermentation between the pH of 5.59 ± 0.09 (0 h) and 6.37 ± 0.15 (6 h). *Leuconostoc*'s presence at the beginning of fermentation may be attributed to their growth condition at pH 6.0 - 6.5. This is identical to the study by Schutte (2013) who identified *L. pseudomesenteroides* from *Oshashikwa*, a traditionally fermented milk in Namibia. The organisms were responsible for the initiation of lactic acid fermentation (Whitman, 2009). These heterolactic organisms produce carbon dioxide and organic acids which rapidly lower the pH of the beverage to 4.0 or 4.5 (Steinkraus, 1992) and inhibit the development of undesirable microorganisms. The carbon dioxide produced replaces the oxygen, making the environment anaerobic (Battock & Azam-Ali, 1998; Dimic, 2006) and suitable for the growth of subsequent organisms such as *Lactobacillus*. In addition, the anaerobic environment created by the CO_2 has preservative effect to the beverage since it inhibits the growth of unwanted contaminants bacteria (Achi & Ukwuru, 2015). As reported by Meslier *et al*. (2012), *L. pseudomesenteroide* is widely present in many fermented foods such as dairy, wine and beans while *L. mesenteroides* is associated with sauerkraut and pickled fermented products (Dimic, 2006). The organism produces dextrans and aromatic compounds (diacetyl, acetaldehyde, and acetoin) which could contribute to the taste and aromatic profile. These organisms were isolated by Doulgeraki *et al*. (2013) from fermented Greek table olive.

Table 3.2 Tentative lactic acid bacteria isolated at different times and pH during fermentation of pearl millet slurry for the preparation of NAPMB

pH and lactic acid bacteria	Fermentation time (h)												
	0	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**	**33**	**36**
pH*	6.37 ± 0.15	6.09 ± 0.13	5.59 ± 0.09	5.41 ± 0.07	4.68 ± 0.09	4.36 ± 0.17	4.06 ± 0.06	3.96 ± 0.03	3.9 ± 0.05	3.84 ± 0.06	3.81 ± 0.04	3.78 ± 0.03	3.77 ± 0.01
Leuconostoc mesenteroides ssp. dextranicum	x												
Leuconostoc pseudomesenteroides	x	x	x										
Pediococcus pentosaceus	x			x			x						x
Streptococcus thoraltensis;	x		x						x				
Enterococcus gallinarum					x	x		x	x		x		
Enterococcus casseliflavus		x				x							
Enterococcus faecium					x	x	x	x	x		x		
Enterococcus faecalis											x		
Enterococcus avium				x									
Enterococcus duran				x									

*Values are mean ± standard deviation. x – Indicates the time the bacteria was isolated. NAPMB – non-alcoholic pearl millet beverage

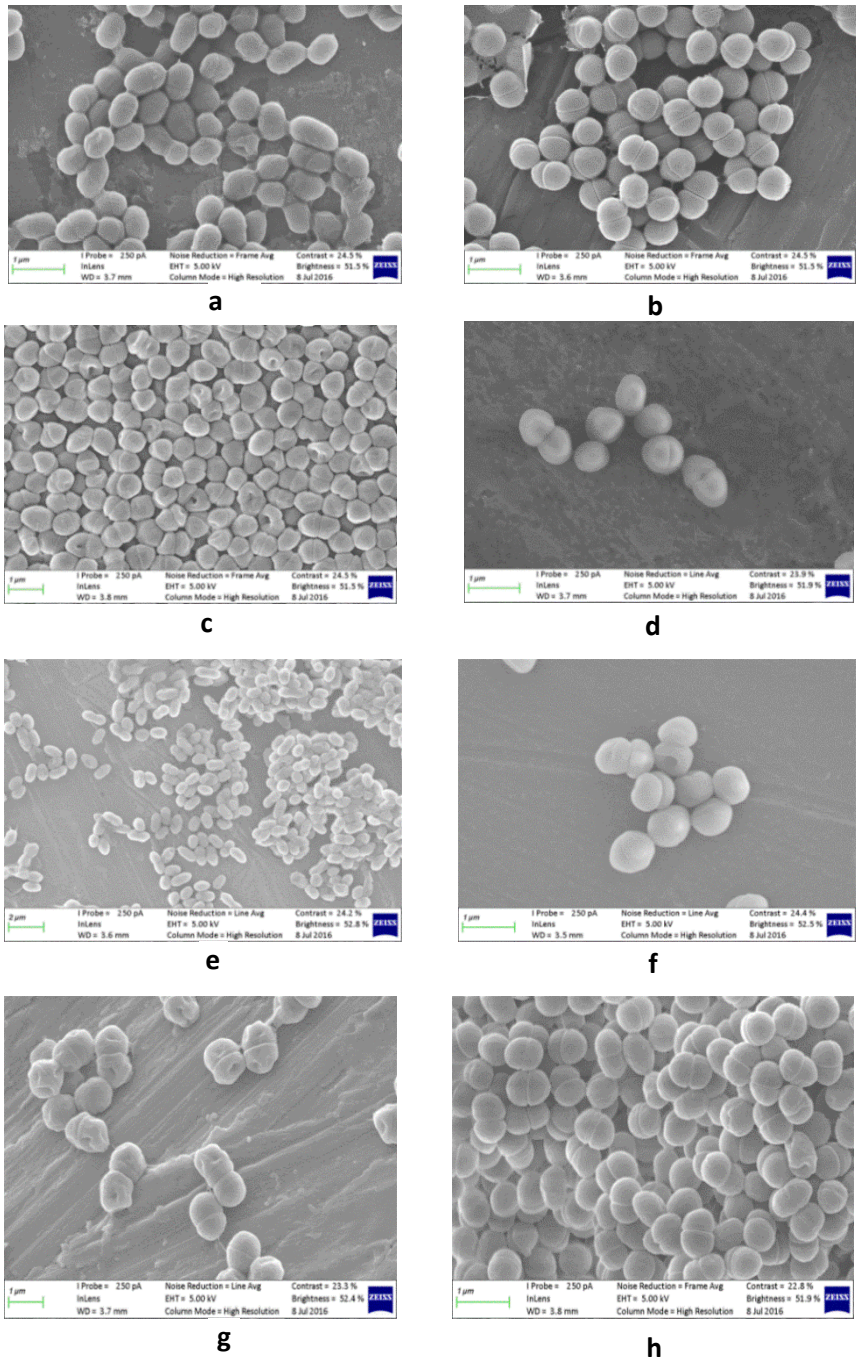

Figure 3.6 Scanning electron microscopy of bacterial cells isolated from pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage. Tentative identities: **(a)** *Leuconostoc mesenteroides ssp. Dextranicum* (1 µm), **(b)** *Pediococcus pentosaceus* (1 µm), **(c)** *Enterococcus durans* (1 µm), **(d)** *Streptococcus thoraltensis* (1 µm), **(e)** *Enterococcus gallinarum* (2 µm), **(f)** *Enterococcus casseliflavus* (1 µm), **(g)** *Enterococcus faecium and* **(h)** *Enterococcus avium* (1 µm)

Pediococcus pentosaceus shown in Figure 3.6b was isolated at 0, 9, 18 and 36 h of fermentation, similar to the report by Abegaz (2007) and Nuraida (2015). The genus *Pediococcus* belongs to the family *Lactobacillaceae* in the order *Lactobacillales* growing at optimum pH of 4.5 - 8.0 (Batt & Tortorello, 2014). They have the ability to produce bacteriocins (antimicrobial agent) which are used as a food preservative (Batt & Tortorello, 2014). The bacteriocins produced inhibit the growth of Gram positive bacteria since they attack the cytoplasmic membranes of the cell of which is protected by the polysaccharide protective layer in Gram negative cells (Achi & Ukwuru, 2015). The organisms were also responsible for producing acid during fermentation. The Pediococci depress the pH to 3.5 before they halt their own growth (Steinkraus, 1992).

Streptococcus thoraltensis was also present after 6 h of fermentation (Figure 3.6d). The presence of *S. thoraltensis* could be through contamination of pearl millet grains and/or utensils. The organism was isolated from animal intestinal tracts of swine (Facklam, 2002).

A number of enterococcus bacteria (Table 3.2) were isolated throughout the fermentation at different times (3 – 30 h) between pH 3.81 and 6.09. They became active between 12 and 30 h and are known to grow well between pH 4 and 9.6 (Gimenez-Perreira, 2005). The organisms are responsible for the development of flavours due to their glycolytic, proteolytic and lipolytic activities. They have probiotic activities and have the potential to be used as bio-preservatives. In general enterococci organisms are ubiquitous and are found in the environment and gastrointestinal tract of healthy animals and humans (Gimenez-Pereira, 2005). These organisms are used as starter cultures in the fermentation of food since they create unique sensory properties (Gimenez-Pereira, 2005); and contribute to texture and safety (Araujo & de Luces Ferreira, 2013). *Enterococcus casseliflavus* shown in Figure 3.6f and *Enterococcus gallinarum* shown in Figure 3.6c were identified after 3 and 15 h. *E. casseliflavus* have been isolated from olive brines and traditional fermented food and used as starter culture (de Castro *et al.,* 2002; Mwale, 2014; Oladipo *et al*. 2015). *E. gallinarum* was isolated between 12 and 30 h at pH 3.81 to 4.68, similar to Oladino *et al*. (2015) who identified the organisms in Nigerian traditional fermented foods. They have lipolysis, proteolysis, bile tolerating and low pH tolerating properties. They have hydrophobic properties and produce bacteriocins which will inhibit food pathogens and spoilage microorganisms (Oladipo *et al.* 2015). *E. faecium* (Figure 3.6g) was detected at 12, 15, 18 and 21 h while *E. faecalis* was detected after 30 h.

E. faecium and *E. faecalis* are reported to be probiotics but their original source may be through contamination. Shutte (2013) also reported isolation of *E. faecium* from traditionally fermented milk *Omashikwa*. However, as reported by Gimenez-Pereira (2005) they are suspected to be pathogenic to humans and are resistant to antibiotics.

The biochemical properties of presumptive lactic acid bacteria (LAB) isolates are shown in Table 3.3. All the isolates were Gram positive, catalase-negative and do not produce gas from glucose (hot-loop test negative). All cells were cocci and cocci-oval in morphology and showed no grow at 4ºC. At 45ºC there was no growth of the isolates except *E. faecium.* The inability of all the LAB isolates to grow at 4°C could demonstrate increased glycolytic activity which could lead to the increased production of lactic acid (Menconi *et al*., 2014). However, the same report by Menconi *et al*. (2014) reported that the growth of *Lactococcus* at low temperature resulted in reduced production of lactic acid due to the reduced glycolytic activity. The inability to grow at high temperature could mean that the LAB strain have a high growth rate and lactic acid production. Their inability to grow at 45°C are in disagreement with Menconi *et al*. (2014)'s report. The differences could be due to the period of incubation which was 2 – 4 h in Menconi *et al*. (2014)'s report whereas this study incubated for 48 h. The isolates grew at 10ºC and 6.5% NaCl concentration except *E. avium.* The growth of all LAB isolates except *E. avium* in 6.5% salt concentration indicated that the LAB strain could be used as commercial starter culture. During the commercial production of lactic acid by LAB strain, alkali could be added to increase the pH and reduce excess decrease in pH (Menconi *et al*., 2014). However, the same report by (Menconi *et al*., 2014) mentioned that LAB strains grown in the presence of salt concentration could lead to the loss of turgor pressure leading to an effect on the physiology, enzyme activity, water activity and metabolism of the cell. These physiological properties could be used to confirm the ability of the LAB isolates to be used as starter cultures. The LAB of importance during fermentation of pearl millet slurry were from the genera *Leuconostoc, Pediococcus* and *Enterococcus.*

3.4.6 Pearson correlation between the total titratable acidity (TTA), pH, total viable count (TVC), yeast and mould (YM) and correlated optical density (OD_{corr})

Table 3.4 summarises the Pearson correlation between TTA, pH, TVC, YM and OD_{corr} during the fermentation of pearl millet slurry for the production of non-alcolic pearl millet beverage (NAPMB). There was a very strong, negative linear relationship between TTA and pH of the beverage during fermentation (r = -0.975, $p < 0.05$). Meanwhile, the TTA

.

Table 3.3 Physiological properties of tentative lactic acid bacteria isolated from pearl millet slurry during fermentation for the production of NAPMB

Lactic acid bacteria	Gram reaction	Catalase test	Morphology	Hot-loop test	4°C	10°C	45°C	6.5% NaCl
L. mesenteroides ssp. dextranicum	+	-	Cocci, groups forming chains	-	-	+	-	+
L. pseudomesenteroides	+	-	Cocci, groups forming chains	-	-	+	-	+
P. pentosaceus	+	-	Cocci, groups forming chains	-	-	+	-	+
S. thoraltensis;	+	-	Cocci, strepto forming chains	-	-	+	-	+
E. gallinarum	+	-	Cocci, groups forming chains	-	-	+	-	+
E. casseliflavus	+	-	Cocci, single, pairs, tetracocci forming small chains	-	-	-	-	-
E. faecium	+	-	Cocci, groups forming chains	-	-	+	+	+
E. faecalis	+	-	Cocci, groups forming chains	-	-	+	-	+
E. avium	+	-	Cocci, single, pairs, groups forming chains	-	-	-	-	-
E. duran	+	-	Cocci, groups forming chains	-	-	+	-	+

NAPMB – non-alcoholic pearl millet beverage

Table 3.4 Pearson correlation of pH, TTA, LAB, YM and OD_{corr} of NAPMB[1,2]

	TTA (%)	**pH**	**LAB (cfu/ml)**	**TVC (cfu/ml)**	**YM (cfu/ml)**	**OD_{corr}**
Total titratable acidity (TTA) [%]						
pH	-0.975**					
Lactic acid bacteria (LAB) [cfu/ml]	0.440**	-535**				
Total viable count (TVC) [cfu/ml]	0.225	-0.272	0.173			
Yeast and mould (YM) [cfu/ml]	-0.085	0.011	0.278	0.341		
Correlated optical density (OD_{corr})	0.383*	-0.305	-0.81	-0.110	-0.713**	

1 **correlation significant at p = 0.01. *correlation significant at p = 0.05. NAPMB – non-alcoholic pearl millet beverage

2 The strength of the absolute correlation value: 0.00 - 0.19 = very weak, 0.20 - 0.39 = weak, 0.40 - 0.59 = moderate, 0.60 - 0.79 = strong and 0.80 - 1.0 = very strong (Evans,1996; Beldjazia & Alatou, 2016)

had a moderate positive relationship between lactic acid bacteria (LAB) count (r = 0.440, $p < 0.05$). The pH had negative moderate relationship with the LAB count (r = -0.535, $p < 0.05$). The LAB count had a positive weak and very weak relationship with the TVC and YM, respectively. A very weak negative relationship was also observed between the LAB and OD. Meanwhile the TVC had a positive weak and negative very weak relationship with the YM and OD respectively. YM had a strong negative relationship with the OD (r = -0.713, $p < 0.05$).

In summary a moderate-strong linear relationship was between the TTA and pH, LAB and pH and YM and OD_{corr}. These results further indicated that during succession fermentation of glucose by LAB to lactic acid, the pH dropped due to the built up of hydrogen ion. The decrease in pH thus resulted in the increase in TTA. The OD of the beverage was also inversely related to the growth of YM. This could be because the increase in bacterial load was mainly from the growth of LAB in succession but not the YM.

3.4.7 Inherent structural grouping on the basis of fermentation time using principal component analysis (PCA)

The pH, total titratable acidity (TTA), correlated optical density (OD_{corr}), lactic acid bacteria (LAB), yeast and mould (YM) and total viable count (TVC) in pearl millet slurry during fermentation for the production of non-alcoholic pearl millet beverage (NAPMB) were subjected to principal components analysis (PCA). Figure 3.7 shows the scores (fermentation time) and loadings (pH, TTA, OD_{corr}, LAB and TVC) during 36 h fermentation. The variations in the data could be explained by two principal components (PC1 and PC2). The cumulative variability was 74% with much variation (54%) explained by PC1 and 20% by PC2. PC1 was highly correlated to LAB and TTA at 36 h of fermentation. Furthermore, PC2 was highly correlated to YM at 30 and 33 h of fermentation. This indicated that the variations in the quality characteristics of NAPMB could mainly be explained by high TTA, LAB, YM and OD between 30 and 36 h of fermentation. During fermentation the LAB breakdown sugars into lactic acid which decreased the pH. Therefore, in future work the TTA could be used to monitor the growth of LAB during fermentation. YM also plays a role during fermentation and could be used as an indicator. OD could be used to monitor the increase in microorganisms during fermentation.

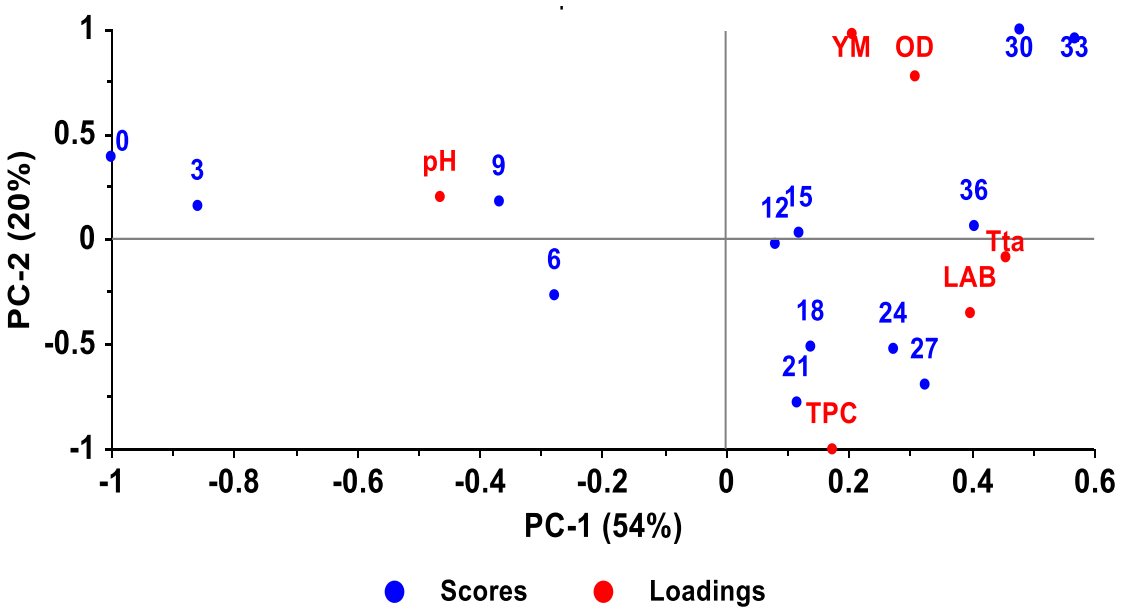

Figure 3.7 Principal component analysis (PCA) score plot for non-alcoholic pearl millet beverage

3.5 Conclusion

Natural fermentation of pearl millet slurry was dominated by lactic acid bacteria (LAB) and contaminants and their survival was in succession due to the increase in lactic acid. *L. pseudomesenteroides, L. mesenteroides ssp. dextranicum, E. gallinarum* and *P. penotosaceus* were the main fermenting LAB. Optimal non-alcoholic pearl millet beverage (NAPMB) could be produced by fermenting the slurry for 18 h at 37°C with expected pH of 4.06 ± 0.06. Principal component analysis (PCA) indicated that two variables are important in monitoring chance fermentation. Lactic acid bacteria (LAB) are associated with total titratable acidity (TTA) which could be used as an indicator for the survival of LAB. Yeast and mould (YM) and correlated optical density (OD_{corr}) were also good indicators to monitor the progress of chance fermentation. The isolation and identification of microorganisms from chance fermentation was achieved.

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CHAPTER FOUR

PRODUCTION OF NON-ALCOHOLIC PEARL MILLET BEVERAGE USING PURIFIED CULTURES OF LACTIC ACID BACTERIA

Abstract

The aim of this investigation was to assess the effect of using purified cultures of lactic acid bacteria (LAB) on the fermentation parameters (pH, total titratable acidity and viscosity) with a view to produce a stable and acceptable non-alcoholic pearl millet beverage. Pearl millet extract (PME) was produced by hydrating pearl millet flour (PMF) with water (1:10, PMF:Water). To the mixture, 15% of sprouted rice flour (SRF) and 10% ground ginger were added. Carrageenan (0.03, 0.05 & 0.6%), pectin (0.6%) and sodium alginate (0.3%) were used to stabilize the extract. The stability was determined using Turbiscan MA 2000. Turbiscan profiles for each stabilisers indicated that sodium alginate and pectin at 0.3% and 0.6%, respectively, produced a stable PME. Pectin was selected for producing a stable PME. PME was pasteurized, cooled to 40°C and inoculated with *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* at 0.05 and 0.025%, respectively, and in combination. The extract was then fermented for 18 h at 37^0C. The pH, total titratable acidity (TTA) and viscosity of NAPMB were determined. In addition, sensory evaluation of the beverage was conducted. Generalized linear model was used to determine the effect of the purified cultures on the pH, TTA and viscosity of the beverage. SPSS Monte Carlo simulation was used to model the influence of the LAB on the pH, TTA and viscosity of the beverage for 1000 cases. The pH of the beverage ranged between pH 3.32 and pH 3.90. *L. mesenteroides*, *P*. *pentosaceus*, *E. gallinarum,* the interaction between *L. mesenteroides* and *P*. *pentosaceus* and the interaction between *L. mesentoroides* and *E. gallinarum* had a significant ($p < 0.05$) effect on the pH of NAPMB. The TTA of the beverage ranged from 0.50 to 0.72%. All cultures had a significant ($p < 0.05$) influence on the TTA of the beverage with the exception of the interaction between *L. mesenteroides* and *E*. *gallinarum* ($p = 0.102$). The viscosity ranged from -88.00 to 11.74 mPa.s. All LAB cultures had a significant influence on the viscosity of the beverage. However, Monte Carlo simulation showed that *E. gallinarum* caused an increase in the pH and a decrease in the TTA of the beverage, an undesirable effect in beverage fermentation. During fermentation, the pH of the beverage is desired to decrease while the TTA increases, hence *E. gallinarum* was removed. The interaction between *P. pentosaceus* and *L. mesentoroides* at 0.025% and 0.05%, respectively produced an acceptable NAPMB.

4.1 Introduction

Cereals play an important role in health and disease prevention in almost every country. This has led to the growing demand in the development of a variety of cereal-based foods. The development of cereal-based probiotic foods has the ability to increase market margin for dairy-free food products. Dairy-free foods are in demand due to the growing number of vegetarians; increase in lactose intolerant individuals; and many dairy products that have high cholesterol (Yu & Bogue, 2013). Fermentation of food has been used in the production of many probiotic foods. The combination of probiotics and cereal can produce functional foods and beverages under controlled conditions with defined consistent characteristics and health promoting properties (Yu & Bogue, 2013).

There are different forms of cereal beverages produced and consumed in Africa such as *Kunu, Ogi* (Gaffa *et al*., 2002), *Uji, Kivunde* and *Motoho* (Ramaite, 2004). Many of these beverages are produced at household level through chance fermentation and few of them are at commercial level. *Mahewu* and *Chibuku* are examples of such industrialized cereal beverages in South Africa and Zimbabwe, respectively (Gadaga *et al*., 1999). Fermentation of beverages, especially lactic acid fermentation, is an important indigenous technology extensively practiced by many people in Africa. This process is cheaper to run and prevent spoilage of food and food-borne diseases especially in areas prone to rapid food deterioration (Sahlin, 1999; Lei, 2006; Katongole, 2008).

Foods and beverages produced through fermentation play an important role in socio-economic and protein requirements of people in many developing countries (Sahlin, 1999; Achi, 2005). However, these foods and beverages are made under unhygienic environment which leads to low yield, poor and inconsistent quality (Achi, 2005). The beverages are produced through traditional fermentation which depends on environmental microorganisms. To control these, isolated and purified microorganisms involved in the fermentation could be used in order to improve the efficiency of the fermentation process, quality and safety of the end-product. In Chapter 3, the lactic acid bacteria (LAB) were isolated and purified and it is of interest to investigate their effectiveness under controlled conditions. The production of beverages under controlled conditions using purified cultures is believed to result in beverages of high quality, which are consistently safe for consumption (Schutte, 2013).

The aim of this investigation was to assess the effect of purified cultures of LAB on some physical properties including viscosity of the beverage with a view to producing a stable non-alcoholic pearl millet beverage.

4.2 Materials and Methods

4.2.1 Sources of materials and equipment

Pearl millet and rice grains were purchased from Agricol in Brackenfell, Cape Town. Ground ginger was purchased from Deli Spices, Cape Town.

Perten 3100 laboratory mill, water-bath (Ecobat 207), cabinet oven, Hanna Edge pH meter (HI - 11310), Colloid mill, Silverson L4RT homogenizer and Geiger & Klotzbucher cabinet oven were provided by the Department of Food Science and Technology, Cape Peninsula University of Technology. Turbiscan MA 2000 (Formulation, Toulouse, France) was provided by the Department of Chemical Engineering, Cape Peninsula University of Technology. Rheolab QC (Aanton Paar) was provided by Agrifood Technology Station, Cape Peninsula University of Technology.

