The effect of processing techniques on the microbiological and nutritional qualities of the leafy vegetables *Vigna unguiculata* and *Moringa oleifera* grown in South Africa

by

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DEDICATION

This project is dedicated to God, the author and the finisher of my faith, for the wisdom, strength and support He's given me to successfully complete this research, and to my husband (Stephen) and my children (King, Anointed and Olive-virtue) for their immeasurable support, love and understanding.

DECLARATION

Student number: 50828576

I declare that **The effect of processing techniques on the microbiological and nutritional qualities of the leafy vegetables *Vigna unguiculata* and *Moringa oleifera* grown in South Africa** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

15.09.2014

SIGNATURE DATE

(Mrs Sarah O. Otun)

ABSTRACT

Cowpea (*Vigna unguiculata*) and moringa (*Moringa oleifera)* are nutritious and medicinal vegetables, but could also harbor harmful microbial contaminants.

The main aim of the project was to determine the effect of each processing techniques on the microbiology, proximate nutrients and shelf life of these vegetables to produce nutritious, tasty, safe and long lasting vegetable products.

The processing techniques used were: washing, blanching, and drying. Leaf samples were collected at each stage of processing and were analysed for total viable count, coliform count, yeast and mould count and nutritional content.

Microbial plate analysis showed the presence, particularly on cowpea leaves, of yeasts and bacteria such as *Pseudomonas, Klebsiella, Staphylococci, Streptococci,* and *enterobacter* including enteropathogens such as *Salmonella spp., Shigella dysenteriae* and *E coli*. The presence of *E.coli* on the leaves was also confirmed using polymerase chain reaction-amplified ribosomal DNA analysis. The most effective processing technique which reduced microbial load to below SABS standards while retaining nutritional quality was the washing of the leaves twice with tap water followed by steam tunnel blanching at 94^{o}C for 12 minutes. Oven drying the leaves at 60^{o}C gave satisfactory and extended shelf life results. Proximate analysis comparison of the two leaf types showed that on average moringa leaves contained more ash (2.37 vs 1.1 g), protein (6,9 vs 3,6 g), fat (0,41 vs 0.2 g) and energy (305,1 vs 70 KJ) but less dietary fibre (0,9 vs 7,5 g) than cowpea leaves. No significant differences were noted in these values following washing and steam blanching. These results indicate that washing of these leaves is effective as to reducing microbial load and maintaining proximate values in the short term (up to 4 days) but that oven drying is effective for longer-term storage.

Key Words: Cowpea*,* moringa, vegetables, nutritional content, microbiological analysis, processing techniques, pathogen, coliform, yeast, shelf life.

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ABBREVATIONS AND ACRONYMS

^{o}C	**degree Celsius**
%	Percentage
+ve	Positive
-ve	Negative
AOAC	Association of Official Analytical Chemists
Ave	Average
CDC	Center of Disease Control
cm	Centimetre
CSIR	Council for Scientific and Industrial Research
f	Frequency
g	Gram
H_2O	Water
hrs	Hours
HPLC	high performance liquid chromatography –
kg	Kilogram
L	Litre
min	Minutes
ml	Millilitre
mM	milli-molar
mg	Milligram
PCR	Polymerase chain reaction
pH	hydrogen ion concentration / measure of alkalinity or acidity
Prep	Preparative
rpm	revolutions per minute
RSA	Republic of South Africa

SABS	South African Bureau of Standards
SANAS	South African National Accreditation System
SANBI	South African National Biodiversity Institute
sec	Seconds
UNISA	University of South Africa
UV	Ultraviolet

CHAPTER 1

1.1. Introduction

Increased health awareness and the fight to eradicate malnutrition have recently led to higher consumer demand for nutritious vegetable products and nutraceuticals which are affordable and widely available (Gleeson and O'Beirne, 2004). Fresh vegetables which are minimally processed, e.g. washed, treated, cut and packed in a convenient way, have been introduced into the market and to guarantee premium quality these natural products require special care during the whole handling process from harvest to consumption (Barth *et al*, 2009).

Cowpea and moringa leaves are good sources of nutrients and these plants are regarded as some of the world's most beneficial plants, as most of their parts (e.g. leaves, fruits, flowers and pods) serve as an important source of nutrients (Okonya *et al*, 2010). According to Fuglie, (1999), moringa leaves contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas, and the protein quality is higher than in milk and eggs. They also act as cardiac and circulatory stimulants and are anti-inflammatory, antihypertensive and antidiabetic agents that are being used for the treatment of several ailments in traditional medicine, mostly in Africa and South Asia (Anwar *et al*, 2007; Okonya *et al*, 2010). Nevertheless, except by a few communities in the Limpopo and Mpumalanga provinces these vegetables remain underutilised in South Africa, (South African National Research Council, 2006).

Cowpea and moringa leaves are available as food throughout the year and, thus, help to improve food security (Okonya *et al*, 2010; Anwar *et al*, 2007). However, research has shown that around a quarter of all harvested leafy vegetables are spoilt prior to being consumed as a result of mechanical damage, microbial contamination and moisture loss. In addition, they also harbour pathogens (Arun and Sarita, 2011). Therefore, these highly perishable vegetables require special processing techniques to reduce microbial loads and the risk of

food poisoning and to prevent post-harvest losses thus prolonging their shelf-life (Barth *et al*, 2009).

1.2. Problem Statement

Cowpea and moringa leaves are nutritious and have many health benefits. Unfortunately, the leaves are still prone to spoilage and could also potentially carry food pathogens such as *E.coli*, *Staphylococcus aureus* and *Salmonella* spp., the ingestion of which may result in gastroenteritis (Barth *et al,* 2009).

In 2006 the South African Department of Health published statistics relating to *E. coli* food poisoning, mainly associated with consumption of green, leafy vegetables. The report revealed morbidity and mortality figures associated with foodborne-disease outbreaks in the Eastern Cape, KwaZulu-Natal and Limpopo provinces of South Africa, between 2001 and 2005. The statistics supplied in this report indicated a significant total number of foodborne illnesses (n = 1886) associated with the deaths of 51 individuals (Agyepong, 2009).
Chemical treatments such as ethylene bromide, methyl bromide, ethylene oxide, aluminium phosphide and malathion and γ-irradiation are effective in controlling pest and microbial contamination. However, these agents have adverse effects on food and the environment as they are associated with ozone depletion and mutagenesis (Falade, 2013).

Therefore, there is a need for an effective and safe processing technique that may be applied to leafy vegetables such as cowpea and moringa leaves, in order to reduce the microbial load on these leaves (FDA, 2000) while, at the same time retaining the nutrient value and desired quality and extended shelf stability of these vegetables. Natural processing has been proposed as the best alternative to both chemical and irradiation treatment, as it is inexpensive, energy saving, non-toxic and has minimal effect on the freshness, sensory and nutritional content of cowpea and moringa leaves.

1.3. Aim

To investigate the effect of various processing techniques on the microflora and nutrient content of cowpea and moringa leaves.

1.4. Objectives

- To determine the effect of processing techniques such as washing and hot water or steam blanching on the microbial load, nutrient content and sensory quality of fresh cowpea and moringa leaves
- To scale up such techniques so as to investigate the most effective commercial processing and preservation techniques that may be used to reduce the microbial load of fresh cowpea and moringa leaves
- To investigate the effect of various leaf drying techniques on microbial load and nutrient content of dried cowpea and moringa leaves
- To study the shelf-life of tap water washed, steam blanched and milled moringa leaves
- To isolate and identify using standard microbiological methods those bacterial and fungal contaminants found on treated and untreated moringa and cowpea leaves
- To confirm the presence of coliform bacteria on moringa leaves using the polymerase chain reaction

A brief description of the research design that was followed in order to attain the above aim and objectives is discussed below in research materials and methods (chapter 3).

CHAPTER 2

Literature Review

2.1. Cowpea (*Vigna unguiculata*)

2.1.1. Scientific Classification

Cowpea is an annual legume and vegetable and it is also known as the Southern pea, lubia (Davis *et al*, 1991). Its scientific classification is listed in **Table 1**.

Table 1: Scientific classification of cowpea

Kingdom:	Plantae
Phylum:	Angiosperms
Class:	Eudicots
Sub-class	Rosids
Order:	Fabales
Family:	Fabaceae
Genus:	Vigna
Species:	*V. unguiculata*
Binomial name	*Vigna unguiculata*
Local Names	
Afrikaans	Akkerboon, koertjie
Sepedi	Monawa
IsiZulu	Imbumba
English	Blackeye bean

2.1.2. General background and History

Cowpea *(Vigna unguiculata)* is an annual legume that was domesticated in West Africa. It is an important grain legume and leafy vegetable in most parts of Africa and Asia. It is produced on some commercial scale in eighteen African countries and seven Asian countries (Davis *et al*, 1991).

The cowpea plant originated and was domesticated in western Africa and was later moved to East and Southern Africa, Asia, Europe, United States and Central and South America. Cowpea is chiefly used as a grain crop for animal fodder or as a vegetable (Davis *et al,* 1999). The name "cowpea" was probably derived from when it was an important livestock feed for cows in the United States.

2.1.3. Background

Cowpea is a yearly leguminous plant with several growth forms. It may be erect, trailing, climbing or bushy, usually indeterminate under favourable conditions. It has a strong taproot and many distributed side lateral roots in topsoil. As shown in **Figure 1**, the leaves are usually dark green in colour, with considerable variation in size (6–16 x 4–11 cm) and shape (long, pointed to oval) depending on the variety (Department of Agriculture, Forestry and Fisheries, South Africa, 2010).

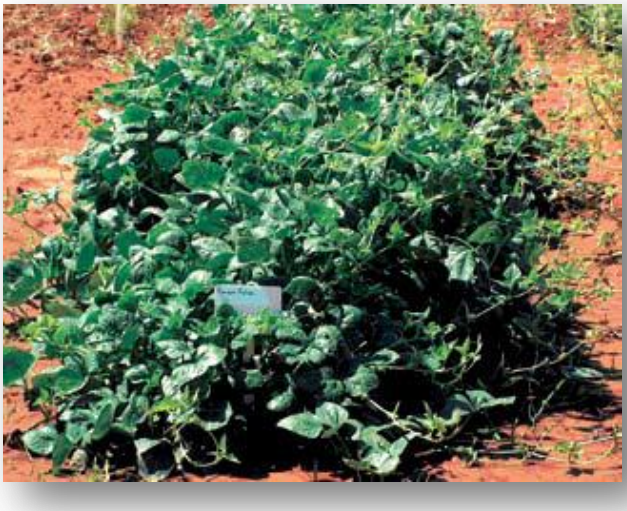


Figure 1: Cowpea leaves. Source: Jansen *et al*, (2007).

2.1.4. Uses of cowpea plant

In addition to consumption as food, there are many uses of cowpea leaves. These include animal foliage and nitrogen fixation into the soil (Davis *et al*, 1991). Fresh cowpea leaves can be cooked like spinach or can be dried and crushed into powder for use in soups and sauces (Nielsen *et al*, 1999). **Figure 2** and **Figure 3** are examples of indigenous cowpea dishes.

Figure 2: South African Morogo - cowpea balls. Source: http://www.CaribbeanPot.com

Figure 3: Chinese - cowpea stir-fry. Source: http://www.CaribbeanPot.com

The leaves are the most nutritious part of the plant and are a significant source of highly bioavailable nutrients required by the body. The nutritional content of cowpea leaves is listed in **Table 2**.

Table 2: Nutritional composition of cowpea leaves (*Vigna unguiculata).* Adapted from Nielsen *et al,* (1999).

Nutrient component	Content per 100 g
Water (%)	88.4
Energy (cal)	3.4
Protein (g)	4.2
Calcium (mg)	110
Iron (mg)	4.7
Carotene (mg)	2.4
Ascorbic acid (mg)	35

2.1.5. Medicinal Value

Research has revealed that countries that preserve indigenous vegetable diets and have high consumption of these vegetables are much less likely to be affected by cardiovascular diseases, diabetes and other effects of malnutrition. Hence, there is a possibility that indigenous vegetables such as cowpea could contribute towards an alleviation of diabetes, gout, hyperlipidemia, gastro-intestinal tract infections, protozoan parasites and other diseases. This motivates for an intervention geared towards encouraging individuals to increase the consumption of indigenous leafy vegetables (Kimiywe *et al*, 2007). There is a need for further investigation to establish the basis of the above-mentioned perceptions.

2.2. Moringa Leaves (*Moringa oleifera*)

2.2.1. General

Moringa oleifera known simply as ‘moringa’ originated in the sub–Himalayan regions of India. The *Moringa oliefera* tree (**Figure 4**) is one of the most widely cultivated and distributed naturalised species of the family *Moringaceae,* especially in parts of Asia, South America and Africa where these plants are now established as a part of the local flora (Arun and Sarita, 2011).

Figure 4: *Moringa oleifera* tree. Source: Plants of Hawaii (2008).

2.2.2. Classification

Moringa oleifera is also known as the horseradish tree, radish tree, drumstick tree, mother's best friend and miracle tree (Fahey, 2005). Its scientific classification is listed in **Table 3**.

Table 3: Scientific classification of *Moringa oleifera*

Source: USDA, 2012

Scientific Classification	
Kingdom:	Plantae - Plants
Subkingdom	*Tracheobionta* - Vascular plants
Superdivision	*Spermatophyta* - Seed plants
Division	*Magnoliophyta* - Flowering plants
Class	*Magnoliopsida* - Dicotyledons
Subclass	*Dilleniidae*
Order	*Capparales*
Family	*Moringaceae*
Genus	*Moringa*
Species	*M. oleifera*
Binomial	***Moringa oleifera***

2.2.3. Cultivation

The *Moringa oleifera* tree is a fast growing tree that is able to survive different climatic conditions of rainfall, seasonal changes (spring, summer, autumn and winter), a wide range of soil types ranging in pH from 5.0 - 9.0 and is rarely affected by drought (Arun and Sarita, 2011). In addition to their natural habitat they are used artificially as hedges and in house yards (Morton, 1991).

2.2.4. *Moringa oleifera* In South Africa

In India and parts of West Africa and Central Africa, use of the moringa plant has become very common among local people who have included it as part of their traditional cuisine (Fuglie, 1999). Although the moringa plant grows in some parts of South Africa (e.g. in the Mokopane district in the Limpopo Province), most South Africans are still unaware of the plant and its numerous uses and benefits (National Research Council, 2006).

The results of a literature study as well as a few informal discussions held in Tshwane in the Gauteng province of South Africa revealed that very few South Africans, except for mostly members of the Indian community, use moringa in their diets. A publication indicated that the moringa plant is listed as a naturalised plant in South Africa and this might indicate that the popularity and general acceptability of the plant in South Africa is on the increase (Agyepong, 2009).

2.2.5. Uses of *Moringa oleifera* plant

In addition to food, there are many products derived from various parts of the moringa tree. These include biomass and biogas production, domestic cleaning agents, fertilizer and manure, honey, medicine and bio-pesticides (Fahey, 2005; Agyepong, 2009). Please refer to **Appendix 1** for a more complete list of products associated with the moringa tree. A list of the nutrient composition of moringa leaves as compared to other foods is shown in **Table 4**.