4.2.2 Production of pearl millet extract (PME) and effect of hydrocolloid on the stability of PME

Pearl millet flour (PMF) and sprouted rice flour (SRF) were prepared as described in Chapter 3, Section 3.2.2 and 3.2.3. PMF was mixed with cold tap water in the ratio of 1:10 (PMF:Water). The slurry was then hand mixed with 10% ground ginger and 15% SRF. The slurry was hand mixed with a plastic spoon for 1 min and further homogenized for 15 min at 6200 rpm using Silverson L4RT homogenizer. The slurry was left to hydrate for 3 h at 25°C in a 25 L bucket and the supernatant decanted carefully into a separate 25 L bucket. The sediments were further mixed with 1000 ml of water and left to stand for 1 h. The supernatant was decanted into the previous 25 L bucket and pooled together. The fluid was then sieved using sterilized cheese cloth followed by 5 µm filter bag using a filtration system, and finally sieved again with a finer sterile cheese cloth. The resulting fluid was then blended using colloid mill for 30 min with clearance adjusted to minimum (about 0.0508 mm). Figure 4.1 depicts the flow diagram for the production of pearl millet extract (PME). Furthermore, an experiment was conducted using lecithin (0.1 – 0.2%), carrageenan (0.03- 0.6%), pectin (0.6%), sodium alginate {0.1%) and 9 different combinations were obtained. To estimate the effect of hydrocolloids on the stability of NAPMB, Turbiscan MA 2000 was used.

4.2.3 Determination of the stability of pearl millet extracts (PME)

The stability of the pearl millet extract (PME) was measured using Turbiscan MA 2000 (Formulation, Toulouse, France). Aliquot samples of PME (9 ml) with different variations of stabilisers at room temperature were separately poured into the cylindrical sample tube

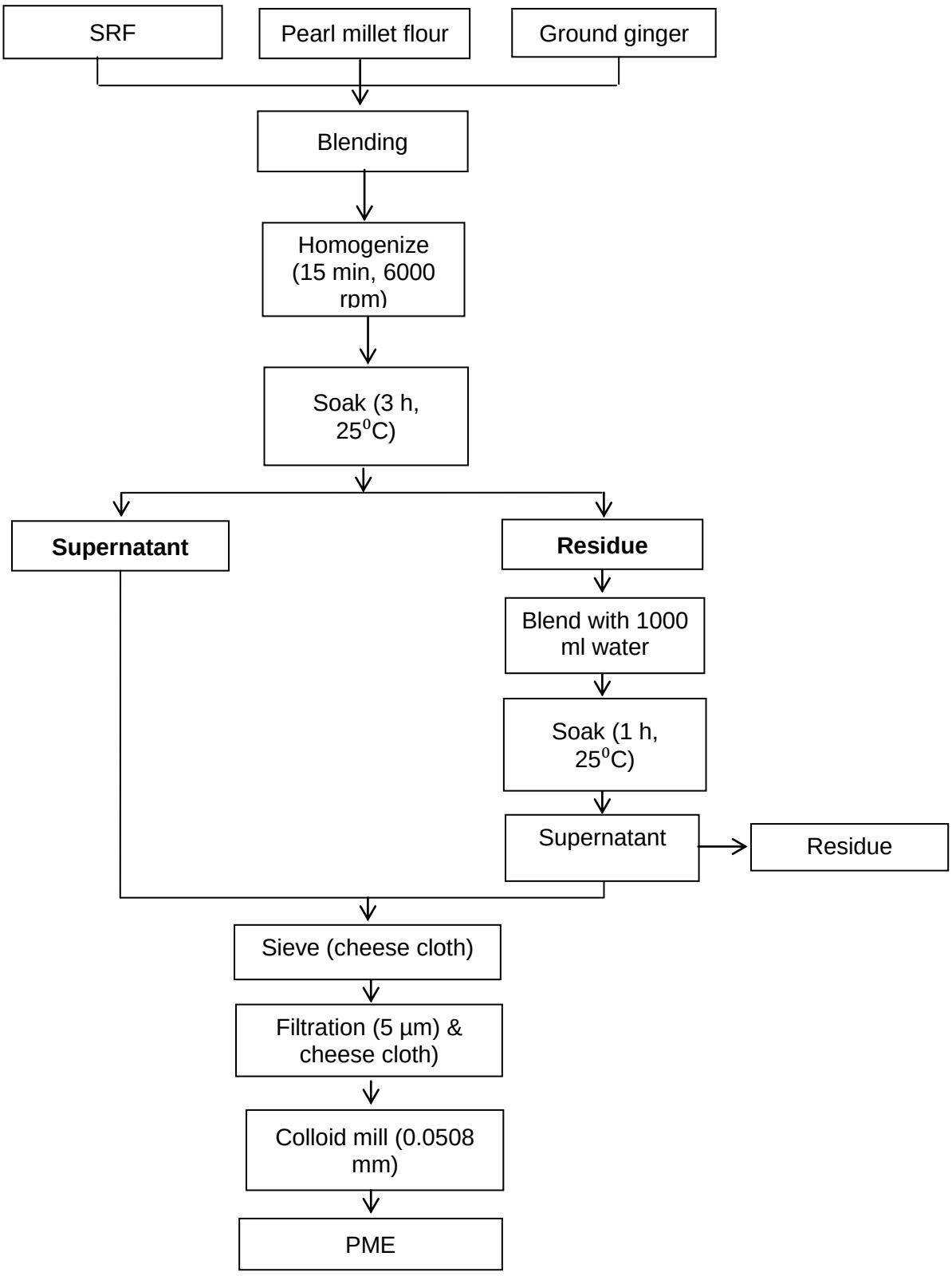


Figure 4.1 Flow diagram indicating the steps used in the production of pearl millet extract (PME). SRF – sprouted rice flour

(65 mm length) and inserted into the optical scanning analyzer. The samples were scanned following the method described by Adeyi (2014), Hardy (2016) and Maphosa (2016). Turbiscan detection head moves along the vertical sample tube length from the bottom to the top while multiple near-infra-red light (860 nm) were scattered through the sample over 40 min at 5 min intervals. The lights reflected backwards (backscattering flux) and the lights that passes through (transmission flux) the sample were measured and backscattering (BS) flux curves generated as a function of sample height in relation to the instrument internal standard.

In addition, the stability of the beverage was also determined by measuring the settled particle height in a 250 ml Schott bottle. This procedure depends on the transition time of the particles to the bottom of the glass bottles. The Schott bottles were filled with 200 g or 100 g PME and kept at 4^0C. After 48 h of storage, the heights of the settled particles were measured from the bottom of the bottle. The stability was expressed as the ratio of particles at the bottom of the bottle to the height of beverage in the bottle expressed in percentage (Equation 4.1). The zero percentage indicates that the product is stable while the high percentage stability (100.00%) represents an unstable product.

$$\text{stability (\%)} = \frac{b}{B} \times 100 \qquad 4.1$$

Where b = Settled particle height (mm) and B = Height of beverage (mm).

4.2.4 Production of stable pearl millet extracts (PME)

Pearl millet extract (1000 ml) was weighed into a 5 L plastic beaker. The dry ingredients [pectin (0.6%), sodium citrate (0.1%) and white sugar (5%)] were mixed separately using a plastic spoon in a plastic bowl. Sunflower lecithin paste (0.1%) was added to the pearl millet extracts (PME) and blended using a spoon. PME was then blended at 6600 rpm for 5 min using a Silverson L4RT homogenizer while slowly adding the dry ingredients and blended for about 1 min using a plastic spoon and further blended using Silverson L4RT homogenizer for 2 min at 6600 rpm. Aliquots (250 ml) of PME in 250 ml Schott bottles were pasteurized in a water bath (Ecobath 207) at 98^0C for 30 min shaking at 40 rpm. The extract was then chilled at 4^0C until use.

4.2.5 Experimental design for the production of non-alcoholic pearl millet beverage (NAPMB) using purified cultures of lactic acid bacteria

A three-level augmented factorial design for the cultures (*L. mesenteroides P. pentosaceus and E. gallinarum*) each at two level (05 and 0.1%) with three center points was used to determine the optimal non-alcoholic pearl millet beverage (NAPMB). Table 4.1 shows the coded independent variables and their levels. The design was randomized

Table 4.1 A full factorial design showing the independent variable and their levels[1] in for optimization of pearl millet beverage

Run	X_1	X_2	X_3
1	1	1	-1
2	1	1	1
3	-1	-1	-1
4	-1	-1	1
5	-1	1	-1
6	0	0	0
7	0	0	0
8	-1	-1	-1
9	1	1	-1
10	1	-1	-1
11	-1	1	1
12	1	1	1
13	-1	1	1
14	1	-1	-1
15	-1	-1	1
16	0	0	0
17	1	-1	1
18	1	-1	1
19	-1	1	-1

[1]Coded variables: -1, 0 and +1 equates to 0.05, 0.075 and 0.10%, respectively, and X_1, X_2 and X_3 equates to *L. mesenteroides P. pentosaceus* and *E. gallinarum,* respectively.

and run in duplicate. Generalized linear model was used to determine the effect of the purified cultures of lactic acid bacteria (LAB) on the pH, total titratable acidity (TTA) and viscosity of the beverage with a view to producing a stable NAPMB. SPSS Monte Carlo simulation was used to model the influence of the LAB on the pH, TTA and viscosity for 1000 cases.

4.2.6 Generalized linear model used to model the effect of each main and interactive probiotic cultures on the pH, total titratable acidity (TTA) and viscosity of the beverage

Generalized linear model (SPSS GENLIN) was used to estimate the effect of each of the main factors (*L. mesenteroides*, *P. pentosaceus* and *E. gallinarum*) and their interaction on the pH, total titratable acidity (TTA) and viscosity. The multivariate linear regression model is given in Equation 4.2.

$$Y = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3 + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{23}X_2X_3 \quad 4.2$$

Where the notation Y represents the estimated parameter response (pH, lactic acid or viscosity). $ß_0$ represent the overall mean (intercept), β_1, β_2 and β_3 are the main coefficient effect for *L. mesenteroides, P. pentosaceus* and *E. gallinarum,* respectively. β_{12}, β_{13} and β_{23} are the interactive coefficient effect for the main factors. X_1, X_2 and X_3 represent independent factors, *L. mesenteroides, P. pentosaceus* and *E. gallinarum,* respectively.

4.2.7 The effect of isolated pure lactic acid bacteria (LAB) on the pH, total titratable acidity (TTA) and viscosity of the beverage

A two-level factorial design for *L. mesenteroides* and *P. pentosaceus* each at two levels (0.05 and 0.10%) augmented with centre point was used to evaluate their effects (individual effects) and interactive effects on the acceptability (bench top sensory), pH, total titratable acidity and viscosity of the beverage. The experimental design was run randomly and the taste of the beverage was not accepted. Thereafter, *L. mesenteroides* and *P. pentosaceus* were used in combination at 0.05% each and in combination at 0.05 and 0.025%, respectively. A bench top sensory was used to evaluate the taste of the beverage. The interactive effect of *P. pentosaceus* and *L. mesenteroides* at 0.025% and 0.05%, respectively was accepted.

4.2.8 Production of non-alcoholic pearl millet beverage (NAPMB)

Pearl millet extract (1000 ml) was weighed into a 5 L plastic beaker. The dry ingredients [pectin (0.6%), sodium citrate (0.1%) and white sugar (5%)] were mixed separately using a plastic spoon in a plastic bowl. Sunflower lecithin paste (0.1%) was added to the PME and blended using a spoon. PME was then blended at 6600 rpm for 5 min using a Silverson L4RT homogenizer while slowly adding the dry ingredients. PME was blended for about 1 min using a plastic spoon and further blended using Silverson L4RT homogenizer for 2 min at 6600 rpm. PME was pasteurised in a pot at 85^0C for 15 min and hot filled into 100 ml Schott bottles. The bottles were rapidly cooled to 25^0C using ice blocks and tap water. The extract was then aseptically inoculated with *L. mesenteroides* (0.05%) and *P. pentosaceus* (0.025%) combined. The extract was then fermented for 18 h at 37ºC. The resulting beverage was then chilled at 4^0C until use. Figure 4.2 shows the production of optimal non-alcoholic pearl millet beverage.

4.2.9 Determination of the pH and lactic acid production in pearl millet beverage

The pH of pearl millet slurry during fermentation for the preparation of non-alcoholic pearl millet beverage (NAPMB) [10 ml] was measured in triplicates using Hanna Edge (HI - 11310) glass electrode pH meter standardized with pH buffer solution of 4, 7 and 10.

The total titratable acidity (TTA) of pearl millet slurry during fermentation for the preparation of NAPMB was determined in triplicates by titrating 10 ml of the beverage with 0.1N NaOH using phenolphthalein as indicator until a light pink colour appears. The TTA was expressed as percentage lactic acid (AOAC, 1980). Equation 4.3 was used to calculate the percentage acidity, with each 0.1M NaOH equivalent to 90.08 mg lactic acid.

$$\text{TTA}\,(\%\ \text{lactic acid}) = \frac{\text{ml NaOH x N NaOH x M.E}}{\text{volume of sample used x 1000}} \times 100 \qquad 4.3$$

Where, ml NaOH = volume of NaOH (ml), N NaOH = molarity of NaOH, M.E = the equivalent factor of lactic acid being 90.08 mg, 1000 = factor used to convert the M.E which is normally in mg to grams, and 100 used to express the lactic acid concentration in percentage.

4.2.10 Determination of the viscosity of non-alcoholic pearl millet beverage

The change in viscosity of pearl millet beverage over time was determined using Rheolab QC (Aanton Paar) with temperature device C-PTD 180/AIR/QC and measuring system

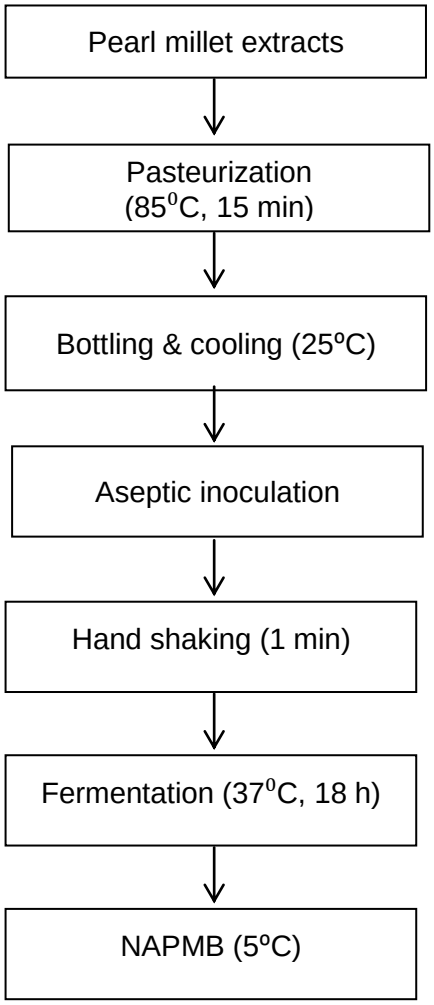


Figure 4.2 Flow diagram for the production of non-alcoholic pearl millet beverage (NAPMB)

CC27. The beverage (18 ml) was poured into an upward projected sample cup and analyzed following the manufacturer's instruction at 5^{0}C and 22^{0}C over 5 min. In all runs, the shear stress (*τ)* was set at 20 Pascal. The average of the triplicates was used.

4.2.11 Monte Carlo simulation of the pH, total titratable acidity (TTA) and viscosity produced using pure cultures

Monte Carlo simulation was used to estimate the effect of each main effect (individual effect) and their interaction effects on the pH, total titratable acidity (TTA) and viscosity of the beverage assuming a uniform distribution for 1000 cases. The model used was SPSS GENLIN, analysis type was 3 Walt, distribution was normal and CI level equals 95.

4.2.12 Data analysis

The results reported are mean of three independent trials. Multivariate analysis of variance (MANOVA) was used to determine mean difference between treatments at p = 0.05. Duncan's multiple range tests was used to separate means where differences exist using IBM SPSS ver 23 (IBM, 2015). Principal component analysis (PCA) was used to summarise and uncover any patterns in the fermentation data set by reducing the complexity of the data.

4.3 Results and Discussion

4.3.1 Effect of stabilizers on the stability of pearl millet extract

Turbiscan profiles of different pearl millet extract (PME) with different stabilisers are shown in Figure 4.3. The backscattering (BS) depends on three main parameters, namely, the particle size, the volume fraction and the relative refractive index between the dispersed and continuous phase (Turbiscan, 2009). Thus, if any of these parameters changes due to particle size the sample is said to flocculate or coalescence; or changes by local variation of the volume fraction then the sample is defined to be creaming or sedimentation. If a product is stable the BS flux profiles overlay on one curve while in unstable samples the profiles varies (Turbiscan, 2009). Figure 4.3a shows the control sample with no added stabilisers. The profiles clearly depicts sedimentation of particles in PME over 40 min. At the beginning of the scan there were particles settling at the bottom of the sample tube and that increased with time. Carrageenan (0.03 – 0.05%), lecithin (0.1 – 0.2%) and disodium phosphate (0.1%) at different factorial levels did not stabilize the PME since the profiles did not overlay each other (Figure 4.3a – 4.3e). These results were in agreement with the ratio of settled particles to the total PME were the stability ranged from 23.33 to 33.85% (Table 4.2). However, the extract was stable with slight sedimentation after carrageenan was increased to 0.3% with lecithin and disodium phosphate at 0.1% each (Figure 4.3f). The extract was still stable with slight sedimentation when disodium phosphate was replaced with dicalcium phosphate (0.1%) [Figure 4.3h]. The instability of PME with carrageenan at 0.03 – 0.05% could be due to the low level used. Carrageenan (0.2%), disodium phosphate (0.1%), lecithin (0.1%) and maltodextrin (0.1%) produced a stable PME with some white precipitate at the bottom and some floating in the extract (Figure 4.3j). Carrageenan at high concentration (>0.3%) formed a gel during storage at 4°C. Carrageenans are linear, sulfated, high molecular polysacharides extracted from different red algae (Stephen *et al*., 2006). Their primary structure is made-up of repeating disaccharides sequence of ß-D-galactopyranose

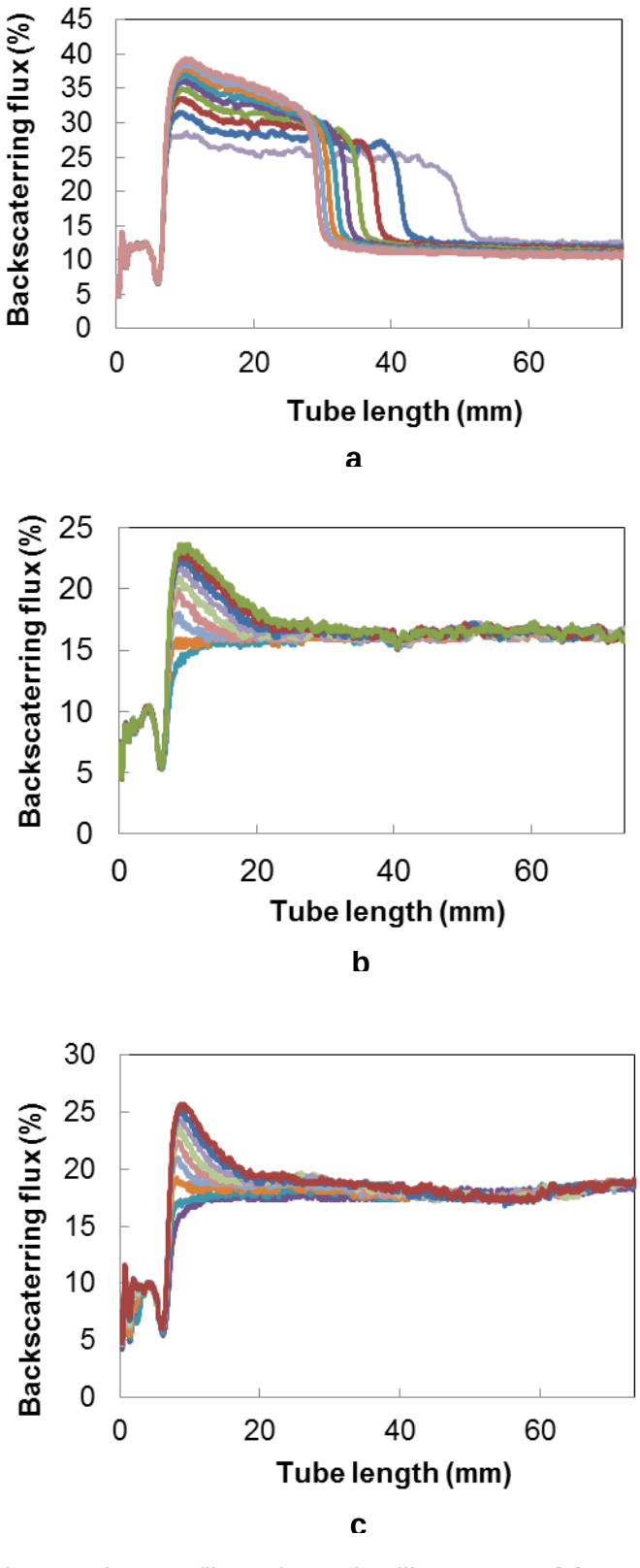
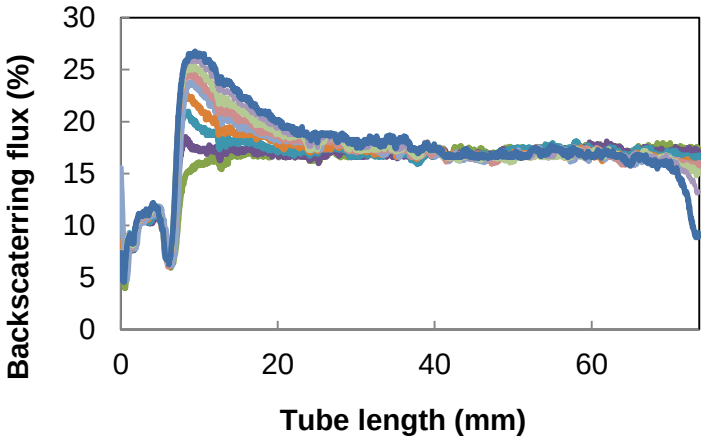


Figure 4.3 Backscattering profiles of pearl millet extract **(a)** no stabilisers – control, **(b)** lecithin – 0.1%, disodium phosphate – 0.1% and carrageenan – 0.03%, **(c)** lecithin – 0.2%, disodium phosphate – 0.1% and carrageenan – 0.03%,



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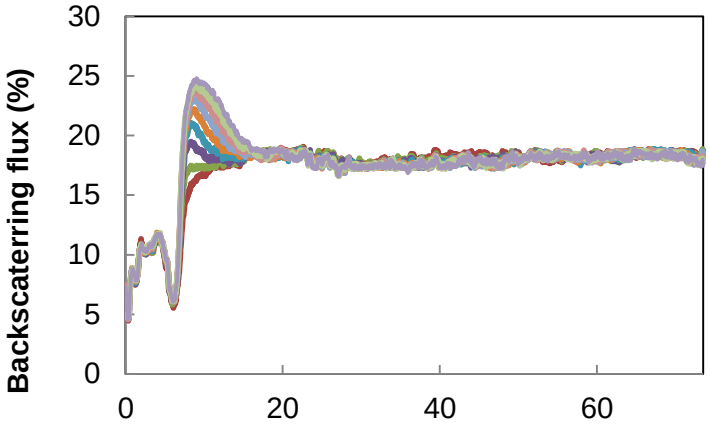

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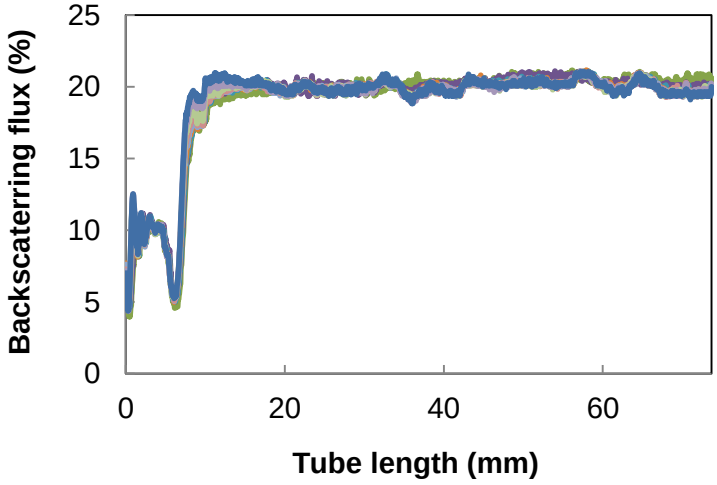

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Figure 4.3 Backscattering profiles of pearl millet extract (d) lecithin – 0.1%, disodium phosphate – 0.1% and carrageenan – 0.05%, (e) lecithin – 0.2%, disodium phosphate – 0.1% and carrageenan – 0.05%, (f) lecithin – 0.1%, disodium phosphate – 0.1% and carrageenan – 0.3% (continued)

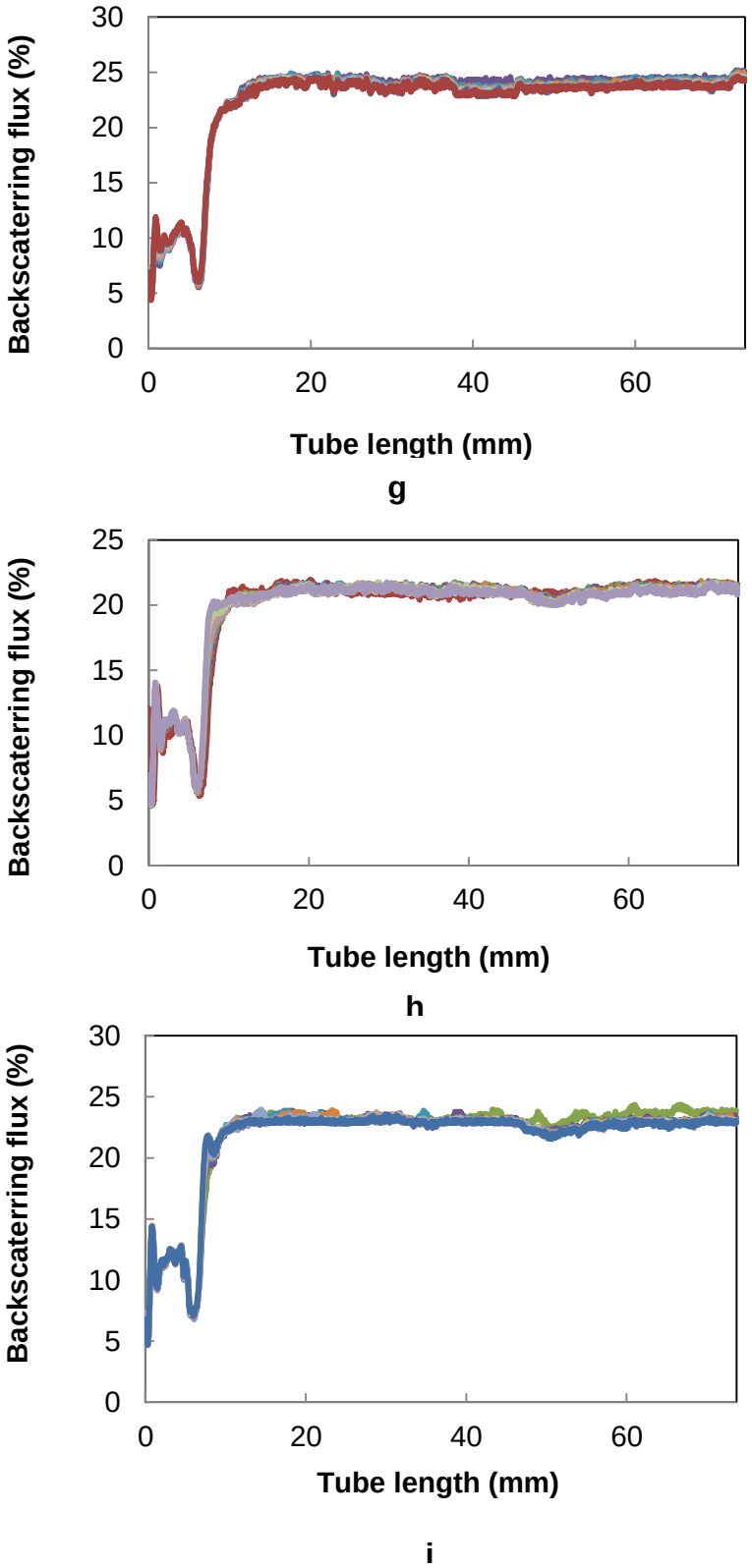

Figure 4.3 Backscattering profiles of pearl millet extract (g) lecithin – 0.1%, disodium phosphate – 0.1% and pectin – 0.6%, (h) lecithin – 0.1%, dicalcium phosphate – 0.1% and carrageenan – 0.3% and (i) lecithin – 0.1%, dicalcium phosphate – 0.1% and sodium alginate – 0.3%

residues linked glycosidically through 1 and 3 positions (A residue) and α-galactopyranose residues linked glycosidically through 1 and 4 positions (B residue). The commecially available carrageenan are kappa (*k*), iota (*i*) and lambda (λ) with different functional properties. The carrageenan used in PME

Table 4.2 Stability of pearl millet extract after 48 h

Sample Identification	Stability (%)
No stabilisers – control	23.08
Lecithin – 0.1%, disodium phosphate – 0.1% and carrageenan – 0.03%	30.77
Lecithin – 0.2%, disodium phosphate – 0.1% and carrageenan – 0.03%	33.85
Lecithin – 0.1%, disodium phosphate – 0.1% and carrageenan – 0.05%	30.77
Lecithin – 0.2%, disodium phosphate – 0.1% and carrageenan – 0.05%	33.85
Lecithin – 0.1%, disodium phosphate – 0.1% and carrageenan – 0.3%	23.33
Lecithin – 0.1%, disodium phosphate – 0.1% and pectin – 0.6%	0.00
Lecithin – 0.1%, dicalcium phosphate – 0.1% and carrageenan – 0.3%	30.00
Lecithin – 0.1%, dicalcium phosphate – 0.1% and sodium alginate – 0.3%	0.00
Lecithin – 0.1%, disodium phosphate – 0.1%, maltodextrin – 0.2% and carrageenan –0.2%.	16.67

contained both *k*- and *i*-residues. *k*-Carrageenan is made up of ß(1→4) D-galactose-4-sulphate and α(1→3) 3.6-anhydro-D-galactose while *i-C*arrageenan consists of ß(1→4) D-galactose-4-sulphate and α(1→3) 3.6-anhydro-D-galactose-2-sulphate (Stephen *et al*., 2006). The mechanisms of stabilisation caused by carrageenan is not well established but is believed that carrageenan forms stability by associating in a quasi-permanent double helix network and/or 'log-jam' network. During double-helix formation, the crosslinking chains are arranged into a three-dimensional network whereas in 'log-jam' network there is no branching of helixes that occurs but the helixes are arrested within a network. Carrageenan forms stability in the presence of potassium and calcium in water and is dissolved by heating and subsequent cooling (Danisco, 2001; Stephen *et al*., 2006). Pearl millet contains Ca^{2+}, $Fe^{2+,}$ Na^{+}, Mg^{2+} and Zn^{2+} as reported by Nambiar *et al*. (2001). Therefore, the carrageenan could have formed a three dimensional network with available Ca^{2+} within the PME and dissolved during the pasteurisation of the PME. However, Tako (2015) reported that *k*-carrageenan caused a least elastic modulus when

mixed with NaCl. This indicated that carrageenan is selective to cation in forming a binding polymer. The report showed that carrageenan gels well in the presence of large univalent cations such as K^+, Rb^+ and Cs^+ but not with small cations such as Na^+ and Li^+. Tako (2015) also reported that *i*-carrageenan form good stability with Ca^{2+} resulting in calcium-salt of *i*-carrageenan during cooling but did not work with K^+ and Na^+. Danisco (2001) also stated that potassium and calcium ions are important for stability strengthening of carrageenan. Therefore, the lack of potassium in pearl millet could have resulted in the lower stabilising capacity of *k*-carrageenan. Thus, Ca^{2+} could have been used by *i*-carrageenan during the stability formation but was not sufficient. In addition, since carrageenan used was a mixture of *k*- and *i*-carrageenan, the *k*-carrageenan could have not participated in stability formation since Ca^{2+} is a divalent ion. Hence, at higher concentration (above 0.3%) a brittle gel was formed due to the absence of *k*-carrageenan participation (Danisco, 2001).

Pectin (0.6%), lecithin (0.1%) and disodium phosphate produced a stable PME as shown in Figure 4.3g where the profiles are linear and overlay each other. These results are in agreement with those reported by Modha & Pal (2011) that a level of 0.6% pectin was selected during the optimisation of *Rabadi*, a fermented milk using pearl millet. Pectin (0.6%) combined with disodium phosphate (0.1%) gave the best taste of the beverage. The ratio of the height of settled particles to the total PME height also indicated that pectin at 0.6% stabilized the PME. Pectin scored 0% which shows the particles were all suspended (Table 4.2). Pectins are linear heteropolysaccharides made up of mostly galacturonic acid units. They contain carboxylic groups found within the uronic acid residue which may be in a free form or salt form with Ca^{2+}, K^+, Na^+ or NH_4^+. Their structure is inconsistent since they can change during isolation from plant. Pectins are divided into low (20-40%) and high (60-75%) methoxyl (ester) pectin depending on their degree of esterification (Raj *et al.*, 2012). Low methoxyl pectin (LMP) are of interest due to their low calorific value (Stephen *et al*., 2006). High methoxyl pectin (HMP) forms gels in the presence of sugars and acid, unlike LMP which requires divalent ions such as Ca^{2+} to precipitate and form gels since they lack sufficient acid groups. The pectin used (Du Pond, SY 640) was a LMP which reacted with the available Ca^{2+} from pearl millet to form a stable PME. The pH of the extract was also low which facilitated the formation of a stable extract. LMP could have formed a stable PME by the formation of side-side associations with galactoranans where specific sequences of galacturonic acid (GalA) monomer which are in parallel or adjacent chains became linked intermoleculary using electrostatic and ionic bonding of carboxyl groups (Raj *et al*., 2012). This mechanisms of

Ca^{2+} binding to the carboxyl groups in the pectin is named the 'egg-box' (Stephens *et al*., 2006).

Sodium alginate (0.3%) mixed with lecithin and dicalcium phosphate each at 0.1% produced a stable PME (Figure 4.3i). The ratio of the height of settled particles to the total PME height also indicated that sodium alginate at 0.3% stabilized the PME (0%) [Table 4.2]. Alginates are polyuronan isolated from the cell wall of many brown seaweed. The alginates are used depending on their biosythesis properties. Their properties are based on their chemical structure. They consists of 1→4 linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) pyranose residues in an unbranched chain. These residues are capable of forming G-blocks, M-blocks or MG-blocks. G-blocks are formed when the solution contains calcium (Ca^{2+}) and/or hydrogen (H^{+}) ions at low temperature (Brownlee *et al*., 2009). The G-blocks are important in alginate structure due to the Ca^{2+} and H^{+} content binding ability. MG blocks are responsible for the flexibility of polyssacharides and tend to reduce the viscosity of alginate solution (Brownlee *et al*., 2009). Alginate has a thickening, stabilising and gel-forming ability. Sodium alginate which is usually used in the food industry forms gels when in contact with divalent ions such as calcium. The sodium ions from sodium alginate formed crosslinks with Ca^{2+} which is capable of forming two bonds with the alginate polymers. Long periods of contact between the alginate and Ca^{2+} also strenthens the stability. Pearl millet contains about 25 - 42 mg/100 g of calcium (Nambiar *et al*., 2011). These Ca^{2+} could have created the network with the sodium, hence a stable PME with sodium alginate at 0.3% level. However, health conscious consumers may not accept the sodium-salt in alginic acid. Therefore, lecithin, disodium phosphate and pectin at 0.1, 0.1, and 0.6%, respectively were selected for stabilising pearl millet extract.

4.3.2 Effect of different lactic acid bacteria on the pH and total titratable acidity (TTA) of non-alcoholic pearl millet beverage

The generalized linear model showing the relationship between lactic acid bacteria (LAB) [*L. mesenteroides*, *P. pentosaceus* and *E. gallinarum*] and pH of non-alcoholic pearl millet beverage (NAPMB) are shown in Table 4.3. Based on the intercept (ß) the LAB were increasing resulting in a decrease in the pH of the beverage. Based on Monte Carlo simulation, the pH of NAPMB ranged between 3.32 and 3.90 with a mean of 3.61 ± 0.17. Cumulative distribution showed a 5% probability of the pH following below 3.32 or above 3.90 and therefore can conclude that the pH was between 3.32 and 3.90. *L. mesenteroides*, *P. pentosaceus, E. gallinarum,* the interaction between *L. mesenteroides*

Table 4.3 The generalized linear model for the effects of *L. mesenteroides P. pentosaceus* and *E.* gallinarum and their interaction on the pH of non-alcoholic pearl millet beverage

Parameter	Coefficient (β)	Std, Error	95% Wald Confidence Interval		Significance
			Lower	Upper	
Linear coefficient effect					
Intercept	3.44	0.06	3.32	3.57	0.000
Main coefficient effect					
L. mesentoroides (X_1)	1.28	0.53	0.24	2.31	0.016
P. pentosaceus (X_2)	4.41	0.53	3.37	5.45	0.000
E. gallinarum (X_3)	-2.73	0.53	-3.76	-1.69	0.000
Interactive coefficient effect					
*L. mesenteroides * P. pentosaceus*	-63.00	4.85	-72.51	-53.49	0.000
*L. mesenteroides * E. gallinarum*	55.00	4.85	45.49	64.51	0.000
*P. pentosaceus * E. gallinarum*	-2.33	4.85	-11.85	7.18	0.631

and *P. pentosaceus* and the interaction between *L. mesenteroides* and *E. gallinarum* had a significant effect ($p \leq 0.05$) on the pH of NAPMB with the exception of the interaction between *P. pentosaceus and E. gallinarum*. The linear model for this design can be presented as in Equation 4.4 using the coded values.

$$Y = 3.44 + 1.28X_1 + 4.41X_2 - 2.73X_3 - 63.00X_1X_2 + 55.00X_1X_3 - 2.33X_2X_3 \quad 4.4$$

Where X_1 = *L. mesentoroides,* X_2 = *P. pentosaceus* and X_3 = *E. gallinarum.*

Based on the coefficients, there was a significant ($p < 0.05$) increase in the pH caused by *L. mesenteroides*, *P. pentosaceus* and the interaction between *L. mesenteroides* and *E. gallinarum*. *E. gallinarum* and interaction effects of *L. mesenteroides* and *P. pentosaceus* had a significant ($p < 0.05$) decrease on the pH. The interaction effects of *P.*

pentosaceus and *E. gallinarum* caused a non-significant ($p > 0.05$) decrease on the pH of the beverage.

Scatterplots shown in Figure 4.4 show the effect of the main coefficient on the pH of the beverage for 1000 cases. Based on the scatterplots generated from Monte Carlo simulation, *L. mesentoroides* caused an increase on the pH, *P. pentosaceus* caused an increase and decrease on the pH whereas *E. gallinarum* caused a decrease on the pH of the beverage. Overall, all the pure cultures of LAB had a significant ($p < 0.05$) effect on the pH of the beverage with *P. pentosaceus* having the highest contribution (973.9%) followed by *E. gallinarum* (655.5%) and lastly *L. mesenteroides* (132.7%). Figure 4.5 show the percentage contribution of each independent variable on the pH of non-alcoholic pearl millet beverage.

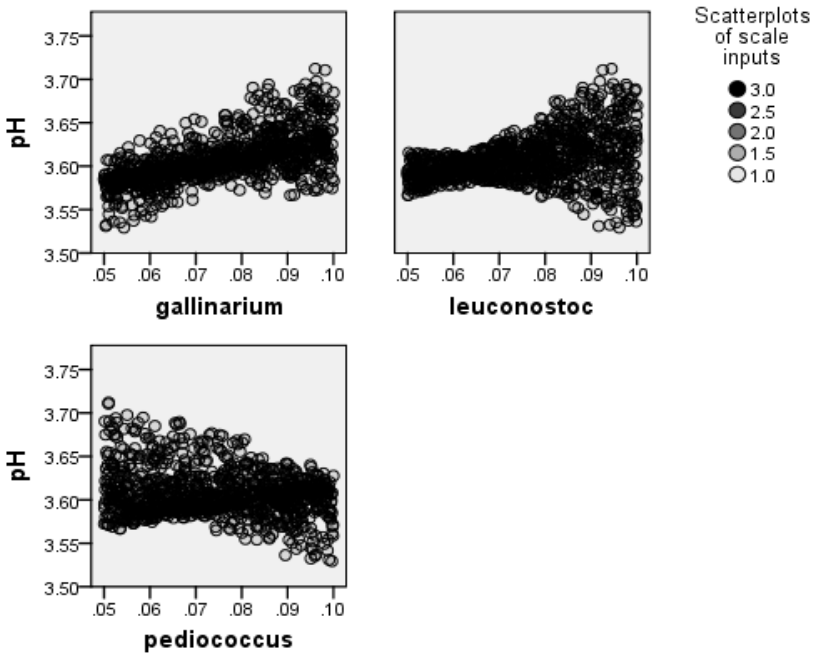

Figure 4.4 Scatterplots showing the effect of lactic acid bacteria on the pH of the beverage.

Lactic acid bacteria (LAB) in general are able to tolerate a wide range of pH in the presence of organic acid such as lactic acid. *L. mesenteroides* grows early during food fermentation and then superseded by the growth of other LAB as seen in Chapter 3, Table 3.2. During LAB fermentation, carbohydrates are broken into lactic acid which

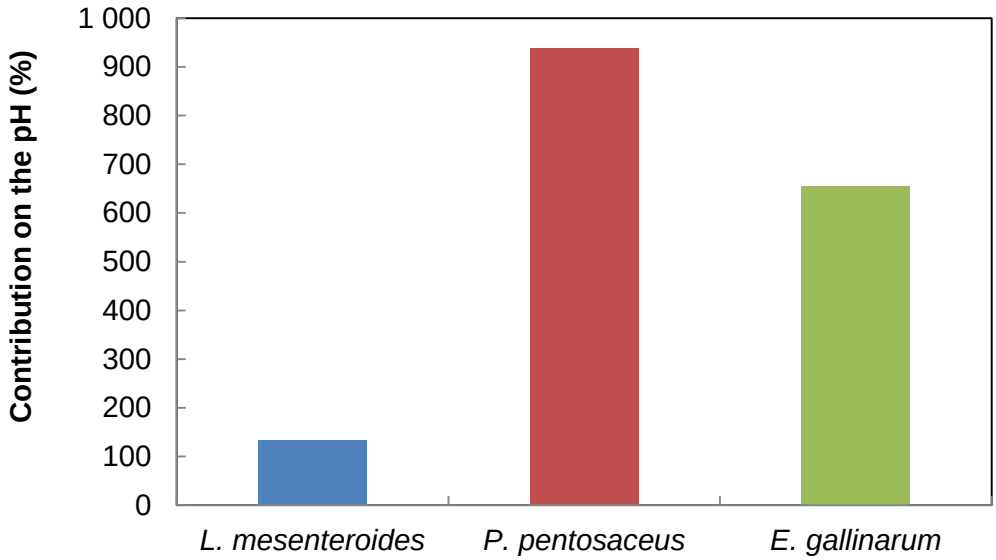

Figure 4.5 Percentage contributions of purified lactic acid bacteria on the pH of non-alcoholic pearl millet beverage

allows the growth of acidophilic bacteria such as *P. pentosaceus* and *E. gallinarum* (McDonald *et al*., 1990). *L. mesenteroides* and *P. pentosaceus* were responsible for the creation of acidic environment while *E. gallinarum* increased the pH of the beverage. The increase in the pH could be caused during the autolysis of *E. gallinarum*. Autolysis occurs when the conditions are unfavourable for microorganisms. During autolysis the hydrolytic enzymes breakdown the peptidoglycan on the bacterial cell wall resulting in cellular lysis (Ramirez-Nunez *et al*., 2011). *Enterococcus spp*. grows in a wide range of pH (4.4 – 10.6) [Curtis & Lawley, 2003]. The pH of the beverage was between 3.32 and 3.90 which could have accelerated the autolysis of *E. gallinarum*.

L. mesenteroides (heterolactic bacteria) produced the least acid unlike the homalactic bacteria *P. pentosaceus* which produced above intermediate amount of acid. This is because heterolactic bacteria primarily produce about 50% lactic acid, 25% acetic acid and ethyl alcohol and 25% CO_2. In contrast, homolactic produces mainly lactic acid (Azam-Ali, 1998). The CO_2 produced replaces oxygen present in the beverage and create an anaerobic environment which gave growth to subsequent anaerobic bacteria (Azam-Ali, 1998). This is in agreement with Kohaldove & Karovicova (2007) who reported the growth of *Pediococcus spp*. dominating the latter stages of fermentation of maize. *P. pentosaceus* was responsible for the rapid acidification of dough. The addition of

sprouted rice flour (SRF) which contained amylase was necessary since chance LAB fermentation requires the enzymes to saccharify the grain starch (Kohaldove & Karovicova, 2007). Since the beverage was fermented, the pH is expected to decrease as more lactic acid accumulates but *E. gallinarum* was increasing the pH of the beverage.

The coefficient and related results from the generalized linear model for the main effect of *L. mesenteroides*, *P. pentosaceus* and *E. gallinarum* and their interactions on the total titratable acidity (TTA) of non-alcoholic pearl millet beverage (NAPMB) are shown in Table 4.4. Based on the intercept (ß) the pure cultures were multiplying resulting in an increase in TTA of the beverage. Monte Carlo simulation showed that the TTA ranged between 0.50 – 0.72% with a mean of 0.58 ± 0.06%. Cumulative distribution showed a 5% probability of the TTA falling below 0.50% or above 0.72%. Therefore, can conclude that the TTA of the beverage falls between 0.50 and 0.72%.

All cultures had a significant influence ($p \leq 0.05$) on the TTA of the beverage during fermentation with the exception of the interaction between *L. mesenteroides* and *E. gallinarum*. The notation model for these interactions is shown by Equation 4.5.

$$Y = 0.69 - 2.38X_1 - 1.15X_2 + 1.05X_3 + 32.00X_1X_2 - 4.67X_1X_3 - 12.67X_2X_3 \qquad 4.5$$

Based on the equation, *L. mesenteroides*, *P. pentosaceus*, interaction between *L. mesenteroides* and *E. gallinarum,* and interaction between *P. pentosaceus* and *E. gallinarum* caused a significant ($p < 0.05$) decrease on the TTA of the beverage. The interaction between *L. mesenteroides* and *P. pentosaceus* caused a significant ($p < 0.05$) increase on the TTA of the beverage. The interaction between *L. mesenteroides*, *P. pentosaceus* and *E. gallinarum* had no significant ($p > 0.05$) effect on the TTA of the beverage. The interaction of the pure cultures on the TTA is shown using scatterplots from the Monte Carlo simulation in Figure 4.6. Based on the scatterplots, *L. mesentoroides* caused a decrease and increase on the TTA, *P. pentosaceus* caused an increase on the TTA while *E. gallinarum* caused a decrease on the TTA of the beverage. Looking at the percentage contribution, *P. pentosaceus* had a high contribution (526.3%) on the influence of TTA followed by *E. gallinarum* (137.3%) then *L. mesentoroides* (72.54%). The effect of the main effects on the TTA of the beverage is shown in Figure 4.7.

Table 4.4 The generalized linear model for the effects of *L. mesenteroides, P. pentosaceus* and *E. gallinarum* and their interaction on the total titratable acidity (TTA) of non-alcoholic pearl millet beverage

Parameter	Coefficient (β)	Std, Error	95% Wald Confidence Interval		Significance
			Lower	Upper	
Linear coefficient effect					
(Intercept)	0.686	0.035	0.616	0.755	0.000
Main coefficient effect					
L. mesentoroides (X_1)	-2.383	0.311	-2.993	-1.774	0.000
P. pentosaceus (X_2)	-1.15	0.311	-1.759	-0.541	0.000
E. gallinarum (X_3)	1.05	0.311	0.441	1.659	0.000
Interactive coefficient effect					
L. mesenteroides * *P. pentosaceus*	32	2.853	26.408	37.59	0.000
L. mesenteroides * *E. gallinarum*	-4.667	2.853	-10.26	0.925	0.102
P. pentosaceus * *E. gallinarum*	-12.667	2.853	-18.26	-7.075	0.000

The TTA was measured as the total lactic acid produced from the fermentation of starch and sugars by LAB. During fermentation, homolactic bacteria *P. pentosaceus* and *E. gallinarum* produced mainly lactic acid whereas *L. mesenteroides* produced lactic acid, CO_2 and acetic acid/ethyl alcohol. Thus, *P. pentosaceus* and *E. gallinarum* contributed highly in the production of lactic acid. The results were similar to those observed by Monilola & Omolara (2013) on the increase in lactic acid (TTA) during the fermentation of starchy-based foods. In addition, this was in agreement with Basinskiene *et al*. (2016) who reported an increase in TTA during the fermentation of non-alcoholic beverages from cereals. However, looking at the generalised linear model and Monte Carlo simulation, *E.*

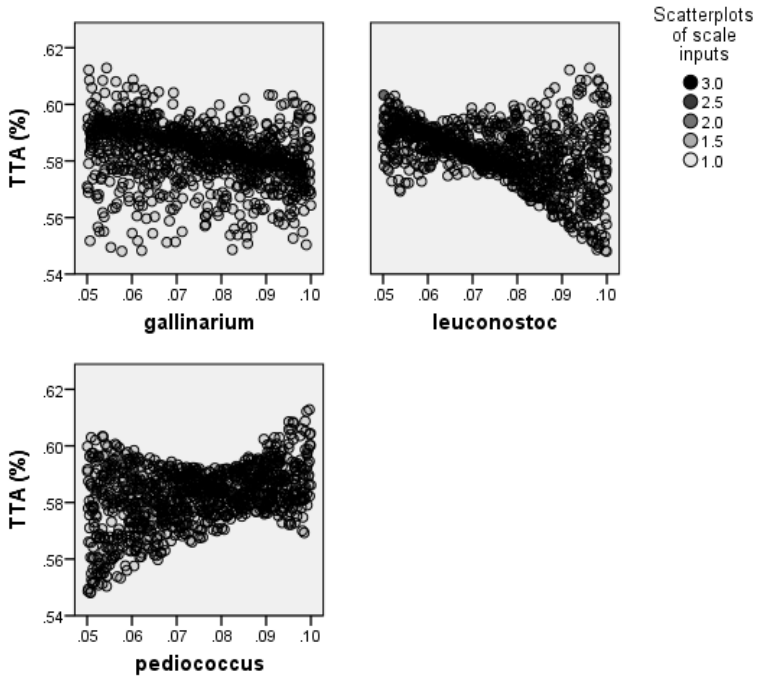

Figure 4.6 Scatterplots showing the effect of lactic acid bacteria (LAB) on the total titratable acidity of the beverage

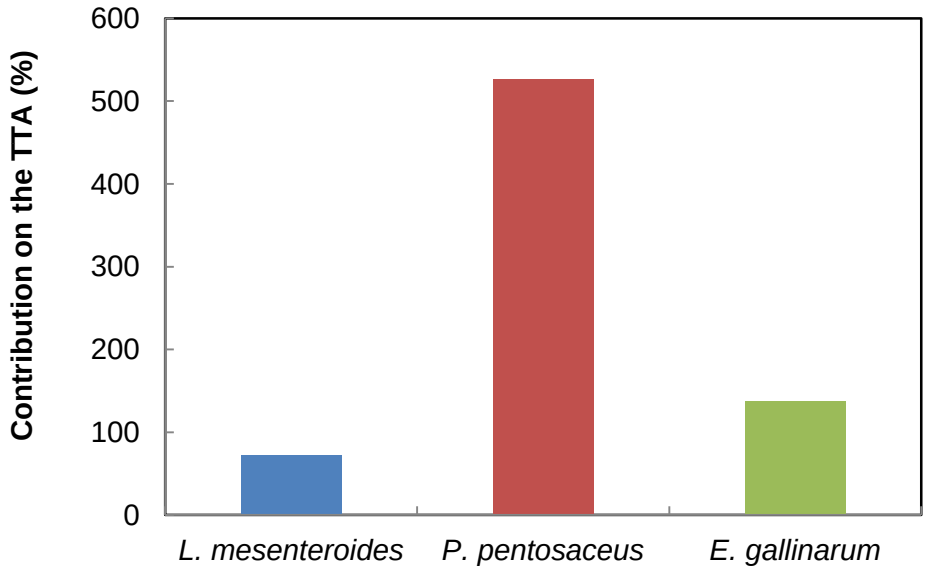

Figure 4.7 Percentage contributions of purified lactic acid bacteria (LAB) on the total titratable acidity (TTA) of non-alcoholic pearl millet beverage

gallinarum caused significant increase and decrease on the pH and TTA of the beverage, respectively. This is not desired during beverage fermentation hence it was eliminated.