Table 4: Nutrient Composition of *Moringa oleifera* leaves (per 100g) as compared to popular nutritious foods. Adapted from: Fahey, (2005), Fuglie, (1999) and McCance and Widdowson (1992).

Nutrient	Content	
	Moringa (raw) Per 100g	Other foods (raw) Per 100g
Vitamin A	6.8mg	1.8mg in Carrot
Calcium	440mg	120mg in Milk
Potassium	259mg	88mg in Banana
Protein	6.7g	3.1g in Yoghurt
Vitamin C	220 mg	30mg in Orange
Phosphorous	70mg	61mg in Curly-kale
Energy	95Kcal	98Kcal in Garlic
Carbohydrates	8.28 g	7.6g in Beetroot (root)
Sodium	9mg	3mg in Cucumber
Zinc	0.6mg	0.6mg in Broccoli
Iron	4.00mg	2.1mg in Spinach
Magnesium	147mg	71mg in Okra
Manganese	0.36mg	0.1mg in Onion
Dietary fibre	2.0g	1.9mg in Cauliflower
Fat	1.40g	1.4g in Brussels sprouts
Protein	9.40g	7.9g in Garlic
Water	78.66g	89.7g in Spinach
Riboflavin (vit. B_2)	0.660mg	0.11mg in Brussels sprouts
Pantothenic acid (B_5)	0.125mg	0.3mg in Cucumber
Vitamin B_6	1.200mg	0.28mg in Cauliflower

The moringa plant also supplies a host of prophylactic and therapeutic health benefits (Arun and Sarita,(2011); Anwar *et al, (*2007); Fuglie, (1999, 2000)) These include:

- Helping to balance cholesterol levels in the body.
- Assisting in balancing sugar levels associated with diabetes.
- Stimulating the immune system.
- Stimulating metabolism.
- Aiding in digestion and acting as a natural laxative.
- Serving as a nutrition booster and a non-sugar based energiser.
- Assisting in weight loss.
- Increasing breast milk production.
- Acting as a skin tonic.

For many years, parts of the moringa plant have been used to treat a number of health conditions or diseases in Ayurvedic medicine as well as other traditional systems where, particularly the roots, seeds, leaves and flowers have been utilised (Arun and Sarita, 2011). A list of these therapeutic uses is provided in **Appendix 2**.

It should be noted that there are potential hazards associated with using moringa as a food source. Thus, consumption of moringa should be avoided by people on blood-thinning medication or pregnant women without medical consent, because of the effect of moringa leaves on improving blood coagulation (Fahey, 2005).

2.3. General background on the processing and analysis of leafy vegetables

2.3.1. Processing Techniques

Based on published statistics (South African Department of Health, 2006) relating to *E. coli* food poisoning that year, mainly associated with consumption of green, leafy vegetables, 51 people died of food borne-related illness out of the 1886 people who experienced food-borne illness (Agyepong, 2009). A similar outbreak of bacterial food-borne disease began in Germany in May 2011 that resulted from the consumption of contaminated leafy vegetables. One *E. coli* strain, *E. coli* O104:H4 was identified as the causative organism. As reported to the WHO, this outbreak was not restricted to Germany, but included 11 other countries including Denmark, France, Netherlands Sweden and the United States of America (www.sciencedaily.com).

Therefore, there is a need for effective processing at every step from planting to consumption of leafy vegetables, in order to reduce microbial contamination which might eventually lead to food poisoning and food spoilage (FDA, 2000).

However, if current processing techniques are not controlled, it could reduce the levels of some of the health-benefiting nutrients, or the availability of these compounds, so minimal processing is desirable to ensure product safety and good quality over a prolonged shelf-life period.

As shown in **Figure 5,** the following processing techniques have been used for cowpea and moringa leaves (CSIR in-house protocol):

2.3.1.1 Sorting

This step involves the picking out of the healthy leaves and detaching them from their stems.

2.3.1.2 Washing

Washing is not only used to remove field soil and surface micro-organisms, but also to remove fungicides, insecticides and other pesticides from leafy vegetables. The [13] g water may contain detergents or other sanitizers that can assist in removing these residues.

2.3.1.3 Blanching

This is a special heat treatment to inactivate enzymes such as catalase and peroxidase present in vegetables, where the leaves are submerged in boiling water 94°C (hot water blanching) or steam at 85-95°C (steam blanching). The leaves are removed after a brief, timed interval (too little heat is ineffective while excess heat damages the vegetables) and finally placed under cold running water to stop the cooking process.

2.3.1.4 Drying

During this step, the moisture is removed from the leaves by simultaneous heat and mass transfer. The drying techniques of vegetables require a dependable model to predict its drying behaviour (Premi, 2010). Methods of drying could involve sun-drying, spray drying, freeze drying and oven drying.

2.3.1.5 Milling

This is the stage where the vegetables are broken down by course milling into a smaller size or into a powdered form.

2.3.1.6 Packaging

At this stage the final product of cowpea and moringa leaves that is required for distribution, storage, sale and use is enclosed or protected and sealed. Exposure of the final products to oxygen hastens leaf degradation and nutritional value loss (Premi *et al,* 2010).

2.3.1.7 Storage

Cowpea and moringa powder needs to be stored correctly to prevent it from losing potency and nutritional quality. The powder must be kept dry to inhibit microbial growth so it is important not to scoop it with a wet or dirty teaspoon as this could reintroduce microorganisms into the powder. It should be stored in the dark in a cool place, because sunlight and heat ages the powder and reduces the nutrient content (Arun and Sarita, 2011).