4.3.3 Effect of different purified lactic acid bacteria on the viscosity of non-alcoholic pearl millet beverage

Table 4.5 shows the generalized linear model for the main effect between *L. mesenteroides, P. pentosaceus* and *E. gallinarum* on the viscosity of non-alcoholic pearl millet beverage (NAPMB). Based on the intercept (ß) the pure cultures were multiplying resulting in an increase in viscosity of the beverage. Monte Carlo simulation showed that viscosity of the beverage ranged from -88.00 to 11.74 mPa.s with a mean of 5.56 mPa.s. Cumulative distribution showed a 5% probability of the viscosity falling below -88.00 mPa.s and/or above 11.74 mPa.s. This gave confidence that the viscosity of the beverage is between -88.00 to 11.74 mPa.s.

L. mesenteroides, P. pentosaceus, *E. gallinarum, the* interaction between *L. mesenteroides* and *P. pentosaceus,* the interaction between *L. mesenteroides* and *E. gallinarum*, the interaction between *P. pentosaceus* and *E. gallinarum* and the interaction between *L. mesenteroides, P. pentosaceus* and *E. gallinarum* had a significant influence ($p \leq 0.05$) on the viscosity of the beverage. The design model is represented by Equation 4.6 using coded values.

$$Y = 34{,}44 - 347{,}18X_1 - 183{,}45X_2 - 400{,}45X_3 + 1742{,}13X_1X_2 + 4020.87X_1X_3 + 2494.20X_2X_3 + 13017{,}33X_1X_2X_3 \quad 4.6$$

The negative coefficient indicated that the viscosity of the beverage was decreasing. The interaction between *L. mesenteroides* and *P. pentosaceus,* the interaction between *L. mesenteroides* and *E. gallinarum*, the interaction between *P. pentosaceus* and *E. gallinarum* and the interaction between *L. mesenteroides, P. pentosaceus* and *E. gallinarum* caused a significant ($p < 0.05$) increase on the viscosity of the beverage. Meanwhile the decrease in the viscosity of the beverage was caused by *L. mesenteroides, P. pentosaceus* and *E. gallinarum*. The interaction between *L. mesenteroides, P. pentosaceus* and *E. gallinarum* caused a thicker beverage than the effect of all other lactic acid bacteria (LAB). The effect of these probiotics on the viscosity of the beverage is shown in Figure 4.8 using scatterplots generated from Monte Carlo simulation. Based on the scatterplots, *E. gallinarum* initially had no influence on the viscosity and as time elapsed started to influence the viscosity by increasing and

decreasing the viscosity. This similar concept was seen in the beverage with *L. mesenteroides* whereas *P. pentosaceus* caused the viscosity of the beverage to increase.

Table 4.5 The generalized linear model for the effects of *L. mesenteroides, P. pentosaceus* and *E. gallinarum* and their interaction on the viscosity of non-alcoholic pearl millet beverage

Parameter	Coefficient (*β*)	Std. Error	95% Wald Confidence Interval		Significance
			Lower	Upper	
Linear coefficient effect					
(Intercept)	34.44	2.41	29.71	39.16	0.000
Main coefficient effect					
L. mesentoroides (X_1)	-347.18	30.41	-406.79	-287.58	0.000
P. pentosaceus (X_2)	-183.45	30.41	-243.05	-123.85	0.000
E. gallinarum (X_3)	-400.45	30.41	-460.05	-340.85	0.000
Interactive coefficient effect					
L. mesenteroides and P. pentosaceus	1742.13	384.66	988.22		0.000
L. mesenteroides and E. gallinarum	4020.87	384.66	3266.95	4774.78	0.000
P. pentosaceus and E. gallinarum	2494.20	384.66	1740.29	3248.11	0.000
L. mesenteroides, P. pentosaceus and E. gallinarum	-13017.33	4865.56	-22553.67	-3481.00	0.007

P. pentosaceus had the highest contribution (74.01%) on the increase in viscosity followed by *E. gallinarum* (50.41%) and *L. mesenteroides* (45.98%) [Figure 4.9].

During cereal fermentation lactic acid bacteria (LAB) breakdown starch into simpler sugars resulting in the decrease in viscosity of the beverage. The viscosity of the beverage was affected by factors such as the pH, type of microorganisms and if the type of microorganisms involved in fermentation have amylase enzymes to hydrolyze starch

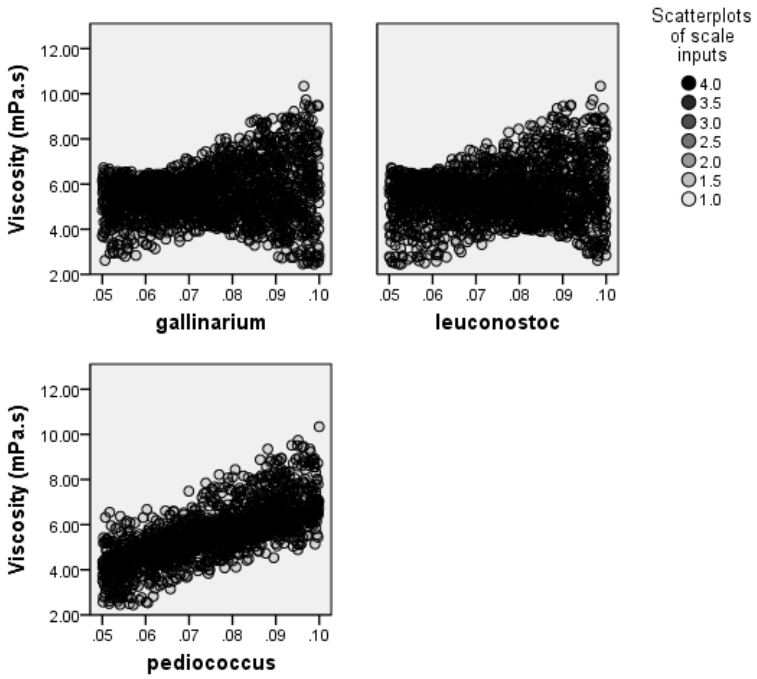

Figure 4.8 Effect of lactic acid bacteria (LAB) on the viscosity of the beverage

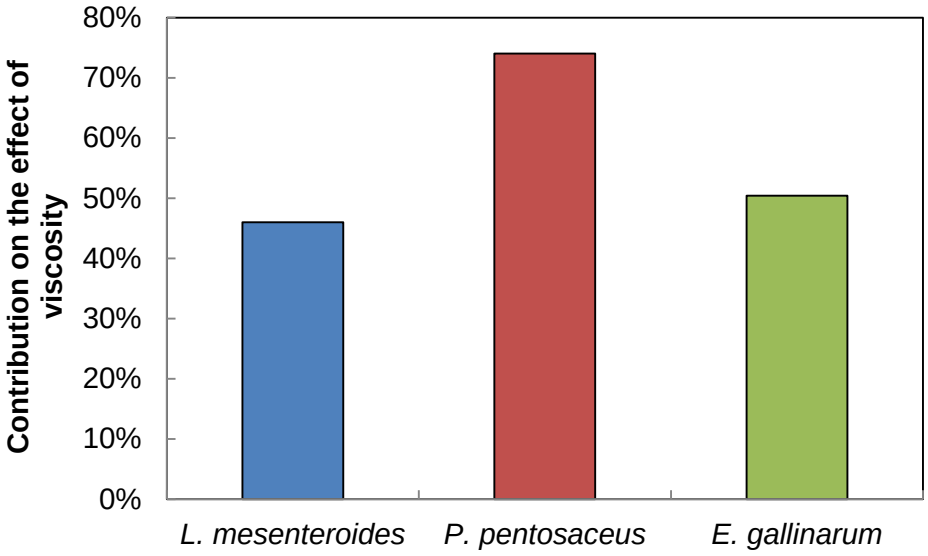

Figure 4.9 Percentage contributions of purified lactic acid bacteria (LAB) on the viscosity of non-alcoholic pearl millet beverage (NAPMB)

into dextrins and sugars. In the case of non-alcoholic pearl millet beverage (NAPMB), sprouted rice flour (SRF) was used which contained high levels of amylase activity. The addition of SRF enhanced the fermentation to decrease the viscosity of the beverage. The decrease of viscosity was important because when the starch is unmodified it becomes hard to digest. The increase in viscosity made the beverage unpalatable. If more water was added to decrease the viscosity of the beverage, the beverage could have lower energy and nutrient content (Wingley *et al*., 2006). Thus, the breakdown of starch by SRF and LAB had several desirable effects on the viscosity and nutritional quality of the beverage.

These results are in agreement with Hayta *et al*. (2001) who reported a decrease in viscosity after fermentation of a traditional fermented beverage (*Boza*) at 20ºC. *L. mesenteroides, P. pentosaceus* and *E. gallinarum* and the interaction *between P. pentosaceus* and *E. gallinarum* caused a decrease on the viscosity of the beverage which is desired for a beverage. However, *E. gallinarum* could not be used since it causes an increase on the pH and a decrease on the TTA of the beverage. Therefore, going forward *L. mesenteroide* and *P. pentosaceus* were selected in the production of the beverage.

4.3.4 Non-alcoholic pearl millet beverage (NAPMB) produced using pure cultures of lactic acid bacteria (LAB)

The interaction between *L. mesenteroides* (0.05%), *P. pentosaceus* (0.10%) and the interaction between *L. mesenteroides* and *P. pentosaceus* (0.05% each) produced a beverage with good taste but a bad after- taste. The interactive effects of *L. mesenteroides* (0.1%) and *P. pentosaceus* (0.05%) and interactive effects of *L. mesenteroides* (0.1%) and *P. pentosaceus* (0.10%) produced a sour beverage with a bad after taste. When cultures (*L. mesenteroides* and *P. pentosaceus*) were used individually at 0.05% each and in combination at 0.05%, *L. mesenteroides* alone produced a beverage that tasted better in comparison to *P. pentosaceus.* A beverage with *L. mesenteroides* (0.05%) and *P. pentosaceus* (0.025%) produced an acceptable beverage. Fugelsang & Edward (2007) reported that *Pediococcus spp*. are responsible for the production of diacetyl compound which results in 'buttery' aroma hence the reduction of *P. pentosaceus* to 0.025% produced acceptable beverage.

The pH of the beverage before fermentation was low due to citric acid added. The use of citric acid at 0.05% and 0.25% resulted in a beverage with a pH of 3.13 and 2.86 on average, respectively. This has resulted in reduced fermentation by the pure cultures of lactic acid bacteria (LAB). Citric acid could have been utilized by *P. pentosaceus*

resulting in increased diacetyl compound responsible for unwanted flavours (Fugelsang & Edward, 2007). The beverage was sweeter with undesired after-taste immediately after pasteurization which became more intense after inoculation and fermentation. The fermentation with LAB could also have resulted in undesired compounds. In addressing these issues, citric acid and disodium phosphate were replaced with sodium citrate to regulate the acidity before inoculation. In addition, phenolic compounds released during fermentation could be responsible for the unacceptable taste (Drewnowski & Gomez-Carneros, 2000). Hence, heat and moisture could be used to treat pearl millet grains before milling into flour. The main purpose will be to inactivate the naturally present lipase enzymes in cereal grains that could lead to rancid off flavours. Furthermore, steaming of the grains will make the starch in grains gelatinise and digestible (Stapley *et al*., 1999). . In addition, the duration of soaking pearl millet grains prior milling could be minimized to avoid any pre-fermentation of grains that could happen.

The increase in the ratio of pearl millet flour (PMF) to water was also important in diluting the unwanted off-taste, bitter, astringent and/or sour taste of the beverage. The ratio of PMF to water was increased from 1:6.5 to 1:10 (PMF:Water). The beverage was pasteurised in an open pot instead of traditionally pasteurising in package. This was to ensure that the grain extract was cooked in order to enhance the aroma, taste and flavour hence removing the undesired taste. Maltodextrin which was used to give the beverage a good body, mouthfeel and aid in the dispersibility of particles caused a bland unpleasant taste and did not completely dissolve in the beverage. Hence, maltodextrin was removed.

In conclusion, pectin (0.6%), sunflower lecithin (0.1%) , sodium citrate (0.1%), interactive effects of *L. mesentoroides* (0.05%) and *P. pentosaceus* (0.025%) were selected in the production of stable NAPMB.

4.3.6 Conclusion

A stable pearl millet extract was produced using pectin at 0.6%. The interactive effects of *L. mesenteroides* and *P. pentosaceus* each at 0.05 and 0.025%, respectively produced an acceptable non-alcoholic pearl millet beverage.

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CHAPTER FIVE

PRODUCTION OF PEARL MILLET BEVERAGE USING BIOBURDEN LACTIC ACID BACTERIA AND ITS PHYSICOCHEMICAL, NUTRITIONAL AND SENSORY PROPERTIES

Abstract

Non-alcoholic cereal beverages (NAPMB) are usually produced through uncontrolled fermentation driven by a cock tail of bacteria. This leads to variability of the final product, hence, in order to commercialize fermented cereal beverages the microorganisms that carry out the fermentation need to be isolated, identified, purified and their role during fermentation determined. The aim of this investigation was to evaluate the physicochemical, nutritional and sensory characteristics of NAPMB produced using pure cultures of bioburden lactic acid bacteria. NAPMB were produced, namely, plain non-alcoholic pearl millet beverage (PNAPMB), moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB). During the production of PNAPMB, pearl millet extract (PME) was pasteurised at 85ºC for 15 min and cooled to 40ºC. The fluid was inoculated with purified cultures of *Leuconostoc mesenteroides* and *Pediococcus pentoseace* at 0.050% and 0.025% (1:0.5), respectively, and fermented at 37ºC for 18 h. MSNAPMB was produced following the same method as PNAPMB but a 4% moringa leaf extract powder was added prior to hydration of pearl millet powder. TNAPMB was prepared by mixing cold water and pearl millet flour (1:1.25; PMF:Water) and hydrated for 3 h at 25ºC. The mixture was divided into ¼ slurry which was mixed with sprouted rice flour (SRF) and ¾ portion which was gelatinized with 1 L of boiling water and cooled to 40ºC. The two portions were mixed together and fermented at 37ºC for 18 h, followed by sieving, dilution with water (1:0.5, filtrate:water) and pasteurization for 15 min at 85ºC. The growth of lactic acid bacteria, pH, total titratable acidity (TTA) and sugars of PNAPMB and MSNAPMB were determined at 3 h intervals during fermentation. The final beverages (PNAPMB and MSNAPMB) were also analyzed in terms of proximate composition, colour, viscosity and metabolites in comparison to TNAPMB. The lactic acid bacteria (LAB) were significantly ($p < 0.05$) affected by the fermentation period and increased from 3.32 to 7.97 log cfu/ml and 3.58 to 8.38 log cfu/ml in PNAPMB and MSNAPMB, respectively. The pH was significantly ($p < 0.05$) affected, in PNAPMB the pH decreased from 5.05 to 4.14 while the pH of MSNAPMB decreased from 5.05 to 3.65 during the 18 h fermentation. The total titratable acidity (TTA) significantly ($p < 0.05$) increased from 0.14 to 0.22% and from 0.17 to 0.38% in PNAPMB and MSNAPMB, respectively, during the 18 h of fermentation. The

total sugar content (sucrose) was significantly affected ($p < 0.05$) during fermentation and ranged from 5.48 to 5.26% in PNAPMB while it ranged from 5.21 to 5.33% in MSNAPMB. The protein, total fat, moisture total sugar and carbohydrates differed significantly ($p < 0.05$) among the samples. PNAPMB, MSNAPMB and TNAPMB had protein content of 1.62%, 2.17% and 1.50%, respectively. The total fat content was low in all beverages accounting for 0.92%, 0.65% and 0.1.54% of total solids in PNAPMB, MSNAPMB and TNAPMB, respectively. The total sugar content was 5.06%, 5.31% and 6.11% in PNAPMB, MSNAPMB and TNAPMB, respectively. The carbohydrates (CHO) were 4.31%, 5.03% and 9.41% in PNAPMB, MSNAPMB and TNAPMB, respectively. The CHO and energy differed significantly ($p < 0.05$) between MSNAPMB and TNAPMB, and between PNAPMB and TNAPMB. The total colour difference (ΔE) was 5.91 and 10.60 in PNAPMB and MSNAPMB, respectively in comparison to the TNAPMB. PNAPMB sample was deemed acceptable in comparison to the MSNAPMB. PNAPMB was preferred by a consumer panel followed by MSNAPMB and TNAPMB. Volatile compounds with beneficial effect such as anti-inflammatory and anti-pathogenic properties were identified in the beverages. Principal component analysis (PCA) indicated that the variations in characteristics of PNAPMB and MSNAPMB could be explained using total fat, saturated fat, total sugar, ash, moisture, proteins, chroma (C), hue and b*.

5.1 Introduction

Beverages are liquid foods that serve as sources of both fluid and nutrients for the body. They provide the body with energy (Ogbonna *et al*., 2013). Traditional non-alcoholic fermented cereal beverages are light to yellowish homogenous suspension with a sweet to sour taste (Bogue & Yu, 2009) consumed by all people including children, pregnant women, sick and old people (Solange *et al*., 2014). They may also serve as breakfast drink, food complement, thirst quencher or refreshment to many people including vegetarians and others with cereal allergies (Gyar *et al*., 2014).

The beverages contain no alcohol. They are considered to be ruined should there be alcohol at high levels. The beverages are traditionally not heat treated after fermentation and thus are considered functional beverages containing high levels of lactic acid bacteria (LAB) [Basinskiene *et al*., 2016]. Fermented non-alcoholic cereal beverages (NACB) are popular in African countries (Terna & Ayo, 2002) as part of tradition and culture. These beverages are fermented by different microorganisms which change the solid or liquid substrates into different products (Weir & McSpadden, 2005). The substrates differ widely, with any material that supports microbial growth being a potential substrate (Chisti, 1999).

Non-alcoholic cereal beverages (NACB) are increasing rapidly as part of the latest trend towards convenient healthy nutrition (Sternenzym, 2011). Beverages are produced from malt or cereal sugar and spices and are sources of antioxidants, vitamins and other health promoting substances. The cereal beverages differ depending on the type of functional constituents used (Sternenzym, 2011). The beverage properties such as viscosity, mouth feel and sweetness can be adjusted to meet the consumer's preferences. The extract of cereal grains can also be mixed with oil emulsion to produce drinks similar to milk or cream but without animal protein, lactose or soy. New flavours can be produced by fermenting with microorganisms (Sternenzym, 2011).

Traditionally, NACB are carried out through spontaneous fermentation involving mixed microflora. However, the spontaneous fermentation of the beverage is difficult to control especially in mass production. Furthermore, unwanted microorganisms that can be found in the beverage can speed-up spoilage after fermentation. This can be worse if the periods between product preparation and consumption are long resulting in premature spoilage. Although numerous technologies (Yang & Seib, 1996; Terna & Ayo, 2002; Oranusia *et al*., 2003; Onaolapo & Busari, 2004; Nkama *et al*., 2010; Uvere & Amazikwu 2011; Oluwole *et al*., 2012; Abimbola, 2013; Adelakan *et al*., 2013; Ayo *et al*., 2013; Olosunde *et al*., 2014) have been developed to address the challenges of underutilization of African cereals, the production of these beverages on large scale is still limited. The limitation of these beverages at large scale could be due to the low acceptability of the beverages due to variability in taste in comparison to traditionally prepared beverages. In order to commercialize these beverages the microorganisms which carry the fermentation needs to be isolated, identified, purified and their role during fermentation determined (Moodley, 2015).

In Chapter 4, a stable pearl millet extract (PME) was produced using pectin (0.6%), while *Leuconostoc mesentooides* and *Pediococcus pentoseace* isolated from the indigenous beverage were selected for the production of acceptable beverage. The effects of these organisms on the modified pearl millet beverage were determined. The aim of this investigation was to produce a stable non-alcoholic pearl millet beverage using the selected bioburden lactic acid bacteria with or without moringa (*Moringa oleifera*) and determine their physical, chemical and sensory properties in comparison to the traditional beverage. Bioburden refers to the number of viable bacteria on the grains that has not been sterilised.

5.2 Materials and Methods

5.2.1 Sources of raw materials and equipment

Pearl millet and rice grains were purchased from Agricol in Brackenfell, Cape Town. Ground ginger was purchased from Deli Spices in Epping, Cape Town. Moringa leaf powder was purchased from Moringa South Africa, Garden Route.

Perten 3100 laboratory mill, Bauermeister Inc., Vernon hammer mill , water-bath (Ecobat 207), Butcherquip junior cooker (Model CCB 0170), Hanna Edge pH meter, Anaerobic Gas-Pack system, incubator, Geiger & Klotzbucher cabinet oven, Sonicator cell disruptor (Heat system-ultrasonic INC. Sonicator cell disruptor, Model W-225R) and compound microscope were provided by the Department of Food Science and Technology, Cape Peninsula University of Technology. The Agilent 1100 HPLC-RID, Biomerieux Vitek 2, BenchTop Pro with omnitronics (VirTis-SP Scientific) freeze-dryer, Ultra-freezer (Glacier, -86ºC ultralow temperature freezer), nitrogen analyser (Leco-TruSpec-N) and UV 1700 Pharmaspec spectrophotometer system were provided by Agrifood Technology Station, Cape Peninsula University of Technology.

5.2.2 Production of pearl millet flour

Pearl millet flour was produced as reported in Chapter 3, Section 3.2.2 but with slight modifications. Dry pearl millet grains were manually cleaned to remove any seeds, broken grains, sand, twigs and any other foreign objects. Excess water after washing was drained off by spreading the grains on a stainless sieve. The grains were steamed for 15 min at 110ºC in a Butcherquip junior cooker. The grains were washed after steaming using cold tap water and soaked in cold water for 6 h (1:2, grains:water). After soaking the grains were dried at 50ºC for 45 h in a cabinet dryer (Geiger & Klotzbucher). The grains were dry milled into 0.8 mm (Falling number/Kjeldal analysis size) powder using a Perten 3100 hammer mill. The resulting pearl millet flour was kept in a clear zipper bag at 4ºC until required.

5.2.3 Preparation of moringa powder extract

Moringa (*Moringa oleifera*) leaf powder was blended with cold tap water at 1:12 ratio (moringa:water) using a Silverson L4R homogenizer at 7000 rpm for 15 min. The mixture was soaked for 30 min and sieved through 850, 355, 250 and finally 125 µm sieves in a descending order. The extract was spread on freeze-dry trays and frozen for 48 h at -76ºC using an Ultra-freezer (Glacier, -86ºC ultralow temperature freezer). The extract was freeze-dried for 72 h using Virtics SP Scientific 35 XL pilot freeze dyer (freeze mode set to -50ºC). The resulting flakes with 96% moisture loss was placed into stomacher bag

and milled using an AES Smasher for 80 sec. The resulting powder was kept in zipper bags until use.

5.2.4 Production of pearl millet extract (PME), plain non-alcoholic pearl millet beverage (PNAPMB) and moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB)

Pearl millet extract (PME) was prepared similar to Chapter 4, Section 4.2.3 with slight modification. Pearl millet flour [PMF] (400 g) was mixed with 15% sprouted rice flour, 12% ground ginger and cold water at 1:2.5 (PMF:Water) ratio. The paste was homogenized using Silverson L4RT blender for 15 min at 7000 rpm. The paste was left to hydrate for 1 h at 25ºC. The supernatant was decanted and the sediments discarded. The liquid was filtered through a sterile cheese cloth followed by a 5 µm filter bag. The extract was mixed with sunflower lecithin (0.1%), sodium citrate (0.1%), pectin (0.6%) and white sugar (5%). The mixture was blended using Silverson L4RT blender at 7800 rpm for 7 min. The resulting PME was pasteurized for 15 min in a pot at 85°C, followed by bottling in sterile 500 ml Schott bottles and cooled immediately in cold water (≤37ºC). The cooled samples were inoculated with *L. mesenteroides* (0.05%) and *P. pentoseaceus* (0.025%) at 1:0.5 ratio and fermented in an incubator at 37ºC for 18 h. During fermentation samples were drawn at 3 h interval for enumeration of lactic acid bacteria, determination of the optical density, analysis of pH, total titratable acidity and sugars. After fermentation the beverage was chilled at 4ºC. The process used in the production of plain non-alcoholic pearl millet beverage (PNAPMB) is shown in Figure 5.1.

Following this method, moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) was produced by mixing moringa concentrate (4%) with ground ginger (12%) and sprouted rice flour (15%). During fermentation samples were drawn at 3 h interval for enumeration of lactic acid bacteria, determination of the optical density, and analysis of pH, total titratable acidity and sugars. After fermentation the beverage was chilled at 4ºC.

5.2.5 Production of traditional non-alcoholic pearl millet beverage (TNAPMB)

The same method as reported in Chapter 3, Section 3.2.3 was followed with slight modification. Pearl millet flour (PMF) [100 g] was mixed with cold water at 1:1.5 (PMF:Water) and left to hydrate for 3 h. The paste was divided into ¼ (62.5 g) and ¾ (187.5 g). To the ¼ portion, 48% sprouted rice flour, 16% ground ginger and 160% cold water were added and mixed with a plastic spoon. The ¾ portion was gelatinized with boiling water at 1:2.7 (paste:water) ratio and cooled to 40ºC in cold water. The two

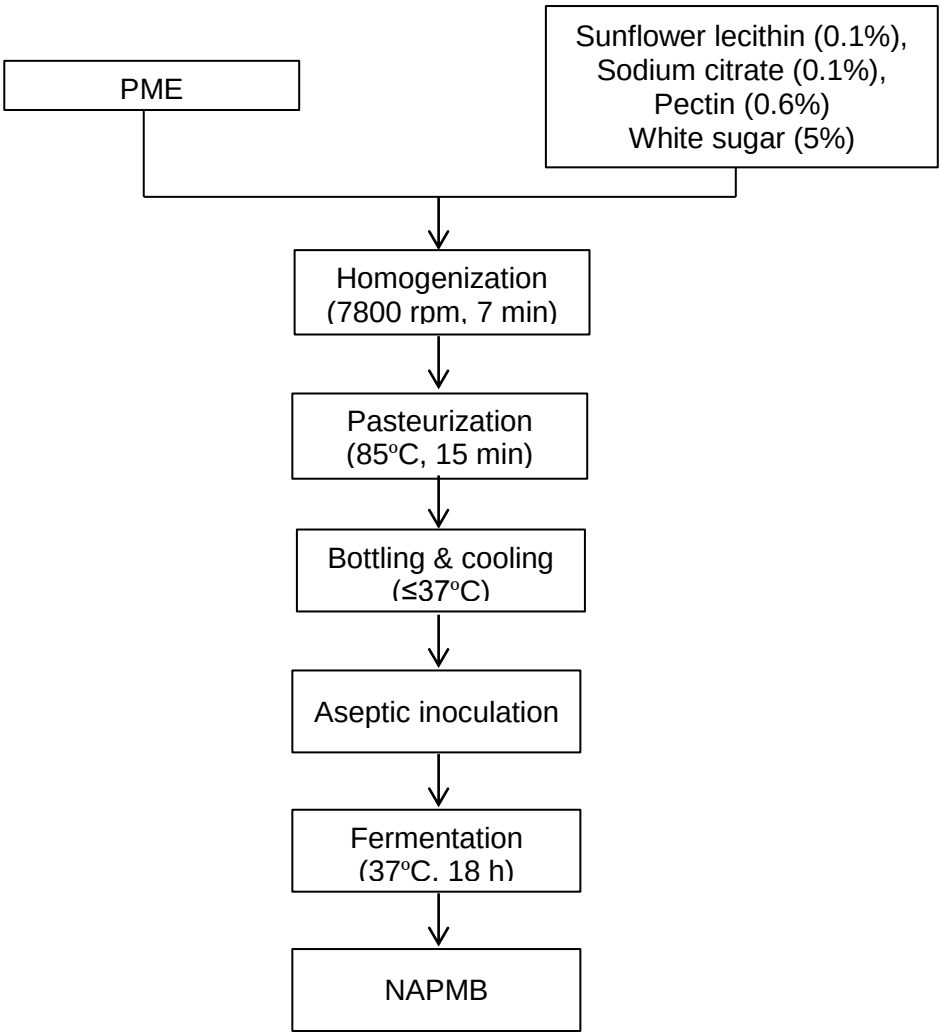


Figure 5.1 Flow diagram for the production of non-alcoholic pearl millet beverage (NAPMB). PME – pearl millet extract

portions were mixed and bottled in sterile 500 ml Schott bottles. The slurry was fermented at 37ºC for 18 h. After fermentation the slurry was sieved through a 106 µm stainless sieve. The filtrate was mixed with cold water at 1:0.5 (filtrate:water) ratio. To the resulting fluid, sugar (6%) and citric acid (0.25%) were added and blended using Silverson L4RT at 2300 rpm for 5 min. The resulting beverage was chilled at 4ºC for sensory evaluation and analysis of viscosity, colour and metabolites. Figure 5.2 shows the production process of TANPMB

5.2.6 Enumeration of lactic acid bacteria in pearl millet extract during fermentation of plain and moringa-supplemented non-alcoholic pearl millet beverages

An aliquot (45 ml) of pearl millet extract (PME) in 100 ml Schott bottles during fermentation of non-alcoholic pearl millet beverage were thoroughly mixed by shaking for 1 min. Pearl millet extract (PME) [10 ml] was transferred to a 10 ml Schott bottle

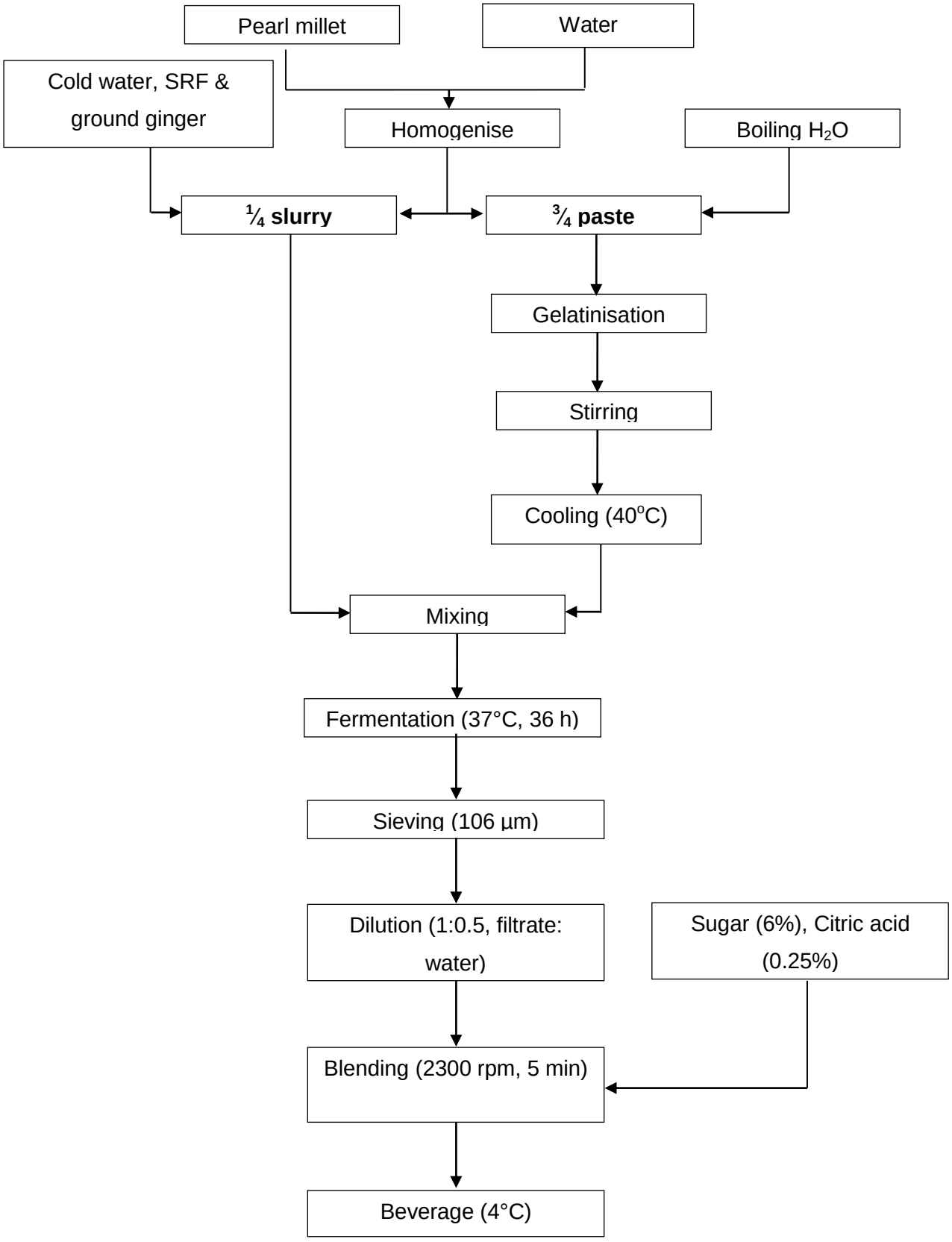


Figure 5.2 Flow diagram for the production of traditional non-alcoholic pearl millet beverage (TNAPMB)

containing 90 ml sterile ¼ strength of Ringer solution (Abegaz, 2007, Kivanc *et al*., 2011) to give 10:100 dilutions followed by a 10 fold serial dilution from 10^{-1} to 10^{-10}. Each dilution was sub-cultured in triplicate. A portion of the sample dilution (1 ml) was added into a 15 x 100 mm plastic Petri plates containing cooled molten deMan Rogosa and Sharpe (MRS) agar [Merck HG00C107.500] (Nwachukwu *et al*., 2010; Temitope & Taiyese, 2012) by means of a pipette and left to solidify (Omemu, 2011). The plates were incubated under anaerobic condition using Anaerobic Gas-Pack system and anaerobic indicator strips at 30ºC for 48 h (Osuntogun & Aboaba, 2004; Nwachukwu *et al*., 2010). All microbiological data were expressed in logarithms of numbers of colony forming unit per ml (log cfu/ml).

5.2.7 Measurement of cells concentration in pearl millet extract by optical density during fermentation of plain and moringa-supplemented non-alcoholic beverages (NAPMB & MSNAPMB)

The growth of lactic acid bacteria (turbidity) was determined using optical density (OD). Pearl millet extract (PME) in 100 ml Schott bottles was thoroughly mixed for 1 min. PME (1 ml) was aseptically transferred into deMan Rogosa and Sharpe (MRS) broth and incubated at 30ºC for 48 h. After incubation, 0.2 ml of the broth was transferred into sterile 3 ml de-ionised water (*d*). The dilution (*d*) was done where necessary (OD above 1.0) since the relationship between microorganisms and OD is non-linear if the OD is above 1.0 (Champagne *et al*., 2007). The sample was mixed by vortexing for 30 sec prior to the OD measurement using UV 1700 Pharmaspec visible spectrophotometer set at 20ºC and 600 nm (OD_{600}) wavelengths (Widdel, 2010). The reference sample used was sterile MRS broth (0.2 ml) mixed with 3 ml sterile de-ionised water (*d*) where necessary. The absorbance was divided by the defined dilution factor (*d*) to get correlated or calculated optical density (OD_{corr}).

5.2.8 Physicochemical analysis of pearl millet extract (PME) during fermentation of non-alcoholic pearl millet beverages (NAPMB)

The pH of pearl millet extract (PMS) [10 ml] during fermentation of non-alcoholic pearl millet beverage (NAPMB) was measured at 3 h interval in triplicates using Hanna Edge (HI - 11310) glass electrode pH meter standardized with pH buffer solution of 4, 7 and 10.

Total titratable acidity (TTA) was assessed at 3 h intervals during the beverage fermentation. The TTA of pearl millet extract during fermentation of NAPMB was determined in triplicates by titrating 10 ml of the sample with 0.1N NaOH using phenolphthalein as indicator until a light pink colour appears. The TTA was expressed as

percentage lactic acid (AOAC, 1980). Equation 5.1 was used to calculate the percentage acidity, with each 0.1M NaOH equivalent to 90.08 mg lactic acid.

$$\text{TTA (\% lactic acid)} = \frac{\text{ml NaOH x N NaOH x M.E}}{\text{volume of sample used x 1000}} \times 100 \qquad 5.1$$

Where, ml NaOH = volume of NaOH (ml), N NaOH = molarity of NaOH, M.E = the equivalent factor of lactic acid being 90.08 mg, 1000 = factor used to convert the M.E which is normally in mg to grams, and 100 used to express the lactic acid concentration in percentage.

5.2.9 Determination of soluble sugars in pearl millet extract during fermentation of plain and moringa-supplemented beverages (NAPMB & MSNAPMB)

The method of AOAC 982.14 as described by Li *et al*. (2002) was used to determine the soluble sugars in pearl millet extract (PME) during fermentation of plain and moringa supplemented non-alcoholic beverages. Sugar extraction was done by mixing 5 g (W_1) of the pearl millet slurry (PMS) with 100 ml (W_2) of 50% ethanol. The mixture was heated in a water bath for 25 min at 85ºC while shaking at 25 rpm to break-up and dissolve the sample. The mixture was cooled to 25ºC ethanol (95%) was used to bring the sample weight to original weight (W_2). The sample was filtered through a 0.45 µm nylon syringe into a 1.5 ml clear screw neck high performance liquid chromatography (HPLC) sample vial. The total sugar content of the extracts was determined in triplicates using HPLC (Agilent 1100 HPLC – RID system) equipped with Zorbax carbohydrates column (4.6 × 150 mm, 5 µm) and Zorbax NH_2 guard column (4.6 × 12.5 mm, 5 µm). The mobile phase used was acetonitrile mixed and de-gassed with milipore distilled water at 75:25 (acetonitrile:water) ratio. The sugar standards were prepared by mixing sucrose (6 mg/ml), fructose (6 mg/ml), glucose (6 mg/ml), maltose (6 mg/ml), lactose (6 mg/ml) and sucrose (30 mg/ml) in a water/ethanol (50:50) solution. The resulting stock solution was used to prepare concentration solutions used for the calibration curve. The concentration used to draw a standard curve were 0.375 (1.875) mg/ml, 0.75 (3.75) mg/ml, 1.5 (7.5) mg/ml and 3.0 (15.0) mg/ml. The values in parenthesis show the sucrose concentration in each solution.

5.2.10 Proximate analyses of plain, moringa-supplemented and traditional non-alcoholic pearl millet beverages

The moisture and ash content in plain, moringa-supplemented and traditional cereal beverages were determined using the oven and muffle furnace method, respectively

(AOAC, 2005). The protein was determined using nitrogen analyser (Leco- TruSpec-N) with furnace set at 950ºC. The protein factor used was 6.31 for millet. Fat was determined using AOAC 996.06 (2005) 18th edition. Total dietary fibre (TDF) was determined using the Fibertec system method. The carbohydrates were determined by difference.

5.2.11 Colour measurement of plain, moringa-supplemented and traditional beverages

A Konica Minolta spectrophotometer (CM-5) was used to measure the colour of plain, moringa-supplemented and traditional non-alcoholic pearl millet beverages. The equipment was set at 10º/D65 illuminate and prior to any measurement the instrument was zero calibrated using the black tile (L* = 5.49, a* = -7.08, b* = 4.66) and white tile (L* = 93.41, a* = -1.18, b* = 0.75). The beverage (10 ml) was poured into a 30 mm diameter Petri dish and the reflectance measured in terms of L*, a* and b*. Each measurement was done three times by doing a quarter-turn of the sample and each sample was measured in triplicates. L* indicates the lightness of the beverage, a* represent the red/greenish of the beverage and b* shows the yellowish/blue of the beverage coordinates. The change in L*, a* and b* can either be negative or positive where -L* = lighter, +L* = darker, -a* = greener and +a* = redder and –b* = blue and +b* = yellow. Chroma (C) shows the quality that differentiates a pure hue from a grey shade and describes the hue saturation or purity. The total colour difference (ΔE) of the beverage was calculated using Equation 5.2. The change in colour is the numerical comparison of the PNAPMB and MSNAPMB to the TNAPMB (control). A colour difference of 1 is defined as just-noticeable difference at which a trained evaluator will notice the differences in colour. If the ΔE is between 4 and 8 the samples are deemed acceptable (Murenanhema, 2012).

$$\Delta E = \sqrt{\left[\left(\Delta L^{*}\right)^{2} + \left(\Delta a^{*}\right)^{2} + \left(\Delta b^{*}\right)^{2} \right]} \qquad 5.2$$

5.2.12 Extraction and identification of volatile compounds in PNAPMB, MSNAPMB and TNAPMB using methanol as a solvent

Aliquot samples (200 ml) of plain non-alcoholic pearl millet beverage (PNAPMB), moringa-supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB) were separately poured in 600 ml freeze-dry jars and frozen at -76°C for 12 h in an ultra-freezer (Glacier -86ºC ultralow temperature freezer) and freeze dried using BenchTop-Pro with omnitronics (VirTis SP Scientific) for

120 h. The dried samples were then milled using AES Smasher XL homogenizer for 40 sec and stored in zipper bags. The method of Azizan *et al*. (2015) was used with slight modification to extract and determine the volatile compounds. Volatile compounds were extracted by mixing 1 g of the beverage powder with cold (5°C) methanol/mili-Q water ($MeOH/H_2O$, 80/20 v/v). The mixture was sonicated for 5 min in ice water using Sonicator cell disruptor (Heat system-ultrasonic INC). Sonicator cell disruptor, (Model W-225R) with H-1 probe set at 7 output, duty cycle at 50 and set on continuously mode. The mixture was then mixed by vortex at high speed for 1 min and filtered through a 0.45 µm nylon syringe into a 1.5 ml clear screw neck Gas-Chromatography Mass-Spectrometry (GC-MS) sample vials.

The volatile compounds of the extracts were determined in duplicates using Agilent Technologies 7890B GC-MS system equipped with HP-5 MS column (5% Phenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm × 0.25 µL). The carrier gas (helium) was set at 0.6 mL/min flow rate, pressure at 3.5105 psi, average velocity at 28.502 cm/sec, hold time at 1.7542 min and the oven temperature at 70°C. The temperature was set to increase at 1°C/min to 76°C after that at 6°C/min to 300°C. The scanning mode was set at a mass range of 50 - 500 m/z, with a solvent delay of 7 min. The spit-splitless inlet was set at a temperature of 250°C, pressure at 3.5105 psi, total flow at 33.6 ml/min and septum purge flow at 3 ml/min. The samples were injected at 50:1 ratio and split flow at 30 ml/min. The peaks were identified using National Institute of Standard and Technology 14 (NIST - 14) mass spectra library.

5.2.13 Sensory evaluation of non-alcoholic pearl millet beverages (PNAPMB, MSNAPMB and TNAPMB)

A total of 50 consumer panellists above 18 years of age were drawn from the Cape Peninsula University of Technology (staff and students). The sensory evaluation was carried out in the sensory laboratory at 25°C. Plain non-alcoholic pearl millet beverage (PNAPMB), moringa-supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditionally non-alcoholic pearl millet beverage (TNAPMB) were prepared 24 h prior to the evaluation and chilled at 4°C. Aliquots (40 ml) of the beverages (PNAPMB, MSNAPMB and TNAPMB) were each served in a white polystyrene foam cup (250 ml) coded with a three-digit random number at 4 - 6°C alongside each other (Figure 5.3). The panellists were asked to sign an approved informed consent form regarding ethical protocol (Appendix A) of research involving Human subjects before starting the evaluations (Ethic approval attached in Appendix B). The panellists were instructed to assess the samples for appearance, colour, aroma, taste, mouthfeel and overall acceptability and rate their preference on 9-point hedonic scale (1 – dislike extremely, 2 – dislike very much, 3 –

Figure 5.3 Coded non-alcoholic pearl millet beverages served to panellists

dislike moderately, 4 – dislike slightly, 5 – neither like nor dislike, 6 – like slightly, 7 – like moderately, 8 – like very much and 9 – like extremely) [Appendix C]. The panellists were asked to rate each sample on own merit based on the attributes and not compare the samples.

5.2.14 Classification of sentiments during the evaluation of non-alcoholic pearl millet beverages (PNAPMB, MSNAPMB and TNAPMB)

Word clouds were used to visually present, identify trends and pattern of the comments from the panellists to make it easier to see word frequencies. Word clouds are graphical representation of the most frequent words that give greater prominence to words that are appearing in a source of text. Most frequent words are displayed larger and in bold. However, the word clouds emphasised on the frequencies of the words but not their importance (negative or positive sense). Thus, sentiment analysis (negative, positive or neutral) was used to discern the meaning/thought of each word from the panellist.

5.2.15 Data analysis

The results reported are mean standard deviation (±) of three independent trials unless stated. Multivariate analysis of variance (MANOVA) was used to determine mean difference between treatments at p = 0.05. Duncan's multiple range tests was used to separate means where differences existed using IBM SPSS ver 23 (IBM, 2015). Principal component analysis (PCA) was used to summarize and uncover any patterns in the fermentation data set by reducing the complexity of the data (The Unscrambler X10.4).

5.3 Results and Discussion

5.3.1 Effect of fermentation time on the viability of lactic acid bacteria (*Leuconostoc mesenteroides* and *Pediococcus pentoseace)* from pearl millet extract during fermentation of plain- and moringa-supplemented non-alcoholic pearl millet beverages (PNAPMB & MSNAPMB)

The effect of fermentation time on the survival of lactic acid bacteria (LAB) [interaction of *Leuconostoc mesenteroides* and *Pediococcus pentoseace*] is shown in Figure 5.4. The growth of the interaction of *Leuconostoc mesenteroides* and *Pediococcus pentoseace* in plain and moringa-supplemented cereal beverages ranged from 3.32 ± 0.04 to 7.97 ± 0.13 cfu/ml and 3.58 ± 0.00 to 8.38 ± 0.02 log cfu/ml, respectively. Initial LAB in plain non-alcoholic pearl millet beverage (PNAPMB) was 3.32 ± 0.004 cfu/ml and significantly increased to 7.96 ± 0.08 cfu/ml in 12 h. The lag phase was not visible as the cells immediately grew exponentially. The cells did not take long to adapt to the new environment. Thereafter, the growth of LAB was not significant between 12 and 15 h. At this point the available nutrients were being depleted and bacteria started to compete for remaining nutrients. *Leuconostoc mesentoroides* which stops growing at a pH of 4.0 - 4.5 could also have been halted as the pH was between 4.71 ± 0.21 and 4.13 ± 0.01. The cells decreased from 8.16 ± 0.02 (15 h) to 7.97 ± 0.13 cfu/ml (18 h). Death phase was setting in after all the nutrients were used up.