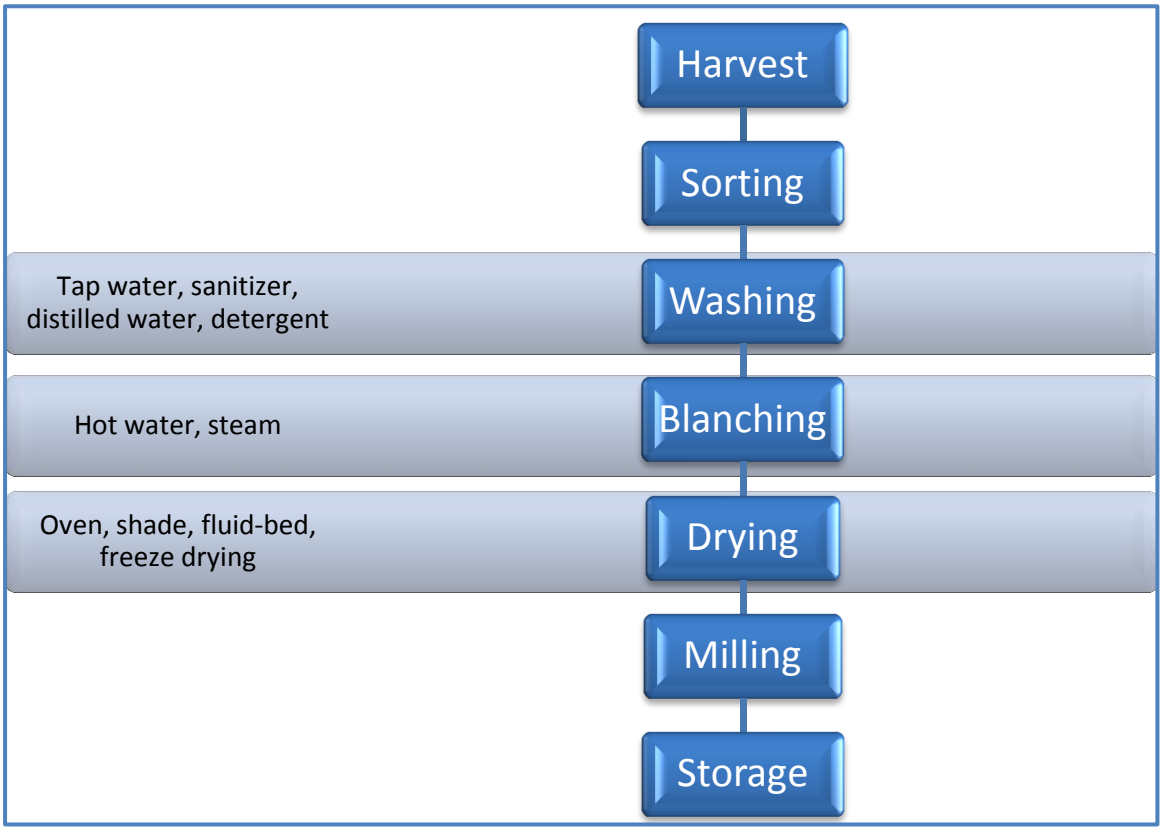


Figure 5: Cowpea and moringa processing techniques. **Source: CSIR in-house protocol.**

2.3.2 Microbiological analysis

2.3.2.1 Microflora:

Vegetables are capable of supporting the growth of diverse species of microorganisms due to their high moisture content and neutral pH (Smith *et al*, 1997). Microbial contamination of

vegetables occur at various stages of production such as the use of contaminated water for irrigation or washing of the vegetables, soil containing manure from human or animal origin, animals including insects and birds, handling of the vegetable product, contaminated harvesting and processing equipment and during transportation (Barth *et al*, 2009).

Microorganisms found on vegetable leaves include bacteria or fungi that have grown on and colonized the leaf surface by utilizing nutrients exuded from plant tissues. These could be pathogenic or spoilage-causing microorganisms. Many of these agents enter the plant tissue through mechanical or chilling injuries, or after the plant surface barrier has been broken down by other organisms (Joshi *et al*, 2010). Besides causing huge economic losses, some fungal species could produce toxic metabolites in the affected sites, constituting a potential health hazard for humans. In order to slow down vegetable spoilage and minimize the associated adverse health effects, great caution should be taken to follow strict hygiene, good agricultural practices and good manufacturing practices during cultivation, harvest, storage, transport, and marketing (Joshi *et al*, 2010; Barth *et al*, 2009).

2.3.2.2 Spoilage Microorganisms

After harvest, vegetables are often spoiled by a wide variety of microorganisms including many bacterial and fungal species. Fungi commonly causing spoilage of fresh vegetables include *Botrytis cinerea* and various species of the genera *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma*, *Phytophthora*, *Pythium* and *Rhizopus*. These affect a wide variety of vegetables causing devastating losses (Joshi *et al,* 2010).

According to a USDA-Economic Research Service study in 2010, 18.9 billion pounds of fresh fruits and vegetables were lost annually due to spoilage, which was 19.6% of all losses of edible foods that year in the United States (Kantor *et al*, 2010). The most common bacterial agents are Gram positive bacteria such as lactic acid bacteria originating mainly from contamination from the hands of employees during harvesting or processing, as well as

Erwinia, carotovora, *Pseudomonas* spp., *Corynebacterium* and *Xanthomonas, campestris* which attack virtually every vegetable type (Garg *et al*, 1990).

2.3.2.3 Pathogenic Microorganisms

There have been a number of human infections associated with the consumption of raw fruits and vegetables. Outbreaks with identified etiology were predominantly of bacterial origin, primarily *Salmonella*, *Escherichia coli*, *Bacillus cereus*, *Clostridium spp*, *Listeria monocytogenes*, *Salmonella* spp, *Shigella* spp and *Staphylococcus* spp which have the potential for growth prior to consumption or which have a low infectious dose (Buck *et al,* 2002). The presence of high coliform bacteria counts are used as indicators of the level of hygiene, the possible presence of pathogens and the quality of the vegetables. Besides causing huge economic losses, some fungal species could produce toxic metabolites in the affected sites, constituting a potential health hazard for humans (Barth *et al*, 2009).

CHAPTER 3

Research Methodology: Part A

3.1. Processing Techniques

Cowpea and moringa leaves were processed using industrial methods performed on a semi-industrial scale as well as on a smaller scale in a controlled environment under aseptic conditions in the laboratory. All experiments were done in triplicate.

3.1.1. Harvesting of leafy vegetables

Cowpea leaves were harvested from plants grown at the Agricultural Research Council (ARC), Roodeplaat, Pretoria, South Africa while *Moringa oleifera* leaves were harvested from plants grown on farms in Atteridgeville and Hammanskraal near Pretoria, South Africa. Harvesting was done by picking the new leaves at the end of the stem while avoiding long and thick stems that would affect the drying process. The harvested leaves were stored in breathable woven bags (not plastic bags) and the harvesters completed a Collectors Form**.** Each bag was weighed and labelled and data were filled in on a transport dispatch note. The bags containing harvested vegetables were transported to the CSIR in Pretoria on the day of harvest.

3.1.2. Receiving of cowpea and moringa leaves:

At the CSIR a batch number was allocated to each received bag containing the cowpea and moringa leaves. Each bag was weighed and the mass was recorded. The moisture content of the vegetables was checked as described below and random samples were aseptically selected from the bags, labelled and then taken to the laboratory for full microbiological and nutrients analyses as well as sensory evaluations. The portions of the cowpea and moringa samples not used for analyses were stored in a cold room at 4^{o}C.

3.1.3. Sorting of leafy vegetables:

The required number of bags containing a sufficient mass (around 50kg) of leaves for processing. The leaves were inspected for fungal and other contaminants and the size, colour and appearance of the leaves was recorded. Defective, insect-infested and contaminated leaves were discarded.

3.1.4. Washing of the leafy vegetables:

Laboratory scale: Approximately 20g of leaves with a healthy appearance was selected and about 5g of these leaves was removed and placed in a sterile petri dish as control. The remaining 15g of leaves were washed for 1min to remove soil and other dirt. These leaves were divided into three portions and each of these was placed into separate petri dishes – around 5g per dish. Apart from the control, unwashed leaves, each portion of leaves was then rinsed for 1min by the addition of washing fluid to the leaves that were then gently swirled for 10 seconds. Three different washing treatments per 5g of leaves included 50ml of tap water, 50ml of distilled water or 50ml of soapy water. The soapy water was prepared by adding 1g of household food-grade detergent to 99ml of tap water. The petri dishes with rinsed leaves were then inverted and allowed to drip dry for 1min. Control and treated leaf samples were then analysed to determine levels of microbial contaminants. The leaves from the tap water washing method were then selected for nutrient analysis and sensory evaluation.

Industrial scale: Leaves were treated as above, but on a larger scale using a total of 5kg of leaves. Approximately 1kg of leaves was placed in a clean plastic bucket for each washing method before 5 litres of washing liquid was added to each bucket that was then gently swirled for 10min. The liquid was then poured off through a small mesh metal sieve (**Figure 6)**. Note that sanitizer (2% sodium bicarbonate (w/v), 2% ammonium bicarbonate, 0.005M calcium chloride, sodium metabisulfite (250 ppm)) was used instead of detergent in the laboratory scale study.

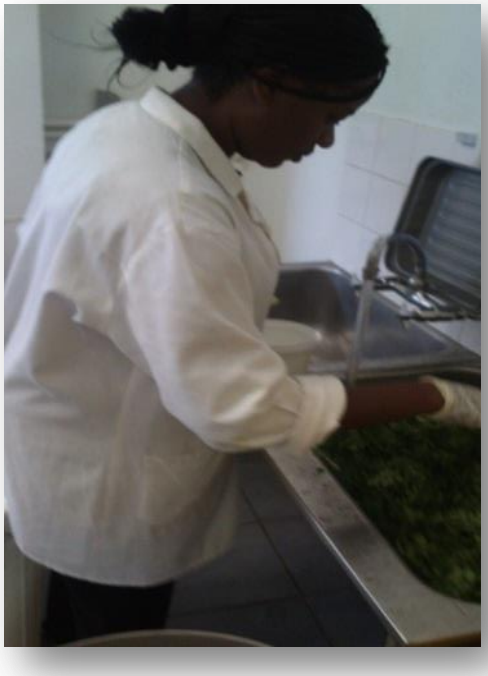


Figure 6: Photograph of leaf washing in tap water. Source: Photograph taken by author

3.1.5. Blanching

Laboratory scale: Approximately 10g of tap water-rinsed leaves were selected (after drip drying for 1min) and a 5g portion of these leaves was moved to a sterile petri dish as a control. Then, 50ml of hot water (95^{o}C) was added to the remaining 5g of leaves before gently swirling for 2min. Control and treated leaf samples were then analysed for microbial contamination, nutrient analysis and sensory evaluation.

Industrial scale: Leaves were treated as above, but at a larger scale using a total of 2kg of leaves. Approximately 1kg of tap water-washed leaves was immersed in hot water at 94^{o}C $\pm$1 for 6-8 minutes (hot water blanching). The other 1kg of tap water washed leaves were divided into approximately 100g portions each of which were placed on perforated stainless

steel trays, which was passed through the steam tunnel machine for 12min (optimum blanching time) at 95^{o}C (Onayemi *et al*, 1987) as shown in **Figure 7**.

Figure 7: Industrial steam tunnel blanching

Source: Taken by author

Note: To determine the optimum blanching time for the industrial process, washed samples were passed through the steam tunnel blanching machine and samples were removed for analysis at different times (e.g. after 0, 3, 6, 9, 12, 18, 24 and 30min) after which peroxidase tests were performed on the samples according to the method described by Arun and Sarita (2011).

3.1.6. Drying

Laboratory scale: Approximately 100g of blanched leaves and tap water-washed leaves as control were drip-dried for 1min. The leaves were then placed in a clean stainless steel tray and placed in direct sunlight for 2 days. The dried leaf samples (control and treated) were then analysed for microbial contamination, nutrient analysis and sensory evaluation.

Industrial scale: Leaves were treated as above, but at a larger scale using a total of 15kg of leaves. The blanched (hot water-blanched and steam-blanched) leaves were divided into three portions of 5kg each. The first portion was dried in an oven at 60^{o}C $\pm$1^{o}C for 48hrs, the second portion was spread on a wooden tray and placed under direct sunlight for 3 days and the third portion was freeze dried. These dried leaves were then collected, weighed, labelled and samples were taken randomly from each portion for full microbial analysis, nutrient analysis and sensory evaluations. The remaining dried samples were milled into fine powder and stored in air-tight thermally sealed plastic containers at room temperature.

Research Methodology: Part B

3.2. Microbiological Analyses

This section describes the microbiological analysis on cowpea and moringa leaves. The following microbiological analyses were carried out on both leafy vegetables after each processing step.

3.2.1. Microbial Enumeration and Isolation

3.2.1.1. Sample treatment

Five grams of the vegetable leaves were diluted with 45ml sterile Maximum Recovery Diluent (MRD) solution (Oxoid LTD, Basingstoke, Hampshire, England) and homogenized for 2min at normal speed in a stomacher. A 10^{-1} to 10^{-8} dilution series was then prepared in MRD and enumerated on each of the different agar media. This serial dilution was performed as follows: 5 g of each sample was diluted by aseptically adding 45ml of MRD and then homogenised in the Stomacher for 2min. One ml (10^{-1} dilution) of each sample was then aseptically transferred into the 10^{-2} dilution and similarly serial dilutions were aseptically prepared up to 10^{-8} in a 76% ethanol sanitised laminar flow cabinet.

3.2.1.2. Determination of total viable count:

The Total Viable Count (TVC) enumerates the total population of viable, non-fastidious microorganisms that grow at mesophilic temperature (i.e. 30^{o}C). Such microorganisms include bacteria, yeast and moulds.

Determination of Total Viable Count in vegetable samples was performed using the Plate Count Method using **T**ryptone soy agar (TSA) (Oxoid LTD, Basingstoke, Hampshire, England) that was prepared according to supplier's instructions.

For pour plating, an aliquot of 1ml of each dilution (10^{-3} to 10^{-8}) was pipetted into a sterile plate and 20ml of TSA (at 48^{o}C) was added to each, before being gently gently mixed. The labelled plates were then left to solidify before transferring to the incubator. The plates were incubated at 30^{o}C for 48hrs before being assessed. This involved counting colonies on plates with between 25 to 250 colonies and the colony count was calculated from dilution factors to give the Total Viable Count according to ISO Method 21527 (Rahman *et al*, 2010).

3.2.1.3. Determination of Yeast and Mould Count

To determine the total viable count of yeast and moulds in vegetable samples using the plate count method, potato dextrose agar (PDA) (Oxoid LTD, Basingstoke,Hampshire, England) was acidified with 10% Tartaric acid (Sigma-Aldrich Inc., St Louis, USA). Serial dilutions were prepared of each sample and 1ml of each dilution was transferred into each plate. Thereafter, approximately 20ml of acidified PDA was added to each plate and gently swirled before allowing the medium to solidify before incubating at 25^{o}C for 5 days. The number of growing colonies was recorded as a combined count according to SABS Method 758.

3.2.1.4. Determination of Coliform Count

Scope: Enumeration of coliforms in vegetable leaves using the Most Probable Number (MPN) method.

Principle: Determination of the number of coliforms by means of a MPN table according to the number of inoculated tubes which gave rise to gas formation in the selective coliform medium. The MPN procedure involves a multiple tube fermentation technique where each of three decimal dilutions of the sample are inoculated into separate tubes of broth medium and incubated at a specific temperature and for a specific time. The method is progressive; i.e., first determining the presence of coliforms in the tubes (presumptive coliforms), then determining if these tubes also contain faecal coliforms (confirmed coliforms), and then

confirming whether *E. coli* is present (Tryptone water Indole test - *E.coli* confirmed). Based on the number of tubes indicating the presence / absence of the three groups of organisms, the most probable number (MPN) present can be estimated from a standard statistical MPN table. The method has been shown to produce satisfactory results with naturally-contaminated foods and water for the detection of coliforms, faecal coliforms and aerogenic *E. coli* (Rahman *et al*, 2010). Briefly, lauryl sulphate tryptose broth (LTB), brilliant green bile broth (BgBB) (Oxoid LTD, Basingstoke, Hampshire, England), were separately dispensed as 9ml aliquots with inverted Durham tubes. Tryptone water was also dispensed in 5ml volumes with inverted Durham tubes.

The MPN Method –Three tube method – involves preparing three serial dilutions of the cowpea samples and for each dilution, inoculate 1ml into each of the tubes of LTB; then after 24hrs of incubation, inoculate 1ml from each positive test tube into BgBB tubes.

E.coli

Positive *E.coli* culture was sub-cultured into Tryptone water and tested for indole production using Kovac's (4(p)-dimethylamino-benzaldehyde) following incubating in a 44^{o}C water bath for 2 days.