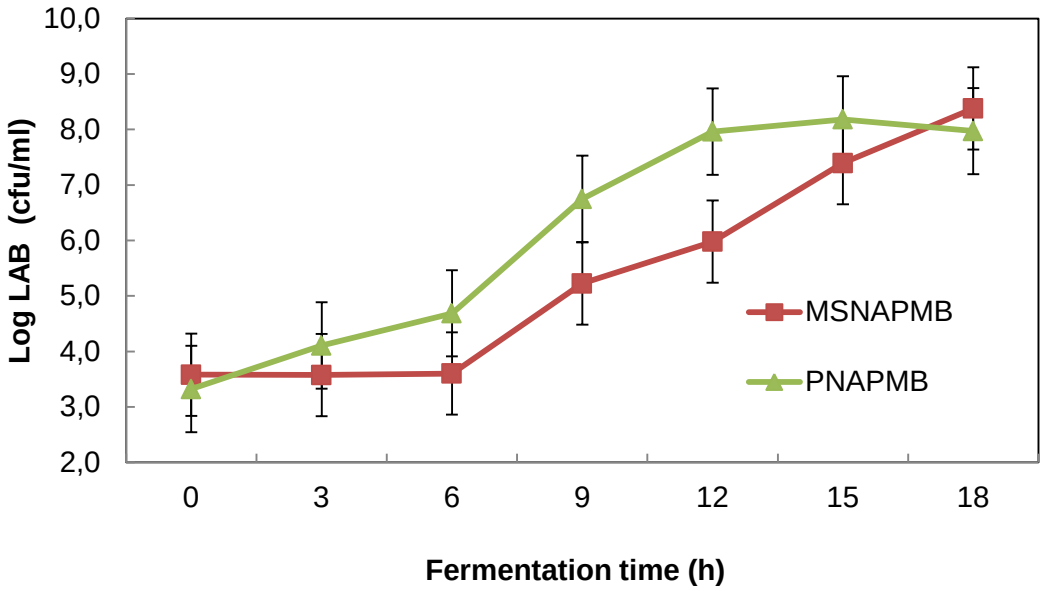


Figure 5.4 Changes in the Lactic acid bacteria (*Leuconostoc mesenteroides* and *Pediococcus pentoseace*) during the fermentation of pearl millet extract for the production of non-alcoholic pearl millet beverage. PNAPMB – plain non-alcoholic pearl millet beverage. MNAPMB – moringa non-alcoholic pearl millet beverage

In moringa-supplemented non-alcoholic pearl millet beverage (MSNAPMB), the LAB started at 3.58 ± 0.00 cfu/ml and remained stationary for 6 h followed by an exponential growth ($p < 0.05$) till a total of 8.38 ± 0.23 log cfu/ml was reached. The cells could have been in lag phase adapting to the new environment during the first 6 h. This indicated that the LAB had to adapt to the MSNAPMB. Thereafter, the cells multiplied at maximal rate utilizing the available nutrients in the beverage. After 18 h, the LAB in MSNAPMB exceeded that of PNAPMB by 0.41 cfu/ml. These results were in agreement with Simango (2002) who reported a sharp increase of LAB in the first 6 h of fermentation and thereafter remained the same during the fermentation of *Mahewu,* a non-alcoholic fermented cereal beverage. The total LAB cells after 18 h were 10^8 and 10^7 cfu/ml in MSNAPMB and PNAPMB, respectively, which is ideal for organisms to confer a health benefits to hosts. However, few studies reported *Moringa oleifera* to have anticynobacterial agent which inhibit the growth of desirable bacteria in food. Since no death phase was visible in MSNAPMB, this could mean that *Moringa oleirefa* supported the growth of LAB, which is in agreement with Hekmat *et al*. (2015) who reported positive effects of *Moringa oleifera* on the survival of *Lactobacillus rhamnosus* GR-1 in yoghurt

with added sugar and MRS broth. The report suggested that added sugar could have acted as a food source to the LAB and countered the antimicrobial effect of *Moringa oleifera*. In addition, this could mean that MSNAPMB can be fermented beyond 18 h should a sour beverage be desired.

5.3.2 Effect of fermentation time on the turbidity in plain and moringa-supplemented non-alcoholic pearl millet beverages

The changes in optical density (turbidity) during the fermentation of pearl millet extract during the production of non-alcoholic cereal beverages is shown in Figure 5.5. The optical density of plain non-alcoholic pearl millet beverage (PNAPMB) ranged from 5.385 ± 0.002 to 4.967 ± 0.004. Fermentation time had a significant effect on the change ($p < 0.005$) in turbidity of PNAPMB. Initial optical density (OD) was 5.385 ± 0.002 and increased significantly to 8.090 ± 0.008 in 3 h. A similar trend of microorganisms increasing significantly from the onset followed by a decrease was reported by Kunasundari *et al*. (2017) during the fermentation of rice starch by *Geobacillus stearothermophilus* which breaks starch directly to lactic acid. *G. stearothermophilus* increased drastically from 9 – 27 h of incubation period and declined after 48 h. Similar to enumeration of lactic acid bacteria (Section 5.4.1), the cells did not take long to adapt to the beverage. Thereafter, the OD decreased until 4.967 was reached in 18 h. The pH could have halted the growth of *Leuconostoc mesentoroides* and the nutrients being all used up during this period. The turbidity of moringa supplemented non-alcoholic pearl millet beverage changed significantly ($p < 0.005$) during the 18 h fermentation. Initial optical density was 5.982 ± 0.011 and slightly decreased to 5.806 ± 0.010 in 3 h. At this point the organisms were still adjusting to the beverage with added moringa extract. There was no replication of cells and injured cells were still recovering. In addition, the cells could have been increasing in size rather than doubling. After 3 h the cells increased significantly ($p < 0.05$) until an OD of 6.421 ± 0.010 (6 h). The LAB were utilizing the nutrients and doubling, reducing the pH of the beverage. After 6 h of fermentation, the LAB decreased to 5.400 ± 0.026. The reduced pH could have halted the growth *L. mesentoroides*. The increase in OD between 12 and 15 h could have been due to the growth of *P. pentoceaus* which tolerated low pH than *L. mesentoroides*. Thereafter, the OD was reduced to 4.732 ± 0.004. The surviving *Leuconostoc* and *Pediococcus* could have been dying due to the lower pH and depleted nutrients.

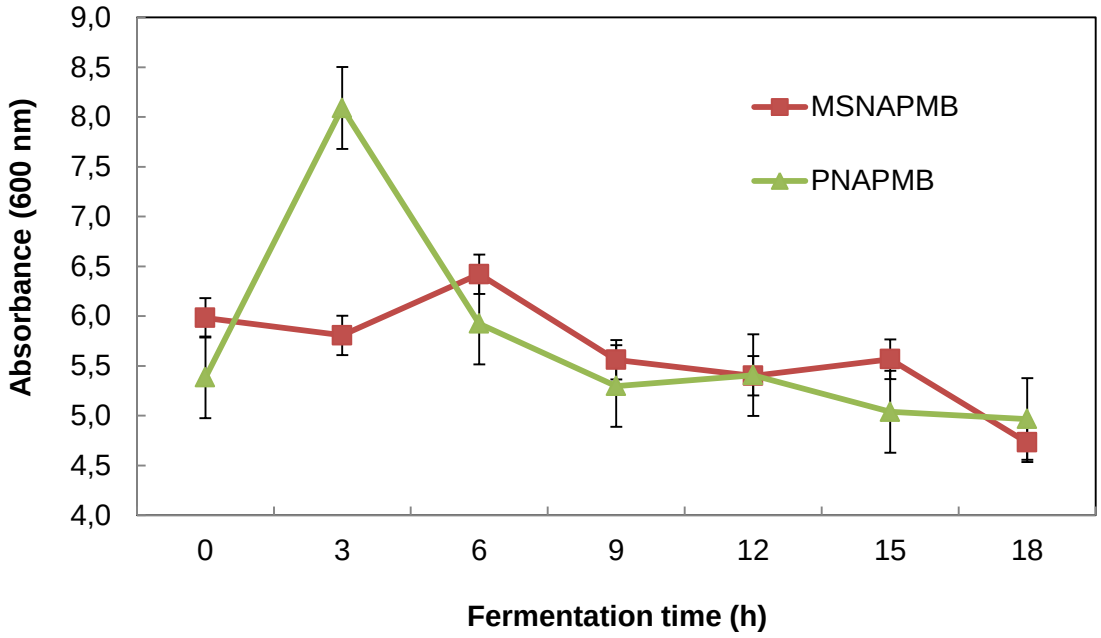


Figure 5.5 Effect of fermentation on the optical density of lactic acid bacteria during the fermentation of pearl millet extract for the production of non-alcoholic pearl millet beverage. PNAPMB – plain non-alcoholic pearl millet beverage. PNAPMB – plain non-alcoholic pearl millet beverage. MNAPMB – moringa non-alcoholic pearl millet beverage.

5.3.3 Effect of fermentation time on the pH and total titratable acidity (TTA) of pearl millet extract during fermentation of non-alcoholic pearl millet beverage (NAPMB)

The effect of fermentation time on the pH and total titratable acidity of pearl millet extract during the production of plain and moringa supplemented non-alcoholic pearl millet beverages is shown Figure 5.6. There was a significant change in the pH of plain non-alcoholic pearl millet beverage (PNAPMB) during the 18 h fermentation period from 4.14 ± 0.01 to 5.05 ± 0.01. The pH did not decrease significantly from the onset of fermentation until 12 h of fermentation elapsed due to the lactic acid bacteria (LAB) which were adapting to the beverage. After inoculation the LAB was in lag phase recovering from any injury and increasing in size but not number. After 12 h the pH decreased significantly to 4.14 ± 0.01 which could be due to the LAB and enzymes breaking down the starch in pearl millet into simpler sugar. The released monomeric sugars were utilised in the production of lactic acid which depressed the pH. Similarly, there was a significant decrease in pH of MSNAPMB after 12 h to 3.65 ± 0.01. However, the pH was lower in

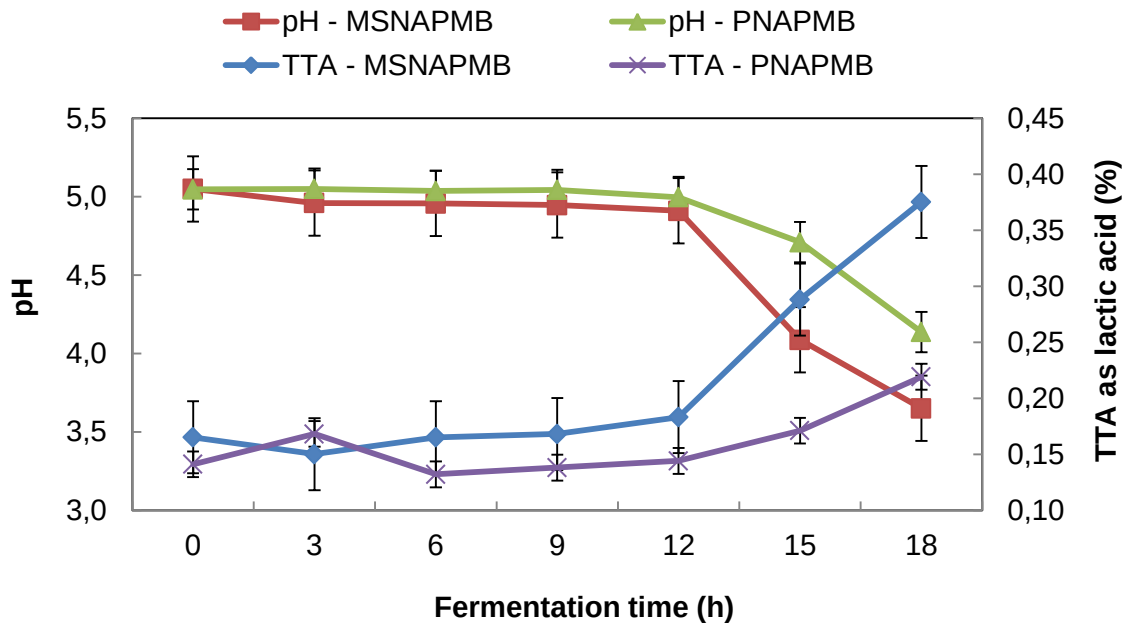


Figure 5.6 Changes in the pH and total titratable acidity of pearl millet extract during fermentation for the production of plain- and moringa-supplemented non-alcoholic pearl millet beverages. PNAPMB – plain non-alcoholic pearl millet beverage. MNAPMB – moringa non-alcoholic pearl millet beverage. TTA – total titratable acidity

MSNAPB compared to PNAPMB which could be due to moringa extract powder which supported the growth of bacteria. The total titratable acidity (expressed as % lactic acid) during the 18 h fermentation period ranged from 0.14 ± 0.01 to 0.22 ± 0.01% and 0.17 ± 0.01 to 0.38 ± 0.04% in PNAPMB and MSNAPMB, respectively. The total titratable acidity (TTA) did not significantly change from the onset of fermentation until 12 h has elapsed. At this point lower amount of lactic acid was produced by LAB. After 12 h, the TTA of PNAPMB and MSNAPMB increased significantly to 0.22 ± 0.01% (18 h) and 0.38 ± 0.04% (18 h), respectively. This was caused by the decrease in pH which increased the acid content of the beverages. The decrease in pH and increase in TTA is in agreement with Magala *et al*. (2015) who reported a decrease in pH from the range of 5.04 - 5.17 to 3.74 – 4.35 and an increase in TTA from 1.28 to 2.59 g/l during the fermentation of rice flour using various lactic acid bacteria. In short, the pH was inversely proportional to the TTA and decreased from 5.05 to 4.14 while the TTA increased from 0.14 to 0.22%.

5.3.4 Effect of fermentation time on the sugar content in pearl millet extract during fermentation of plain, moringa-supplemented non-alcoholic beverages

Sucrose was the main sugar identified in pearl millet extract during fermentation. The sucrose ranged from 4.93 ± 0.12 to 5.48 ± 0.10% and 4.65 ± 0.03 to 5.33 ± 0.12% in plain and moringa supplemented non-alcoholic pearl millet beverage, respectively during the 18 h of fermentation time. The significant change in sucrose content during the 18 h fermentation is shown in Figure 5.7. In plain non-alcoholic pearl millet beverage (PNAPMB) sucrose content significantly decreased from 5.26 ± 0.06% (9 h) to 4.93 ± 0.12% (15 h) followed by an increase to 5.26 ± 0.10% (18 h). There was a significant decrease in sucrose content of moringa supplemented non-alcoholic pearl millet beverage (MNAPMB) between 3 (5.32 ± 0.06%) and 6 h (5.04 ± 0.09%), 9 (5.13 ± 0.07%) and 15 h (4.65 ± 0.03%), and thereafter it significantly increased to 5.33 ± 0.12% (18 h). Parawira *et al*., (2012) also reported a decrease in sugar (Brix) from the onset during the fermentation of *Urwangwa*, a Rwandanese banana beer. The decrease in sucrose could be caused by the utilisation of sugars by lactic acid bacteria (LAB) to produce lactic acid. The apparent increase after 15 h could be due to the inactivity of *L. mesentoroides* when the pH was depressed succeeded by *P. pentoceaus* alone. These results are in agreement with the pH and total titratable acidity which did not significantly change from 0 to 12 h of the fermentation period. This indicated the LAB were still adjusting to the new environment after inoculation. In addition, the sucrose content was higher in MNAPMB than in PNAPMB which also indicated that moringa favoured the growth of LAB. Therefore, during anaerobic fermentation carried out by *L. mesenteroides* and *P. pentoseace* sucrose is broken down to release energy and lactate.

5.3.5 Proximate composition of plain, moringa-supplemented and traditional non-alcoholic beverages

The proximate composition of plain non-alcoholic pearl millet beverage (PNAPMB), moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB) is shown in Table 5.1. The moisture content differed significantly ($p < 0.05$) between the beverages and was 91.74 ± 0.10%, 91.03 ± 0.04 and 87.59 ± 0.06% in PNAPMB, MSNAPMB and TNAPMB, respectively. This is due to the volume of water added during the production of the beverages. The ash content was 2.00 ± 1.55%, 1.56 ± 0.67 and 1.18 ± 0.49% in PNAPMB, MSNAPMB and TNAPMB, respectively. The lower ash content in MSNAPMB could be due to the utilization of minerals element by *L. mesenteroides* and *P. pentoseace (*lactic acid bacteria) during fermentation. The lower ash content could also be due to the lactic acid

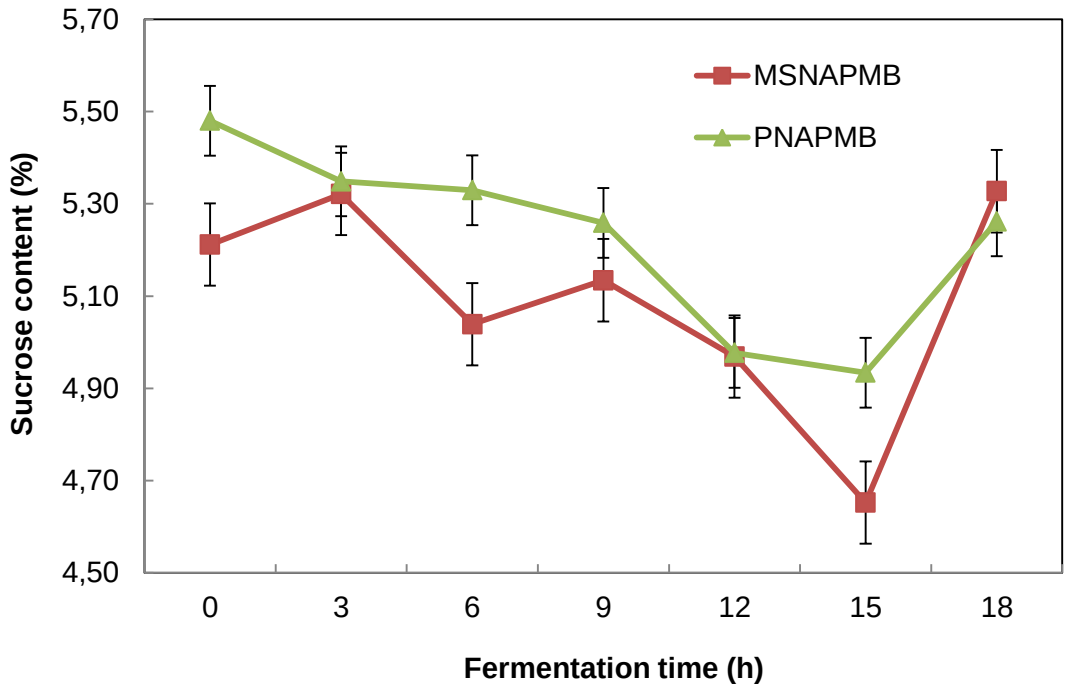


Figure 5.7 Effect of fermentation time on the sucrose content in pearl millet extract during the preparation of plain and moringa supplemented non-alcoholic pearl millet beverages. PNAPMB – plain non-alcoholic pearl millet beverage. MNAPMB – moringa non-alcoholic pearl millet beverage

bacteria whose enzymatic activity resulted in the breakdown of the beverage components into absorbable forms caused by enrichment of the beverage with moringa leaf flour (Igbabul *et al*., 2014). Nour & Ibrahim (2014) reported the decrease in ash content caused by supplementation of beverage with moringa leave powder.

The protein content differed significantly ($p < 0.05$) between PNAPMB and MSNAPMB and was 1.62 ± 0.18% and 2.17 ± 0.02%, respectively. TNAPMB had the lowest protein content of 1.50 ± 1.17% in comparison to PNAPMB and MSNAPMB. The higher proteins in MSNAPMB could be attributed to moringa leaf extract which contains about 19.95% protein as reported by Garba *et al.* (2015). However, protein content of 2.17% in MSNAPMB was lower than that reported by Olosunde *et al*. (2014) [4.63 ± 0.26% crude protein] at 5% moringa seed flour. This could be due to the level of moringa used and/or moringa seed flour instead of moringa leaf flour. This is also an indication that the increase in moringa level caused an increase in protein. This is supported by Nour & Ibrahim (2014) and Abioye & Mo (2015) who reported that the addition of moringa seed flour and moringa leaf flour increased the protein content of pearl millet flour and maize-*ogi*, respectively. In addition, the increase of protein content could be related to the solubilisation of insoluble proteins of raw pearl millet and rice flour and synthesis of protein

Table 5.1 Proximate composition (g/100 ml beverage) of PNAPMB, MSNAPMB and TNAPMB

Nutrient	Proximate composition* (%)		
	PNAPMB	**MSNAPMB**	**TNAPMB**
Moisture	91.74 ± 0.10[a]	91.03 ± 0.04[b]	87.59 ± 0.06[c]
Ash	2.00 ± 1.55[a]	1.56 ± 0.67[a]	1.18 ± 0.49[a]
Protein	1.62 ± 1.62[a]	2.17 ± 0.02[a]	1.50 ± 1.17[b]
Total fats	0.92 ± 0.01[a]	0.65 ± 0.01[a]	1.54 ± 0.09[a]
Saturated fats	0.23 ± 0.00[a]	0.16 ± 0.02[a]	0.48 ± 0.01[a]
Palmitic acid (C_{16})	0.19 ± 0.01	0.14 ± 0.00	0.41 ± 0.03
Stearic acid (C_{18})	0.05 ± 0.00	0.12 ± 0.03	0.07 ± 0.02
Monounsaturated fats	0.24 ± 0.00[a]	0.17 ± 0.01[a]	0.45 ± 0.03[a]
Oleic acid (C18: 1n9c)	0.237 ± 0.00	0.17 ± 0.01	0.45 ± 0.03
Polyunsaturated fats	0.45 ± 0.00[a]	0.32 ± 0.01[b]	0.61 ± 0.05[a]
Linolelaidic acid (C_{18}: 2n6t)	0.45 ± 0.00	0.32 ± 0.01	0.61 ± 0.05
Total sugars	5.06 ± 0.03	5.31 ± 0.02	6.11 ± 0.06
Sucrose	5.06 ± 0.03[a]	5.31 ± 0.02[b]	3.78 ± 0.08[a]
Glucose	0.00	0.00	2.05 ± 0.03
Fructose	0.00	0.00	0.28 ± 0.02
Carbohydrates	4.31 ± 1.42[a]	5.03 ± 0.66[a]	9.41 ± 0.39[b]
Energy (kJ/100 ml)	113.23 ± 25.36[a]	130.23 ± 12.61[a]	197.48 ± 8.07[b]

* results are expressed as mean ± standard deviations. PNAPMB – plain non-alcoholic pearl millet beverage, MSNAPMB – moringa supplemented non-alcoholic pearl millet beverage and TNAPMB – traditional non-alcoholic pearl millet beverage. Values with different superscripts in each row are significantly ($p < 0.05$) different from each other

by lactic acid bacteria during fermentation (Nour & Ibrahim, 2014; Nour *et al.,* 2016). Non-soluble protein tends to aggregate and settle depending on the pH of the beverage. If these proteins become soluble in the beverage during fermentation the protein content could increase apparently.

The total fat content was 0.92 ± 0.01%, 0.65 ± 0.01% and 1.54 ± 0.09% in PNAPMB, MSNAPMB and TNAPMB, respectively. Saturated fats were high in TNAPMB (0.48 ± 0.01%) in comparison to PNAPMB (0.23 ± 0.00%) and MSNAPMB (0.16 ± 0.02%). The polyunsaturated fats in PNAPMB (0.45 ± 0.00) and TNAPMB (0.60 ± 0.05%) differed

significantly to that of MSNAPMB (0.32 ± 0.01%). The fatty acids identified in the beverages were palmitic acid and stearic acid [saturated fats (SFA)], oleic acid [monounsaturated fats (MUFA)], and linolelaidic acid [polyunsaturated fats (PUFA)] . Oleic acid and palmitic acid were the highest in TNAPMB followed by PNAPMB. MSNAPMB had the highest amount of stearic acid followed by TNAPMB. These fatty acids (SFA, MUFA and PUFA) are prime in pearl millet and occur naturally (Sarita, 2016). The presence of palmitic acid was low in PNAPMB (0.19%) and MSNAPB (0.14%) in comparison to the TNAPMB (0.41 ± 0.03%) and stearic acid was also low in PNAPMB (0.05 ± %) and MSNAPMB (0.02 ± 0.03%) when compared to TNAPMB (0.07 ± 0.02%). Palmitic acid is associated with increased risk of coronary heart diseases and tumors while stearic acid is associated with neutral effect on blood total and low density lipoprotein (LDL) cholesterol levels of 1 – 5. However, these saturated fatty acid are lower in cereal beverages in comparison to yoghurt where palmitic and stearic acid account to 16.54% and 11.73%, respectively (Sumarmono *et al*., 2015). Oleic acid (omega-9) was 0.45 ± 0.03% in TNAPMB, 0.24% in PNAPMB and 0.17 ± 0.01% in MSNAPMB. Oleic acid lowers the risk of heart attacks and artherosclerosis and helps in the prevention of cancer (Win, 2005). Linolelaidic acid is an essential fatty acid which is not synthesised by the body and should be provided through a meal. They are essential in the prevention of diseases related to cardiovascular and cancer (Ovando-Martinez *et al*., 2014). This makes the beverages a source of this essential nutrient. The increase in fat during fermentation could be due to the transformation of carbohydrates to fat, meanwhile, the decrease could be caused by the utilization of fat by lactic acid bacteria present in the beverage during fermentation (Nour & Ibrahim, 2014). In contrast, Olosunde *et al*. (2014) reported an increase in fat content from 1.67 ± 0.10% to 2.20 ± 0.05% when 5% moringa seed flour was added to the beverage. However, Nour & Ibrahim, (2014) reported a decrease in the oil content in fermented sorghum with 5% moringa seed flour. Sarita (2016) also reported that fermentation decreases the long-chain fatty acid content in finger millet.

The beverages differed significantly ($p < 0.05$) in terms of sucrose content which was 5.06 ± 0.03%, 5.31 ± 0.02% and 3.78 ± 0.08% in PNAPMB, MSNAPMB and TNAPMB, respectively. In addition to sucrose identified in TNAPMB, glucose and fructose were identified at 2.05 ± 0.02% and 0.28 ± 0.02%, respectively. The sucrose could mainly be from the added sugar during production of the beverages and available free sucrose found in millet (Bora, 2013). The fibre in the beverages could have been utilized by fermenting LAB (Nour & Ibrahim, 2014).

The carbohydrates (CHO) differed significant ($p < 0.05$) between PNAPMB and TNAPMB, and MSNAPMB and TNAPMB. The CHO in PNAPMB, MSNAPMB and

TNAPMB was 4.31 ± 1.42%, 5.02 ± 0.66% and 9.4 ± 0.39%, respectively. The energy content in PNAPMB, MSNAPMB and TNAPMB was 113.23 ± 25.36 kJ/100 ml, 130.23 ± 12.61 kJ/100 ml and 197.48 ± 8.07 kJ/100 ml, respectively. The energy differed significant ($p < 0.05$) between PNAPMB and TNAPMB, and MSNAPMB and TNAPMB. There was no significant increase in CHO and energy as a result of supplementation with 4% moringa leaf extract. The lack of an increase in CHO and energy could be due to moringa flour which is a poor source of CHO. The higher energy in TNAPMB could be due to the presence in carbohydrates in the beverage. Overall, the proximate composition of all beverages did not differ significantly.