3.2.2. Gram Staining

Protocol:

A loopful of sterile distilled water was placed on a slide; using a sterile cool loop a small sample of an isolated bacterial colony was transferred to the drop, and emulsified. The film was allowed to air-dry by passing it briefly through the Bunsen flame two or three times without exposing the dried film directly to the flame. The slide was flooded with crystal violet solution for up to one minute, washed off briefly with distilled water and drained. This was

followed by flooding the slide with Gram's Iodine solution, and allowed to act as a mordant for one minute, washed off with distilled water and drained. The slides were then flooded with 95% acetone for 10 seconds, washed off with distilled water and allowed to drain. Lastly the slides was flooded with safranin solution and allowed to counterstain for 30 seconds, washed off with distilled water, drained and blotted dry. All slides were examined under the oil immersion lens (CSIR in-house protocol).

3.3. Shelf – life study

Approximately 200g of unwashed, tap water-washed and steam blanched moringa samples were separately packaged in 5g portions in sterile, perforated, thermally sealed, plastic bags for storage at 4^{o}C. Each bag was used for microbiology and sensory analysis after 3 - 5 days for a storage period of 14 days. Then 1kg of milled samples were placed separately in 20g portions in sterile thermally sealed plastic bags and stored at -16.5^{o}C, 4^{o}C and at room temperature. Every 30 days over a six months period, a 20g portion of leaves was removed from each bag and analysed for microbial contamination.

Figure 8: Shelf life sensory evaluation of moringa leaves. Photographs (left to right) show leaves that were steam-blanched, washed with tap water, fresh unwashed and steam tunnel blanched. Source: Taken by author

3.4. Biochemical Analysis

The following tests are importance in order to determine the effectiveness of vegetable blanching treatments (temperature and time). Incomplete enzyme inactivation has a negative effect on the finished product quality (Rahman *et al*, 2010). The biochemical analyses carried out were:

- Indole test

This is a confirmatory test for the presence of *E.coli*. Tryptone water was prepared according to manufacturer's instruction. Briefly, 5ml of broth was dispensed per test tube and autoclaved at 121^{o}C for 15min. The bacteria was aseptically inoculated into the broth and incubated for 24hrs at 37^{o}C (Rahman *et al*, 2010). A pinkish-reddish colour change after the addition of a few drops of Kovac's reagent indicates a positive reaction while no colour change indicates a negative reaction (Prabhun *et al*, 2011).

- Peroxidase test

A few drops of 1% guaiacol solution and 0.3% peroxide solution were added directly onto blanched and crushed vegetables leaves. A rapid and intensive brown-reddish tissue colouring indicates a high peroxidase activity (positive reaction) while no colour change after 5min indicates inactivation of the enzyme peroxidase (negative reaction). These results are indicated in **Figure 9** and **Figure 10**, respectively (Rahman *et al*, 2010).

- Catalase test

Two grams of dehydrated vegetables were well crushed and mixed with about 20ml of distilled water. After 15min softening, 3% peroxide solution was poured on the prepared vegetables. The formation of bubbles of free oxygen gas after 2-3 minutes represents a positive reaction (Prabhun *et al*, 2011, Rahman *et al*, 2010).

Figure 9: Positive peroxidase test on cowpea leaves. Source: Photograph taken by author.

Figure 10: Negative peroxidase test on cowpea leaves. Source: Photograph taken by author.

Research Methodology: Part C

3.5. Proximate Analysis

The moisture, ash, fat, carbohydrate, protein and fibre content of cowpea and moringa samples were determined at the CSIR Food Science Analysis Laboratory in Pretoria, South Africa.

3.5.1. Moisture Content

The moisture content of cowpea and moringa samples were checked using a halogen moisture analyser (HX204 Mettler Toledo) where 3g of each sample was weighed into an aluminium dish and placed in the automatic halogen moisture analyser. The results were recorded as a percentage (CSIR-AM020)

3.5.2. Ash content

Clean crucibles were dried inside a muffle furnace for 2hrs at 55^{o}C after which the crucibles were placed in the desiccator to cool. Each crucible was weighed and 3g of the vegetable samples were dispensed per crucible and placed in a muffle oven (Furnace Nabertherm, Germany) at 550^{o}C overnight as shown in and Fig 11, after which the crucible was weighed and the ash (Fig 12) percentage was calculated (AOAC, 1990).

$$\% \text{ Ash content} = \frac{\text{Mass of crucible after ashing} - \text{Mass of crucible before ashing}}{\text{Mass of sample}} \times 100$$

Figure 11: Photograph of muffle oven containing crucibles with moringa leaves

Source: Photograph taken by author

Figure 12: Photograph of Vegetable ash remaining in crucibles following ashing of moringa leaves. Source: Photograph taken by author.

3.5.3. Fat Content

The fat content was determined by weighing 5g of vegetable samples into extraction thimbles and weighing the labelled extraction cups before and after the extraction. The thimbles were connected to a Soxhlet extractor (Gerhadt, Germany) and extracted with petroleum ether for 4hrs. The residue in the extraction bottle after solvent removal represents the fat content of

the samples (Sanjukta *et al*, 2013). The result was reported as a percentage and was calculated as:

$$\% \text{ Fat content} = \frac{\text{Mass of thimble after extraction} - \text{Mass of thimble before extraction}}{\text{Mass of sample}} \times 100$$

3.5.4. Protein Content

The protein analysis was performed in duplicate using a Trumac Nitrogen Analyser according to the manufacturer's instructions (LECO Africa). The nitrogen value is an indicator of the protein content of a substance and was determined by digestion, distillation and finally titration of the sample. The nitrogen value was converted to protein by multiplying by a factor of 6.25 (AOAC, 1990).

3.5.5. Carbohydrate Content

Carbohydrate content was determined by subtracting the total sum of the moisture, ash, crude fibre, fat and crude protein from 100.

3.5.6. Crude Fibre Content

Crude fibre was determined by treating oil-free sample with sulphuric acid (0.26 N) and potassium hydroxide (0.23 N) solution in refluxing systems, followed by oven drying and muffle furnace incineration (AOAC, 1990).

3.5.7. Energy Content (by calculations)

The energy value was calculated by multiplying the mean values for the crude fat, protein and total carbohydrates by 37, 17 and 17, respectively (Akinyele *et al*., 2011).

3.6. Sensory Evaluation

The main reason for sensory testing was to provide data on which sound decisions may be made. It is an integrated, multi-dimensional measure with three important advantages: it rapidly identifies the presence of notable differences, identifies and quantifies important sensory characteristics and identifies specific problems that cannot be detected by other analytical procedures, such as consumer preference.

Sensory evaluation was carried out in the laboratory by 10 panellists, who were used to evaluating the qualities of colour, texture (crispness), odour, and general acceptability of fresh, blanched, dried and milled vegetable (moringa) samples. A simple questionnaire was given to each panel to record their observations about the samples. Panellists were asked to compare the quality attributes of the vegetables using a preference scale with 1= extremely unacceptable and 5= extremely acceptable (Onayemi *et al*, 1987). **Appendix 3** is a copy of sensory evaluation questionnaire used.

Research Methodology: Part D

MOLECULAR BIOLOGY

The molecular biology aspect of this study was carried out in the molecular laboratory at UNISA Florida campus, Johannesburg under the supervision of Dr.Khayaletu Ntushelo.

3.7. Identification and Isolation of Coliforms

Presumptive coliforms were cultured in Lauryl Tryptose Broth (LTB) and Brilliant Green Bile Broth (BgBB) to confirm the presence of coliforms, as described in section 3.2.1.4.

A sterile, flamed loop was dipped into the cultured BgBB after 24hrs of inoculation and was transferred into a tube containing 5ml of Tryptone water (prepared according to manufacturer's instructions) and incubated in a water bath at 44^{o}C for 48 hrs, after which a loop of the broth was plated on Tryptone Soy agar (TSA) for 24hrs at 37^{o}C (3.2.1.4). This protocol is selective only for coliforms, *Enterobacteriaceae* and *E.coli*.

3.7.1. Molecular Analysis

Strain conservation of coliforms and yeasts

Surface coliforms and yeasts from fresh unwashed sample and milled sample were plated and incubated and counted. From these enumeration plates, ten coliform and ten yeast colonies were selected at random. The coliform colonies were inoculated in 10ml of Tryptone soy broth (TSB) (Oxoid LTD, Basingstoke, Hampshire, England) and incubated at 37˚C overnight. Cells were harvested by centrifugation at 4500x*g* for three minutes and the pellet was suspended in cryoprotective medium [K_2HPO_4 0,82 g.l^{-1}, KH_2 PO_4 0,18 g.l^{-1}, $C_6H_5Na_3O_7.H_2O$ 0,67 g.l^{-1},$MgSO_4.7H_2O$ 0,25 g.l^{-1} and sterile glycerol to 15% of the total volume (all substances from Merck KGaA, Darmstadt, Germany)] and frozen at -70˚C.

The yeast colonies from these samples were inoculated in 5ml Yeast Peptone D-glucose (YPD) broth [yeast extract 10g.l^{-1} (Oxoid LTD, Basingstoke, Hampshire, England),

bacteriological peptone 20g.l^{-1} (Oxoid Ltd)) and D glucose 20g.l^{-1} and incubated overnight at 25˚C. One ml of cell suspension was mixed with an equal volume of sterile glycerol and frozen at -70˚C.

3.7.2. DNA extraction

Coliform bacterial DNA was isolated by using the DNeasy tissue kit (Qiagen, Hilden,Germany) following the manufacturer's instructions.

Procedure:

A volume of 180µl of extraction buffer 1 (ATL) and 20µl of Proteinase K was added to 2ml tubes containing pure overnight cultured bacteria cells, which was then vortex mixed and incubated at 56^{o}C for 1hr after which it was micro centrifuged briefly. Then, 200µl of lysis buffer (AL) was added to each tube, vortex mixed and incubated at 70^{o}C for 10min.The mixture was centrifuged and 200µl of 100% ethanol was added to precipitate the DNA. Using a micropipette, the entire contents (~600 µl) of tube was transferred to a labelled spin column. This was followed by centrifuging at 8000rpm for 1min. The eluate was discarded and 500µl of wash buffer 1 (BW1) was added above the filter. This was followed by centrifuging at 8000 rpm for 1min. The process was repeated using wash buffer 2 (BW2) and centrifuging at 14000rpm for 3min. The eluate was also discarded and the filter was placed in a new 1.5ml tube (labelled accordingly). Lastly, 200µl of elution buffer (AE) was added and it was centrifuged at 18000 for 3min. The elution buffer eluted the DNA into the clean tubes that were then stored appropriately (4°C for short term and -20°C for long term storage).

3.7.3. DNA quantification

DNA Quantification by Fluorometer

The fluorometer used at UNISA's molecular laboratory is the Quantus^{TM}from Promega. The following instructions were used for this specific fluorometer.

Procedure:

Preparation of a working solution was performed by diluting the supplied QuantiFluor® dsDNA Dye with I x TE buffer in a ratio 1:200. A blank solution was also prepared by adding 100µl of QuantiFluor® dsDNA Dye working solution and 100µl of 1X TE buffer to an empty 0.5ml PCR tube, mixing and while protecting from light. A standard solution was prepared by diluting the DNA Standard to 2ng/µl by adding 2µl of the provided DNA Standard to 98µl of 1X TE buffer, and mixed. Then, 100µl of QuantiFluor® dsDNA Dye working solution was added, and mixed.

Finally, preparation of the DNA samples was done by adding 100µl of the DNA sample and 100µl of QuantiFluor®dsDNA Dye working solution to a 0.5ml PCR tube, and mixing. The prepared samples were incubated at room temperature for 5 min, protected from light.

The dsDNA protocol on the Quantus™ Fluorometer was selected and the Quantus™ Fluorometer was calibrated by reading the blank and standard samples in the calibration screen and then selecting “Save”. The volume of the unknown sample and desired concentration units was entered and fluorescence of the DNA samples were determined.

3.7.4. Polymerase Chain Reaction

Repetitive-DNA-element PCR fingerprinting made use of the following primers:

- pA Forward primer (5' AGA GTT TGA TCC TGG CTC AG 3',)
- pH reverse primer (5' AAG GAG GTG ATC CAG CCG CA 3')

These primers were purchased locally (Inqaba Biotechnology, South Africa) and used to target the 16S rRNA to obtain genotypic differences as described by Bruce *et al*, (1992). The PCR samples were prepared in a total of 25µl and mixed according to the supplier’s recommendations (Inqaba Biotech, Pretoria, South Africa). Each contained 7.5µl of nuclease free water, 1µl each of forward and reverse primers, 3µl of crude DNA extract and 12.5µl of *Taq* Master Mix (Qiagen, Hilden, Germany).

Thermal cycling was performed in an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories Inc., USA). The reaction conditions for coliform amplification was modified from Sakallah *et al,* (2013) and Bruce *et al,* (1992) started with denaturation at 95˚C for 1 min, followed by 45

cycles of 95˚C for 30 seconds, 62˚C for 30 seconds, 72˚C for 30 seconds, with a final extension of 72˚C for 10 min then stored at 4^{o}C. The results of such a PCR are shown in **Figure 17**.

3.7.5. Electrophoresis

Electrophoresis is a method of separating substances based on the rate of movement of charged molecules while under the influence of an electric field. During electrophoresis, the gel is submerged under buffer in a chamber that has a positive and a negative electrode. After attaching a power pack to the electrodes and applying the appropriate electrical current, the DNA molecules move through the pores of the gel towards the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA is visualized by the use of a dye that binds to DNA.

Protocol

Coliform PCR amplification products were electrophoresed through 1% agarose gels. These gels were prepared by adding 1g of agarose powder to 100ml of 1 x Tris-acetate (TAE) buffer (pH 8), and dissolving by microwaving for 2min until the solution was clear. The gel was allowed to cool about 45˚C with gentle swirling of the flask. Then, 3µl of ethidium bromide was added to the liquid agarose and was mixed thoroughly but gently to avoid bubbles in it (Sakallah *et al*, 2013). The gel was then poured into the gel casting tray that has prepared with rubber gates and the appropriate gel comb. The gel was allowed to cool until it set before the comb and rubber gates were carefully removed from the gel casting tray and the gel placed into the electrophoresis chamber ensuring that the wells were placed at the negative

electrode charge. The chambers were then filled with 1X TAE running buffer so that the gel was just covered with buffer.

Loading the gel

A volume of 1μl of loading dye was added to each sample before 3μl of each sample was loaded into each well of the gel. The solution containing the DNA ladder was appropriately diluted with loading dye before the DNA ladder was carefully loaded into the first and last well of the gel.

Running the gel

The lid was placed on the gel box, the electrode wires were connected to the power supply, making sure the positive (red) and negative (black) are correctly connected. The power supply was switched on, and calibrated as 100 amp, 65volts and 60min. The whole process was monitored ensuring that;-

- the current is running through the buffer by looking for bubbles forming on each electrode.
- that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
- the power run until the dye approaches the end of the gel.

After completion of the process the power was switched off, the wires disconnected and the lid was removed. Using gloves, the tray and gel were carefully removed and the gel was visualized with UV light and photographed with a Polaroid Photo documentation camera. The gel was then appropriately disposed of.