5.3.6 Colour difference of plain and moringa-supplemented non-alcoholic pearl millet beverages in comparison to traditional non-alcoholic pearl millet beverage

The colour of plain non-alcoholic pearl millet beverage (PNAPMB), moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB) is shown in Table 5.2. The L^*, a^* and b^* values between PNAPMB, MSNAPMB and TNAPMB showed that the beverages differ significantly in colour. The values indicated that MSNAPMB is lighter, less reddish and bluer in colour in comparison to the TNAPMB while the PNAPMB is less lighter, more reddish and more yellowish to the TNAPMB. The total colour difference (ΔE) between the MSNAPMB and PNAPMB in comparison to the TNAPMB was 10.60 and 5.91 respectively. The differences in colour of MSNAPMB and PNAPMB in comparison to the TNAPMB samples will be noticed by the consumers, since ΔE is above one for both samples. However, the PNAPMB sample could be acceptable by the consumers since the ΔE falls between 4 and 8. The MSNAPMB may not be acceptable since the ΔE is greater than 8. Figure 5.8 shows the beverages.

5.3.7 Viscosity of plain, moringa-supplemented and traditional non-alcoholic pearl millet beverages over time at different storage conditions (5 and 20°C)

The change in viscosity over time of plain non-alcoholic pearl millet beverage (PNAPMB), moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB) is shown in Figure 5.9. The viscosity at 5°C on average was 3.12 ± 1.12, 42.23 ± 2.10 and 70.67 ± 2.46 mPa.s over 2.58 min in PNAPMB, MSNAPMB and TNAPMB, respectively. The viscosity at 20°C on average was -1.899 ± 1.125, 0.916 ± 0.873 and 54.214 ± 1.696 mPa.s over 2.583 min in PNAPMB, MSNAPMB and TNAPMB, respectively. At both 5 and 20°C, TNAPMB had the highest viscosity followed by MSNAPMB. The viscosity of the beverage decreased when the

Table 5.2 Colour of PNAPMB, MSNAPMB and TNAPMB

Attribute	PNAPMB	MSNAPMB	TNAPMB
L*	58,44 ± 0.05[a]	52,70 ± 0.07[b]	59,67 ± 0.02[c]
*a**	-1,03 ± 0.03[a]	0,64 ± 0.03[b]	2,45 ± 0.19[c]
b*	15,11 ± 0.02[a]	27,48 ± 0.03[b]	19,71 ± 0.05[c]
Lightness*	16,54 ± 0.05[a]	10,81 ± 0.07[b]	17,77 ± 0.02[c]
Chroma (C*)	15.14 ± 0.02[a]	27.49 ± 0.03[b]	19.86 ± 0.08[c]
Hue (h*)	93.91 ± 0.13[a]	88.66 ± 0.06[b]	82.91 ± 0.52[c]
ΔE	5.91	10.60	

*results are expressed as mean ± standard deviations. PNAPMB – plain non-alcoholic pearl millet beverage, MSNAPMB – moringa supplemented non-alcoholic pearl millet beverage and TNAPMB – traditional non-alcoholic pearl millet beverage. Values with different superscripts in each row are significantly ($p < 0.05$) different from each other.

storage temperature was increased. When the temperature of the beverage was increased, the molecular interchange within the beverage also increased. As the molecular interchange increased the molecules became excited and started to move faster. In the beverage, there are substantial attractiveness of molecules and cohesion forces between the molecules which contributes to the viscosity of the beverage. Hence, when the temperature was increased the cohesive forces decreased while simultaneously increasing the rate of molecular interchange. Thus, the increase in temperature caused a decrease in the shear stress, and the decrease in temperature caused an increase in the shear stress. This resulted in lower viscosity of the beverage at higher temperature and higher viscosity at lower temperature. This indicates that those who prefer a thicker beverage could store the beverages at room temperature.

5.3.8 Characterisation of chemical composition and colour of non-alcoholic cereal beverages using principal component analysis (PCA)

The proximate composition and colour parameters of the plain non-alcoholic pearl millet beverage (PNAPMB), moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB), were subjected to principal components analysis (PCA). The variations in the data could be

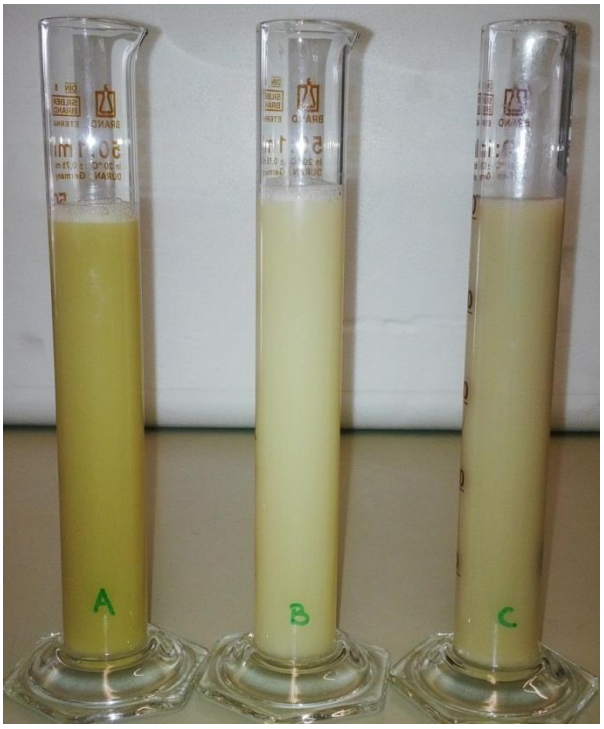


Figure 5.8 Non-alcoholic pearl millet beverages. A: MSNAPMB – moringa supplemented non-alcoholic pearl millet beverage. B: PNAPMB – plain non-alcoholic pearl millet beverage and C: TNAPMB – traditional non-alcoholic pearl millet beverage.

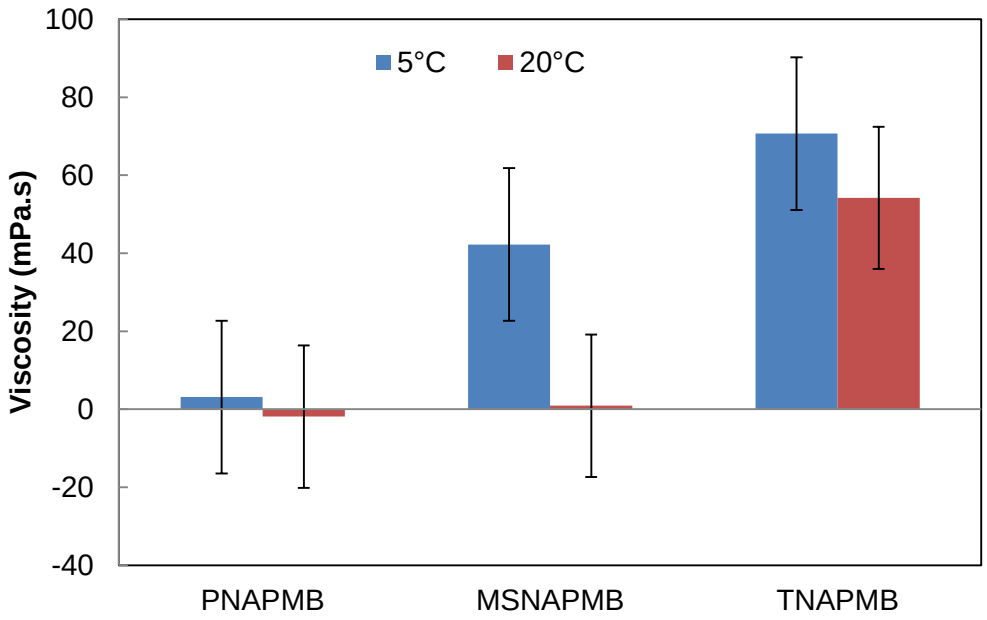


Figure 5.9 Changes in viscosity of PNAPMB, MSNAPMB and TNAPMB at 5 and 20°C. PNAPMB – plain non-alcoholic pearl millet beverage, MSNAPMB – moringa supplemented non-alcoholic pearl millet beverage and TNAPMB – traditional non-alcoholic pearl millet beverage

explained by two principal components (PC1 and PC2). The cumulative variation was 82% with much variation (51%) contributed by PC1 and 31% by PC2. PC1 was highly correlated to MSNAPMB high in moisture, total sugar, proteins, hue and yellowish/blue (b*). Furthermore, PC2 was highly correlated to PNAPMB having high total fat, saturated fat, total sugar, ash, moisture and chroma. Figure 5.10 shows the scores (beverages) and loadings (proximate composition and colour parameters) of the non-alcoholic cereal beverages. This indicated that the variations in characteristics of PNAPMB and MSNAPMB could be explained using total fat, saturated fat, total sugar, ash, moisture, proteins, chroma (C), hue and b*.

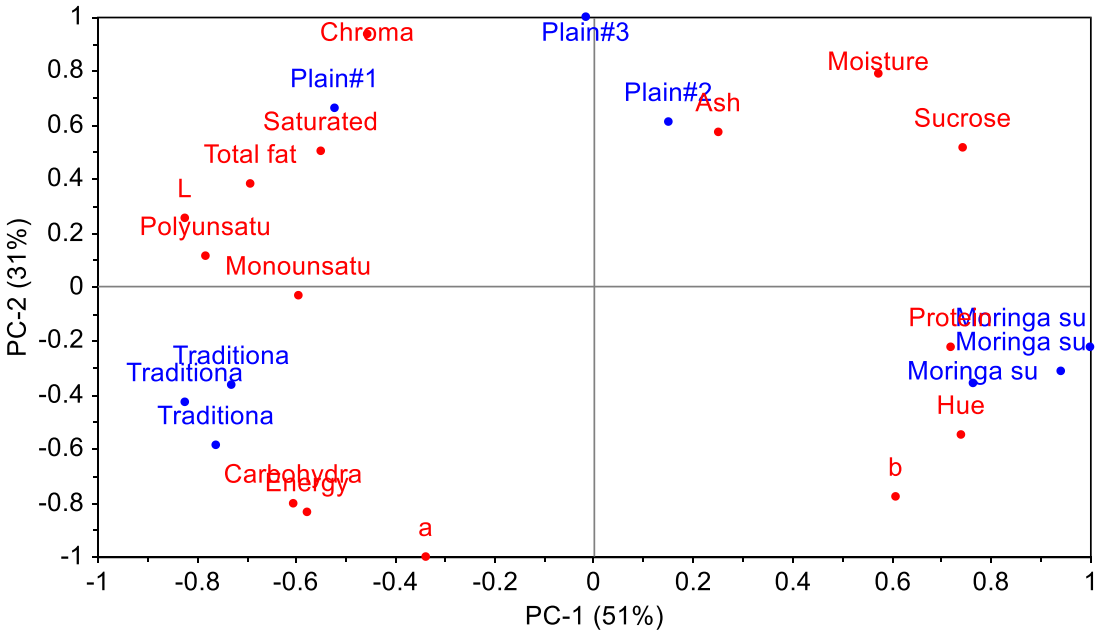


Figure 5.10 Principal component analysis (PCA) score plot for non-alcoholic pearl millet beverages in terms of their chemical composition and colour

5.3.9 Volatile compounds in PNAPMB, MSNAPMB and TNAPMB

The volatile compounds identified in PNAPMB, MSNAPMB and TNAMPB included sugars, alcohols, alkanes, ketones, esters, fatty acids, carbonyl compounds and organic acids. The identified major compounds with their retention time, molecular formula and molecular weight are shown in Table 5.3. The chromatograms for the samples are shown in Figure 5.11 between the abundance and retention time. In PNAPMB the compounds identified between 10 and 43 min of the retention times include: 3H-pyrazol-3-one (10.778 min), 3,4-furandiol, tetrahydro-, trans- (12.758 min), 2,4-dihydro-2,4,5-trimethyl (12.798 min), , DL-arabinose (12.798 min), 5-hydroxymethylfurfural (15.332 min, melezitose (15.367 min), 2-ethyl-oxetane (20.791 min), lactose (21.175 min), 3-deoxy-d-mannoic

Table 5.3 Compounds tentatively identified in methanol extract of PNAPMB, MSNAPMB and TNAPMB

Compound	Chemical formula	Retention time (min)	Molecular weight	Nature of compound
PNAPMB				
3H-pyrazol-3-one, 2,4-dihydro-				
2,4,5-trimethyl	$C_6H_{10}N_{2O}$	10,778	126,079	alcohol
DL-arabinose	$C_5H_{10}O_5$	12,798	150,053	sugar
Melezitose	$C_{18}H_{32}O_{16}$	15,367	504,169	sugar
Lactose	$C_{12}H_{22}O_{11}$	21,175	342,116	sugar
3-deoxy-d-mannoic lactone	$C_6H_{10}O_5$	24,465	162,053	ester
3,4-Furandiol, tetrahydro-,				
trans-		12,758	104,047	
5-Hydroxymethylfurfural		15,332	126,032	aldehyde
N,N'-dibutyl-N,N'-dimethyl-		20,791	200,189	
2-Ethyl-oxetane		21,163	86,073	
Sucrose		32,247	342,116	
Tetrasiloxane, decamethyl-		42,175	310,127	
Methyltris(trimethylsiloxy)silane		42,329	310,127	
MSNAPMB				
Clindamycin	$C_{18}H_{33}ClO_5S$	10,806	424,18	
D-mannopyranose	$C_6H_{12}O_6$	12,74	180,063	
D(+)-talose	$C6H_{12}O_6$	12,797	180,063	sugar
Melezitose	$C_{18}H_{32}O_{16}$	15,418	504,169	sugar
6,10,13-trimmeltetradecanol	$C_{17}H_{36}O$	18,548	256,277	fatty alcohol
Lactose	$C_{12}H_{22}O_{11}$	21,151	342,116	sugar
3-deoxy-d-mannoic lactone	$C_6H_{10}O_5$	24,196	162,053	ester
4,5-Diamino-2-				
hydroxypyrimidine		10,783	126,054	
1,3,5-Triazine-2,4,6-triamine		16,202	126,065	

Table 5.3 Compounds tentatively identified in methanol extract of PNAPMB, MSNAPMB and TNAPMB (continued)

Compound	Chemical formula	Retention time (min)	Molecular weight	Nature of compound
MSNAPMB				
2-Ethyl-oxetane		21,197	86,073	
3-Deoxy-d-mannoic lactone		23,841	162,053	
beta-D-Glucopyranose, 4-O-beta-D-galactopyranosyl-		32,241	342,116	
TNAPMB				
D-mannopyranose	$C_6H_{12}O_6$	12,689	180,063	sugar
3,4-furandiol, tetrahydro-,trans	$C_4H_8O_3$	13,084	104,047	alcohol
Isosorbide dinitrate	$C_4H_8N_2O_8$	14,36	236,028	
D-fructose, 1,3,6-trideoxy-3,6-epithio	$C_6H_{10}O_3S$	20,539	162,035	sugar
Melezitose	$C_{18}H_{32}O_{16}$	23,801	504,169	sugar
Hexadecanoic acid	$C_{16}H_{33}O_2$	29,512	256,24	saturated fatty acid
3-(prop-2-enoloxy) dodecane	$C_{11}H_{14}O$	31,508	240,209	alkeny
Tetradecane, 2,6,10-trimethyl	$C_{17}H_{36}$	33,208	336,303	
Undec-10-ynoic acid, undercyl ester	$C_{22}H_{40}O_2$	32,229	240,282	
Methoxyacetic acid, 2-tetradecyl ester	$C_{17}H_{34}O_3$	33,208	286,251	
Octatriacontyl pentafluoropropionate	$C_{41}H_{77}F_5O_2$	34,392	34,392	
2-hexyl-1-octanol	$C_{14}H_{30}O$	34,644	214,23	fatty alcohol
Eicosane	$C_{20}H_{42}$	35,09	282,329	alkane
Eicosane, 7-hexyl	$C_{26}H_{54}$	36,012	366,423	

Table 5.3 Compounds tentatively identified in methanol extract of PNAPMB, MSNAPMB and TNAPMB (continued)

Compound	Chemical formula	Retention time (min)	Molecular weight	Nature of compound
TNAPMB				
Octasiloxane	$C_{16}H_{50}O_7Si_8$	36,378	578,171	
Di-n-decylsulfone	$C_{20}H_{42}O_2S$	36,773	346,291	
Benzoic acid, 4-methyl-2-trimethylsilyloxy-,trimethylsilyl ester	$C_{14}H_{24}O_3Si_2$	38,06	296,126	
Cyclotrisiloxane, 2,4,6--trimethyl-2,4,6-triphenyl	$C_{21}H_{24}O_3Si_3$	40,446	408,103	
	$C_{29}H_{46}O_7Si_7$	40,664		
4,4,6-Trimethyltetrahydro-1,3-thiazin-2-one		12,723	159,072	
2-Thiophenecarboxylic acid, 5-(1,1-dimethylethoxy)-		13,003	200,051	
5-Hydroxymethylfurfural		14,96	126,032	aldehyde
Propanal, 2-methyl-, 2-propenylhydrazone		16,345	126,116	
Lethane		20,585	203,098	
3-Deoxy-d-mannoic lactone		23,847	162,053	
d-Glycero-d-ido-heptose		26,101	210,074	
n-Hexadecanoic acid		29,517	256,24	fatty acid
Tetracosane		34,392	338,391	
Methyltris(trimethylsiloxy)silane		44,589	310,127	
Octasiloxane	$C_{16}H_{50}O_7Si_8$	37,694	578,171	

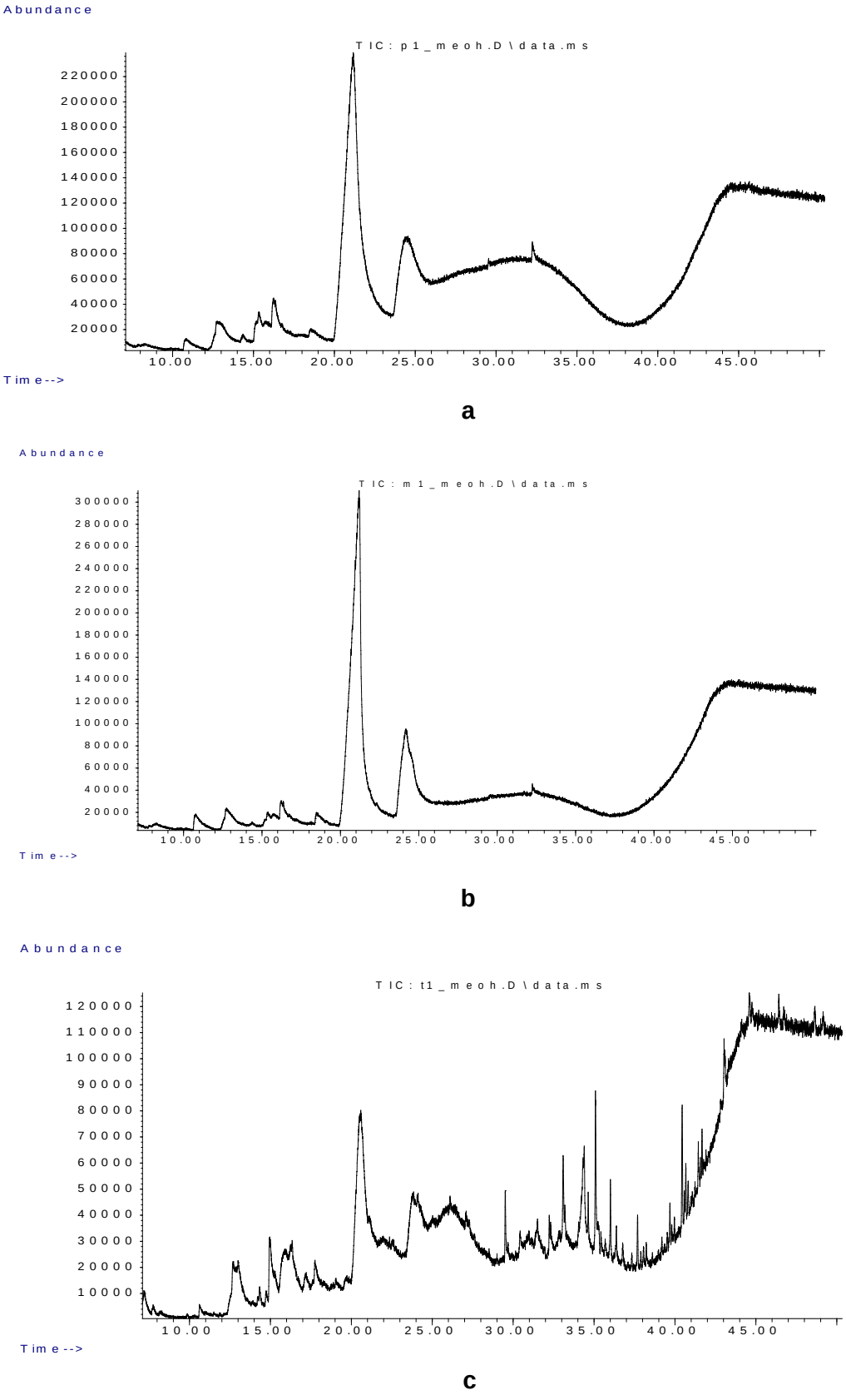


Figure 5.11 GC-MS chromatogram of methanol extract (a) plain non-alcoholic pearl millet beverage, (b) moringa-supplemented non-alcoholic pearl millet beverage and (c) traditional non-alcoholic pearl millet beverage

tetrahydro-, trans- (12.758 min), 2,4-dihydro-2,4,5-trimethyl (12.798 min), DL-arabinose (12.798 min), 5-hydroxymethylfurfural (15.332 min, melezitose (15.367 min), 2-ethyl-oxetane (20.791 min), lactose (21.175 min), 3-deoxy-d-mannoic lactone (24.465 min), urea, N,N'-dibutyl-N,N'-dimethyl-, sucrose (32.247 min) and tetrasiloxane, decamethyl- (42.175 min) among others. Some of the compounds in MSNAPMB were thymine (10.777 min), clindamycin (10.806 min), D-mannopyranose (12.740 min), D(+)-talose (12.797 min), 4,5-diamino-2- hydroxypyrimidine (10.783 min), melezitose (15.418 min), 1,3,5-triazine-2,4,6-triamine (16.202 min), 6,10,13-trimmeltetradecanol (18.548 min), lactose (21.151 min), 2-ethyl-oxetane (21.197 min), 3-deoxy-d-mannoic lactone (24.196 min), 3-deoxy-d-mannoic lactone (23.841 min) and beta-D-glucopyranose, 4-O-beta-D-galactopyranosyl- (32.241 min).

The identified compounds are of importance as they contribute to the taste, aroma, biological and medicinal potential of the beverage. For instance, the ester (3-deoxy-d-mannoic lactone) contributes to the flavour of the beverage during fermentation and has antibacterial effect which results in a safer product (Ghosh *et al*., 2015). The N,N'-dibutyl-N,N'-dimethyl- have immune modulating properties while 5-Hydroxymethylfurfural have antioxidant and antiproliferative properties (Ghosh *et al*., 2015). n-Hexadecanoic acid (palmitic acid) is a fatty acid with anti-inflammatory activities (Thomas *et al*., 2013) andIsosorbide dinitrate is used to treat heart failure and chest pains. Lactose which is present in PNAPMB and MSNAPMB could be from the skim milk used during the freeze drying of isolated lactic acid bacteria. This is supported by the absence in TNAPMB which was not inoculated with isolated probiotics.

The organic acids produced preserve the beverage through the inhibition of pathogenic microorganisms. The nutritional content of the beverage is also improved. The identified compounds with their biological and medical uses prove that the beverages are not meant for refreshing only but have many benefits to consumers. In TNAPMB some of the compounds identified were 4,4,6-Trimethyltetrahydro-1,3-thiazin-2-one (12.723 min), 2-Thiophenecarboxylic acid, 5-(1,1-dimethylethoxy)- (13.003 min), 5-Hydroxymethylfurfural (14.960 min), Propanal, 2-methyl-, 2-propenylhydrazone (16.345 min), Lethane (26.101 min), 3-Deoxy-d-mannoic lactone (23.847 min), d-Glycero-d-ido-heptose (26.101 min), n-Hexadecanoic acid (29.517 min), Tetracosane (34.392 min), Methyltris(trimethylsiloxy) silane (44.589 min) etc.

5.3.10 Sensory characteristics of non-alcoholic pearl millet beverages

The demography of the panellists who evaluated the non-alcoholic pearl millet beverages (NAPMBs) is shown in Table 5.4. There were 50 panellists made-up of 24 and 71% of

Table 5.4 Demography of panellists used in the evaluation of the beverages

Item	Frequency (percentage)*
Gender	
Male	38 (24)
Female	106 (71)
No response	8 (5)
Race	
Black	126 (85)
Coloured	15 (10)
White	3 (2)
Other	3 (2)
No response	4 (1)
Status	
Staff	24 (16)
Student	123 (82)
No response	3 (2)
Nationality	
National	78 (52)
International	36 (24)
No response	36 (24)
Age group	
Less than or equal to 29 years	115 (77)
30 - 39 years	15 (10)
40 and above	15 (10)
No response	5 (3)

*Numbers shows frequency and percentage in bracket.

males and females, respectively, of which 52% were black, 10% coloured and 2% white; 16% were staff members and 82% were students;52% were South African citizens and 24% were international students; 77% less or equal to 29 years, 10% between the age of 30 – 39 and 10% were 40 years old or above.

The panellists differed significantly when rating the beverages in terms of appearance ($p = 0.037$), colour ($p = 0.007$), aroma ($p = 0.020$), taste ($p = 0.001$) and overall acceptability ($p = 0.000$) while the panellists did not significantly differ in rating the mouthfeel ($p = 0.094$) of the beverages.

Figure 5.12 shows the sensory parameters of NAPMBs. The mean rating for appearance of the plain non-alcoholic pearl millet beverage (PNAPMB), moringa-supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB) were 5.90, 5.46 and 4.47, respectively. The beverages differed significantly ($p = 0.037$) in appearance with PNAPMB being the most preferred. The mean rating for colour of PNAPMB, MSNAPMB and TNAPMB were 5.77 (liked slightly), 5.61(liked slightly) and 4.84 (neither liked nor disliked), respectively. There was a significant ($p = 0.007$) difference in the colour of the beverages.

There was no artificial colorants added in all samples, the PNAPMB was golden brown, MSNAPMB was greenish-and-golden in colour while TMNAPMB was milky in colour and appearance. The panellists preferred PNAPMB followed by MSNAPMB and this could be because the beverages were made from pearl millet extract while the appearance of TNAPMB could have been affected by starch in the beverage. In addition, moringa extract powder used in MSNAPMB could have affected the ratings of the beverage colour and appearance. This is in agreement with Olosunde *et al*. (2014) report that a beverage supplemented with moringa seed powder differed significantly in appearance to a beverage with no moringa seed powder. However, this study used moringa seed powder instead if moringa leaf powder. In addition, Olosunde *et al*. (2014) prepared the beverage using same method for TNAPMB and not PNAPMB. PNAPMB and MSNAPMB made using pearl millet extract appeared similar to commercial soft drinks, hence they were preferred. In contrast, TNAPMB still contained particles of starch, proteins and minerals which could have affected the colour. TNAPMB beverage was made with no stabilizers and the sedimentation of particles could be attributed to the lower scores.

The mean score for aroma of PNAPMB was 5.26 (neither liked nor disliked), 4.68 (neither liked nor disliked) for MSNAPMB and 4.23 (disliked slightly) for TNAPMB, hence the beverages differed significantly ($p = 0.020$) in aroma. The organic acids and metabolites produced during fermentation by *Leuconostoc mesentoroides* and *Pediococcus pentosaceaus* could be responsible for the aroma of the beverages. Indigenous cereal beverages lack flavour which develops during fermentation when volatile substances (diacetyl, acetic acid, butyric acid, amino acids, aldehydes etc.) are developed. The unique development of aromas and/or flavour depend on the chemical composition of substrate (type of cereal, sprouted etc.), environmental condition during fermentation (pH, temperature, anaerobic/aerobic) and starter culture (Kohajdová & Karovičová, 2007). TNAPMB was carried out by chance fermentation made up of a diversity of lactic acid bacteria and other bacteria which could have resulted in the unacceptable aromas thus

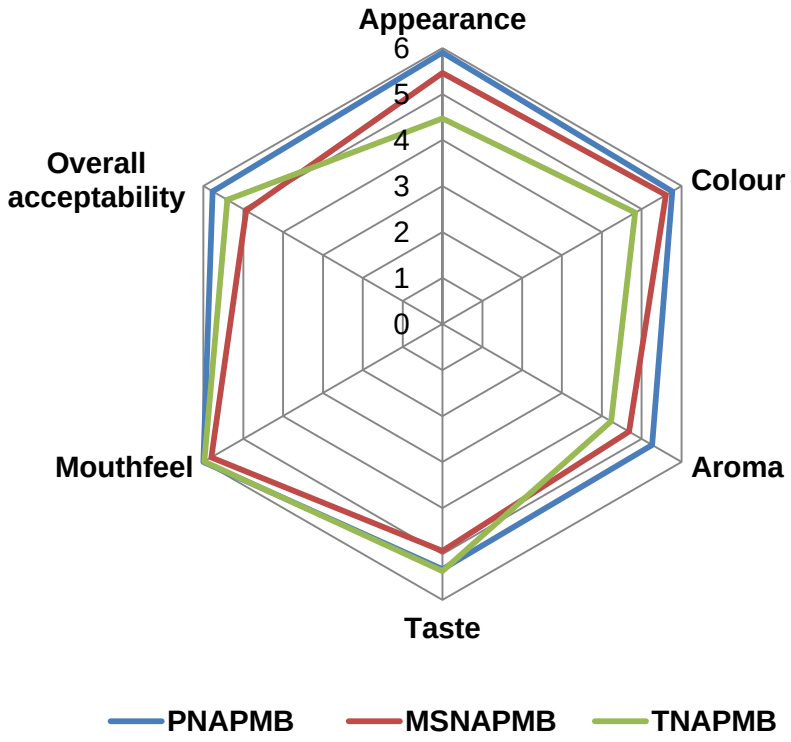


Figure 5.12 Acceptability ratings of sensory attributes of pearl millet beverages. Values used are mean of triplicates. PNAPMB – plain non-alcoholic pearl millet beverage, MSNAPMB – moringa-supplemented non-alcoholic pearl millet beverage and TNAPMB – traditional non-alcoholic pearl millet beverage.

lower rating unlike PNAPMB and MSNAPMB which were fermented using known purified cultures. The slight differences between PNAPMB and MSNAPMB could be due to moringa extract powder which could have released other volatile compounds during fermentation. The beverage was fermented in a closed vessel unlike traditionally beverage which is simply covered with a cloth to exclude foreign matters. During closed fermentation CO_2 is not allowed to escape and is dissolved in the beverage, this may be ideal for anaerobic lactic acid bacteria but it may lead to spoilage and creation of unwanted flavours or aroma. The closed system could also have caused all the released metabolites to be contained within the beverage. The level of diacetyl compound (2,3-butandione) could be high due to the lack of aeration. Hence, when citric acid was used in the beverage the 'off-like' flavour became intense because diacetyl is synthesized well during utilization of citric acid. *Pediococcus pentosaceus* could also be responsible for the production of diacetyl at high levels (Fugelsang & Edward, 2007). Some of the panellists related TNAPMB to *mahewu* and porridge since the beverage is fermented as a whole and the flour was cooked through gelatinization.

The beverages differed significantly ($p = 0.001$) in taste. The mean rating for taste was 5.33 for PNAPMB, 4.93 for MSNAPMB and 5.37 for TNAPMB, meaning the beverages were neither liked nor disliked in taste. The taste of the beverages was the sensation of flavour in the mouth. TNAPMB was rated high followed by PNAPMB then MSNAPMB. The cocktail of bacteria could be responsible for the sweet sour taste profile of TNAPMB whereas only selected lactic acid bacteria (*L. mesentoroides* and *P. pentosaceaus*) were used in PNAPMB and MSNAPMB. The preference for TNAPMB could have been due to cultural preferences by people who are familiar with natural fermented ethnic beverage. The lower rating of MSNAPMB could be due to the fresh leaf earthy flavour of moringa leaf extract in the beverage. Majority (77%) of the panellists were youth (≤ 29 years old market segment) and are loyal supporters of carbonated drinks in South Africa. According to StatsSA (2016), the South African population reported an increasing in the growth rates of the elderly people meaning the beverages have the potential for growth among older generation which is familiar with non-alcoholic cereal beverages such as *mageu*.

The mean score for mouthfeel of NAPMB, MSNAPMB and TNAPMB was 6.00, 5.80 and 5.98, respectively. The beverages did not differ significantly ($p = 0.094$) in terms of the physical and chemical interactions in the mouth. The similarity could be because the beverages were all fermented. Phytates, phenols and tannins found in pearl millet could be responsible for the mouthfeel of the beverages. Murevanhema (2012) also reported the influence of tannin on mouthfeel of fermented bambara milk. However, lactic acid bacteria during fermentation resulted in low pH (3.65 – 4.14) and built-up of lactic acid (0.22 – 0.42%) and pasteurisation of the beverages at high temperature could have resulted in the reduction of these antinutrients.

PNAPMB, MSNAPMB and TNAPMB had a mean score for overall acceptability of 5.77, 4.93 and 5.40, respectively. The beverages differed significantly ($p < 0.05$) in overall acceptability. PNAPMB was rated high followed by TNAPMB then MSNAPMB. The overall acceptability was influenced by all the other attributes of appearance, colour, aroma and taste. PNAPMB had a bright golden-brown appearance resembling most grape flavoured beverages hence it was preferred. TNAPMB had a creamy-milk appearance the panellist could have related to *umgqomothi* (African beverage) which they are familiar with. MSNAPMB was scored lower which could be explained by the greenish-colour and fresh earthy leaf aroma from moringa leaf powder. The beverage was rated low in taste which explains the lower overall-acceptability.

In general, a beverage was produced with isolated and purified cultures of lactic acid bacteria. The hypothesis of producing acceptable beverage was tested and accepted; however, the taste of the beverages could be improved in future work. The

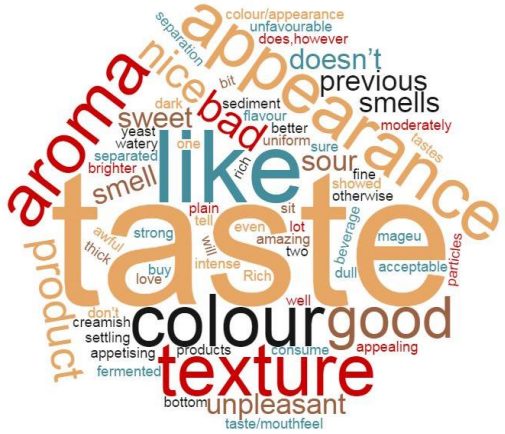
beverages could be flavoured, carbonated and blended with other cereal to boost the taste and nutritional content to suit the young generation. International students are mostly familiar with a lot of fermented beverages in comparison to South African citizens.

5.3.11 Sensory sentiments for non-alcoholic pearl millet beverages

Figure 5.13 shows the word cloud for plain non-alcoholic pearl millet beverage (PNAPMB), moringa supplemented non-alcoholic pearl millet beverage (MSNAPMB) and traditional non-alcoholic pearl millet beverage (TNAPMB). The cloud shows that taste (13%) was the main comment the panellists mentioned as negative sentiments followed by smell, appearance, colour and texture among others. These main negative sentiments are guide in improving the plain beverage. In contrast, the main positive sentiments were sweet, nice and good among others. Overall, PNAPMB had 63 sentiments of which 30.16% (19) were positive and 69.84% (44) were negative. The panellists noted taste, smell, watery and product as the negative sentiments on MSNAPMB among others. The total sentiments for MSNAPMB were 44 and 40.91% (18) were positive while 59.09% (26) were negative. The positive sentiments include like, appearance, colour and nice among others. In terms of TNAPMB, the panellists noted taste, texture, colour and bad as the negative sentiments while appearance, like, aroma and product were noted as the positive sentiments. TNAPMB had 70 sentiments of which 55.71% (39) were positive and 44.29% (31) were negative. The taste, smell (aroma), texture and appearance of the beverages which were the main negative sentiments need to be improved for the beverages to be commercially acceptable. In general, PNAPMB and MSNAPMB received most negative sentiments compared to TNAPMB. The use of citric acid in PNAPMB and MSNAPMB could have resulted in diacetyl compounds when utilised by *P. pentoseace*, hence the negative sentiments. In future, the taste of the beverages could be improved by using aerobic fermentation instead of anaerobic fermentation using sodium citrate instead of citric acid.

a

b



c

Figure 5.13 Word cloud based on the comments from panellists on (a) plain non-alcoholic beverage (PNAPMB), (b) moringa-supplemented non-alcoholic beverage (MSNAPMB) and (c) traditional non-alcoholic beverage (TNAPMB)

5.3.12 Conclusion

Two formulations of non-alcoholic pearl millet beverages were produced, namely, plain non-alcoholic beverage (PNAPMB) and moringa supplemented plain non-alcoholic pearl millet beverage (MSNAPMB). The two beverages were produced similarly although moringa concentrate powder was added to one variation to produce MSNAPB. In addition, traditional non-alcoholic beverage was produced as a control using commonly followed traditional method. The beverages were produced using *Leuconostoc mesenteroides* and *Pediococcus pentoseace* isolated from traditionally prepared non-alcoholic pearl millet beverage. The beverages differed in colour with PNAPMB and MSNAPMB deemed acceptable (liked slightly) by the consumers while PNAPMB was neither liked nor disliked. The beverages may have biological and medicinal benefits due to the compounds present in the beverages. Overall, the beverages were accepted by the consumers; however, the taste of the beverage could be improved. The nutritional content of the beverages did not differ significantly. The modified non-alcoholic pearl millet beverages, PNAPMB and MSNAPMB provide 113.23 and 130.23 kJ/100 ml of energy, respectively. The successful use of isolated and purified cultures of lactic acid bacteria from the indigenous beverage is an indication that a stable acceptable beverage could be produced for the commercial market. An acceptable beverage could be produced using isolated and purified lactic acid bacteria by fermenting pearl millet extract for 18 h at 37ºC.

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CHAPTER SIX
GENERAL SUMMARY AND CONCLUSION

The aim of this study was to evaluate the physicochemical, nutritional and sensory characteristics of non-alcoholic pearl millet beverage produced using pure cultures of bioburden lactic acid bacteria. The objectives in the study were:

1. Isolation and identification of the microorganisms involved in the fermentation of pearl millet beverage.
2. Obtain pure cultures of lactic acid bacteria involved in the natural fermentation of pearl millet beverage.
3. Produce a beverage using isolated and purified lactic acid bacteria.
4. Establish the physical, chemical and viscosity properties of the beverage.
5. Establish the sensory properties of the beverage.

The first and second objectives were successfully accomplished from traditionally prepared pearl millet beverage. A traditional pearl millet beverage was produced through spontaneous fermentation at 37°C for 36 h. During fermentation the number of total viable organisms and lactic acid bacteria were determined at 3 h interval over 36 h fermentation period. In addition, the lactic acid bacteria were isolated based on the colony colour and size, and purified by subsequent growth on fresh MRS agar, and identified using Vitek 2 system. The optimum fermentation time of the beverage was 18 h since there was not significant growth beyond this period. There was a total of 10 species identified from traditionally prepared beverage which included *Leuconostoc, Pediococcus, Streptococcus* and *Enterococcus*. These included probiotics and pathogens which may have a health risk to consumers of the beverage.

The third objective was also achieved successfully using *L. mesenteroides ssp. Dextranicum* and *P. penotosaceus* to produce a beverage. Stable pearl millet extract was produced by mixing pearl millet flour with water, spices and malted rice flour and thereafter sieving the mixture. The extract (filtrate) was stabilized using pectin (0.6%) and lecithin (0.1%). The stability was verified using Turbiscan MA. The extract was pasteurised and inoculated with pure cultures of *L. mesenteroides ssp. Dextranicum* and *P. penotosaceus* and fermented for 18 h at 37°C. The resulting beverage was chilled at 4°C.

The fourth and last objectives of establishing the physical, chemical, viscosity and sensory properties of the beverage produced using *L. mesenteroides ssp. Dextranicum* and *P. penotosaceus* was achieved. The beverage was characterised by determining the proximate composition, colour measurement, volatile compounds produced by the

probiotics used. In addition the viscosity of the beverage, total titratable acidity, pH, growth of probiotics (*L. mesenteroides ssp. Dextranicum* and *P. penotosaceus)*, determination of the total sugar content during fermentation and sensory evaluation of the PNAPMB and MSNAPMB in comparison to traditionally prepared beverage (TNAPMB). The new beverage was also fortified with extract of moringa leaf powder to increase the nutritional composition. Since cereals have lower protein content the addition of moringa increased the protein content of the beverage although not significantly, hence the concentration of moringa leaf powder could be increased. The beverages have many benefits beyond hydrating; the volatile compounds identified include antimicrobial, anti-inflammatory and anti-pathogenic substances. In terms of colour, the beverages differed significantly. PNAPMB and MSNAPMB had a low viscosity which is beneficial since the new generation prefer drinks similar to commercially available soft-drink. All the beverages were neither liked nor disliked, this proves that the new beverage have the potential to replace the traditional beverage. The taste and aroma could be improved by adding other cereals and flavourings to remove bad odour produced by the lactic acid bacteria.

The following challenges and limitation were experienced in the study:

1 Isolation, identification and purification of lactic acid bacteria from pearl millet slurry

1.1 It was difficult to differentiate lactic acid bacteria (LAB) grown by spread plate on MRS agar, hence pour and spread plating were used.

1.2 New LAB were isolated from each batch of pearl millet slurry fermentation; hence only microorganisms identified were used in the study.

2 Production of non-alcoholic pearl millet beverage using purified cultures

2.1 The sieving of pearl millet slurry was difficult and the slurry had sediments of starch. Pectin was used to stabilise the pearl millet extract.

2.2 Addition of citric acid lowered the pH of the beverage before fermentation. This has led to reduced fermentation by purified cultures. Sodium citrate was then used to replace citric acid.

2.3 Maltodextrin was used to give the beverage a rich body but it did not dissolve fully in the extract and resulted in white precipitate and unacceptable taste. Therefore, maltodextrin was removed during the production of the beverage.

2.4 Some cells of LAB precipitated in pearl millet extract during fermentation.

3 Production of non-alcoholic pearl millet beverage using purified cultures and its physicochemical, nutritional and sensory properties

3.1 Supplementation of the beverage with moringa leaf powder produced a beverage with leafy earthy taste and was acceptable by panellist.

3.2 The sour fermented flavour of the beverage as unacceptable by panellists.

4 In future the following could be of interest

4.1 The use of yoghurt cultures during the fermentation of pearl millet extract could be used to determine the acceptability of the beverage if commercially available starter cultures are used.

4.2 Aerobic fermentation instead of anaerobic fermentation could be used to determine the acceptability of the beverage if different metabolites are released.

4.3 Supplementation of pearl millet with other cereals such as sorghum and maize.

4.4 The use of colourants and flavours to mask the unacceptable appearance and smell of the beverage.

4.5 The use of a better filtration system for extraction of pearl millet extract.

Appendix A: Approved ethical clearance

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

4 October 2016
REC Approval Reference No:
CPUT/HW-REC 2016/H37

Faculty of Applied Science

Dear Mr Mmaphuti Ratau

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 15 September 2016 to Mr Ratau for ethical clearance. This approval is for research activities related to student research in the Department of Applied Science at this Institution.

TITLE: Chemometrics and sensory characteristics of pearl millet beverage produced with bioburden lactic acid bacteria pure cultures

Supervisor: Professor V Jideani
Co-Supervisor: Dr Okudoh

Comment:

Approval will not extend beyond 5 October 2017. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Appendix B: Informed consent form signed by volunteers prior tasting

Department of Food Technology
P. O. Box 1906
Bellville 7535

INFORMED CONSENT FOR PEARL MILLET RESEARCH

Dear

We are scientists from Cape Peninsula University of Technology. We are conducting a research to find new food use for Africa's indigenous cereal. No value will be added to any produce except the consumers endorse it. Hence, we are approaching you to be part of this study. We realize you need to make an informed decision whether or not to be part of this study, hence we have provided below further details with regards to the research to assist in your decision process.

Title of Research Project:
Chemometrics and sensory characteristics of pearl millet beverage produced with bioburden lactic acid bacteria pure cultures.

Mmaphuti Ratau (Student) Tel: 079 6739 639
Victoria Jideani (Supervisor) Tel: 021 9538 749
Vincent Okudoh (Co-supervisor) Tel: 021 460 3507/3216

Purpose of the Research:
Pearl millet is an indigenous cereal grown in Africa and Asia as a source of energy and proteins. The crop is adapted to growing regions characterized by drought, low soil fertility and high temperatures. In South Africa pearl millet is known as leotja in Pedi and bulrush in English. Despite its nutritional value and adaptive growth conditions much of its use is limited at household level in South Africa. We have developed a non-alcoholic fermented beverage from pearl millet. The aim of this study is to evaluate the sensory characteristics of non-alcoholic pearl millet beverage (NAPMB).

Description of the Research:
This is an invitation to participate in the sensory study. The procedure to be adopted in the study as well as the terminologies on the score form will be explained to the panelists prior to tasting sessions. You will receive three 40 ml non-alcoholic beverage samples processed at differently. You will not be bound to finish the 40 ml serving size of the beverage. The samples contain water, rice malt, ginger, sugar, emulsifier, stabilizer, and cultures (found in fermented foods such as sauerkraut and pickles). This is the base from traditionally fermented beverage from pearl millet. During the production of these samples strict good manufacturing practices (GMP), standard operating procedures (SOPs) and microbiological analysis was carried to ensure the samples are safe for consumption. You will be required to test them and rate your preference (on a simple questionnaire) for each based on appearance, colour, taste, aroma, mouthful and overall acceptability. Each tasting session will last for 15 - 30 minutes depending on an individual.

Potential Harm, Injuries, Discomforts or Inconvenience:
Pearl millet is a staple food for thousands of Africans; its consumption does not pose any hazard to human health. Therefore, there is no known harm associated with tasting pearl millet products in this study. There are also no known risks of ingesting the ingredients used for the beverage. However, participants are allowed to decline participation should they have concerns.

Potential Benefits:
You will not benefit directly from participating in this study.

Confidentiality:
Confidentiality will be respected and no information that discloses the identity of the participant will be released or published.

Participation:
Participation in this research is voluntary. If you choose to participate in this study you may withdraw at any time.

Contact
If you have any questions about this study, please contact:

Mmaphuti Ratau (Student)	Tel:	079 6739 639
Victoria Jideani (Supervisor)	Tel:	021 9538 749
Vincent Okudoh (Co-supervisor)	Tel:	021 460 3507/3216

Consent:
By signing this form, I agree that:

1. The study was explained to me and all my questions answered.
2. I have the right to participate and the right to stop at any time.
3. I have been told that my personal information will be kept confidential.
4. There is no likely harm from tasting non-alcoholic beverage from pearl millet.
5. I am 18 years or above.

I hereby consent to participate in this study:

.....	.....
Name of Participant	**Signature & Date**
.....	.....
Name of Researcher	**Signature & Date**

Appendix C: Score card used to rate the beverages

SCORE CARD- HEDONIC RATING SCALE
Non-alcoholic pearl millet beverage (NAPMB)

Instruction:
You are provided with 3 samples of beverages processed differently. Please take a sip of water before you start tasting and in between tasting the different samples. Please rate (✓) each sample on its own merit based on the given attributes. Do not compare the samples.

Name of product: …….. **Code:……**

	Appearance	**Colour**	**Aroma**	**Taste**	**Mouthfeel**	**Overall acceptability**
Like extremely						
Like very much						
Like moderately						
Like slightly						
Neither like nor dislike						
Dislike slightly						
Dislike moderately						
Dislike very much						
Dislike extremely						

Comments:

.....

.....

We would like to obtain information about you. Kindly complete this brief questionnaire appropriately:

1. **What is your Gender:** Female ☐ Male ☐

2. **What is your race?** Black ☐ Coloured ☐ White ☐ Indian ☐ Other ☐

3. **Are you a student or staff?** Student ☐ Staff ☐

4. **If you are a student, are you an international student?** Yes ☐ No ☐

5. What is your age group?
☐ Less than or equal to 29
☐ 30-39
☐ 40 & above

Thanks for assisting us!!!!

Appendix D: Research outputs presented at national and international conferences

1. Ratau, M.A. & Jideani, V. A. (2014). Dietary fibre extraction from plant materials - A review U6 Consortium 2nd International Conference, Cape Town, South Africa, 6 - 10 September 2014. Pp. 26. (Paper presentation).

2. Ratau, M.A. Okudoh, V. I. & Jideani, V.A. Identification, Isolation and Purification of Lactic Acid Bacteria from African Fermented Non-Alcoholic Cereal Beverage. Sorghum in the 21st century. Cape Town, South Africa. 9 – 12 April 2018. (poster presentation).

3. Ratau, M.A. Okudoh, V. I. & Jideani, V.A. Fermentation profile of pearl millet extract with purified bioburden cultures of lactic acid bacteria. Cereals & Grains 18. London. United Kingdom. 9 – 12 April 2018. (oral presentation).

Appendix E: Manuscripts submitted for publication in peer reviewed journals

From:Journal of Cereal Science <EviseSupport@elsevier.com>
Subject:Received resubmission YJCRS_2018_787

This message was sent automatically. Please do not reply.

Ref: YJCRS_2018_787
Title: ISOLATION, IDENTIFICATION AND PURIFICATION OF LACTIC ACID BACTERIA FROM PEARL MILLET SLURRY DURING FERMENTATION FOR NON-ALCOHOLIC CEREAL BEVERAGE
Journal: Journal of Cereal Science

Dear Mr. Ratau,

Thank you for resubmitting your manuscript for consideration for publication in Journal of Cereal Science. Your resubmission was received in good order.

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Journal of Cereal Science

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From:COMET-FTB <comet@sdewes.org>
Subject:FTB journal: New submission status

Dear Mr. Ratau,

Thank you for uploading the manuscript:

FTB-5998 Technological Approaches to Improving the Quality, Shelf life and Acceptability of African Traditional Fermented Non-Alcoholic Cereal Bevera
-
Mmaphuti Ratau*, Cape Peninsula University of technology, South Africa
Vincent Okudoh, Cape Peninsula University of technology, South Africa
Victoria Jideani, Cape Peninsula University of Technology, South Africa

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