CHAPTER 4

RESULTS

4.1. The effect of processing techniques such as washing and hot water or steam blanching on the microflora of cowpea and moringa leaves

The washing technique is not only used to remove field soil and surface microorganisms, but also to remove fungicides, insecticides and other pesticides from the leafy vegetables. Tap water, sanitizer, 1% detergent solution and distilled water were used to wash the different batches of the cowpea and moringa leaves, so as to determine the most effective washing techniques (**Table 5**). Washing the vegetables twice was found to be the most effective washing technique because it gave a relatively lower total viable microbial count of vegetables, when compared to other washing techniques. Although washing with 1% detergent water resulted in the lowest total viable microbial count, it affected the sensory quality of the vegetables negatively, making it slimy and discoloured. Blanching is a short heat treatment to inactivate enzymes catalase and peroxidase present in the vegetables, wherein a portion of the 2x tap water washed leaves was plunged into boiling water 94°C (hot water blanching) for 5min and the second portion was passed through a steam tunnel at 85^{o}C-94°C (steam blanching), removed after 12min the optimum steam blanching time (the shortest treatment time which gave a negative result to the peroxidase and catalase test). These results are indicated in **Table 6.**

Table 5: The effect of washing and blanching techniques on the total viable microbial count of cowpea and moringa leaves as compared to SABS standard.

	Treatment	Laboratory scale Total viable count (Ave.log cfu/g)	Industrial scale Total viable count (Ave.log cfu/g)	SABS Acceptable limit (Ave.log cfu/g)
Cowpea	Unwashed control	2.62±0.02	7.3±0.03	6.0
	Tap water washed	1.55±0.08	6.16±0.04	6.0
	Distilled water wash	1.50±0.06	6.09±0.06	6.0
	1% Detergent wash	0	Nd	6.0
	Sanitizer wash	Nd	7.0±0.05	6.0
	Hot water blanched	1.06±0.04	5.18±0.02	6.0
	Steam Blanching	Nd	4.15±0.03	6.0
Moringa	Unwashed	2.61±0.02	5.82±0.01	6.0
	Tap water wash	1.37±0.05	4.12±0.04	6.0
	Distilled water wash	1.30±0.04	4.19±0.05	6.0
	1% Detergent wash	0	Nd	6.0
	Sanitizer wash	Nd	5.0±0.05	6.0
	Hot water blanched	1.13±0.05	3.98±0.07	6.0
	Steam blanching	Nd	2.75±0.04	6.0

(n = 3) , Nd = not done

Mean values ± Standard deviation values ($p < 0.05$).

Table 6: Determination of optimum steam blanching time using catalase and peroxidase activities

Sample Treatment period	**0 min**	**3 min**	**6 min**	**9 min**	**12 min**	**18 min**	**24 min**	**30 min**	**36 min**
Total Viable count	7.75	7.57	7.9	7.34	3.57	4	3.7	3	3.33
Catalase test	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
Peroxidase test	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-

(n = 3)

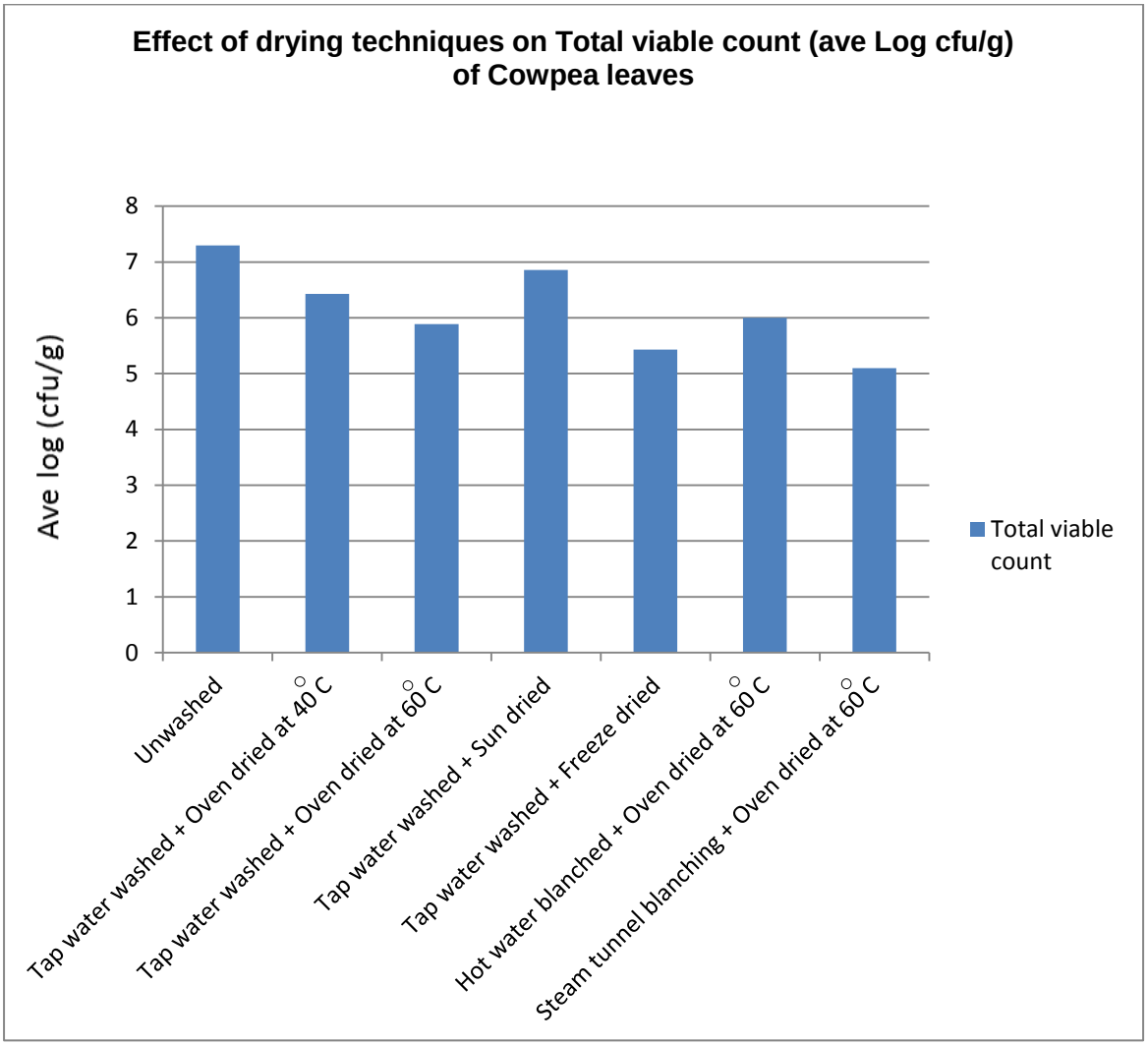
4.1. Up-scaled processing techniques:

Washing twice with tap water and steam blanch techniques gave best results for both lab and pilot-scale which conforms to SABS standard on the level of vegetables microbial load. **Table 5** shows the results of the average log of the total viable microbial count.

Table 6 reveals the results of peroxidase and catalase test on cowpea leaves after steam blanching at different time internals. At 12min the leaves tested negative for both catalase and peroxidase test, indicating the optimum blanching time for enzyme inactivation.

4.2. The effect of various leaf drying techniques on microbial load of cowpea and moringa leaves

During this processing technique, moisture was removed from the leaves by simultaneous heat and mass transfer. The drying techniques of the vegetables required a dependable model to predict its drying behaviour (Premi, 2010). Various drying techniques used were: shade-drying, freeze drying and oven drying and their effect on the microbial load of cowpea leaves are shown in **Figure 13**. Similar results were found also for moringa leaves.



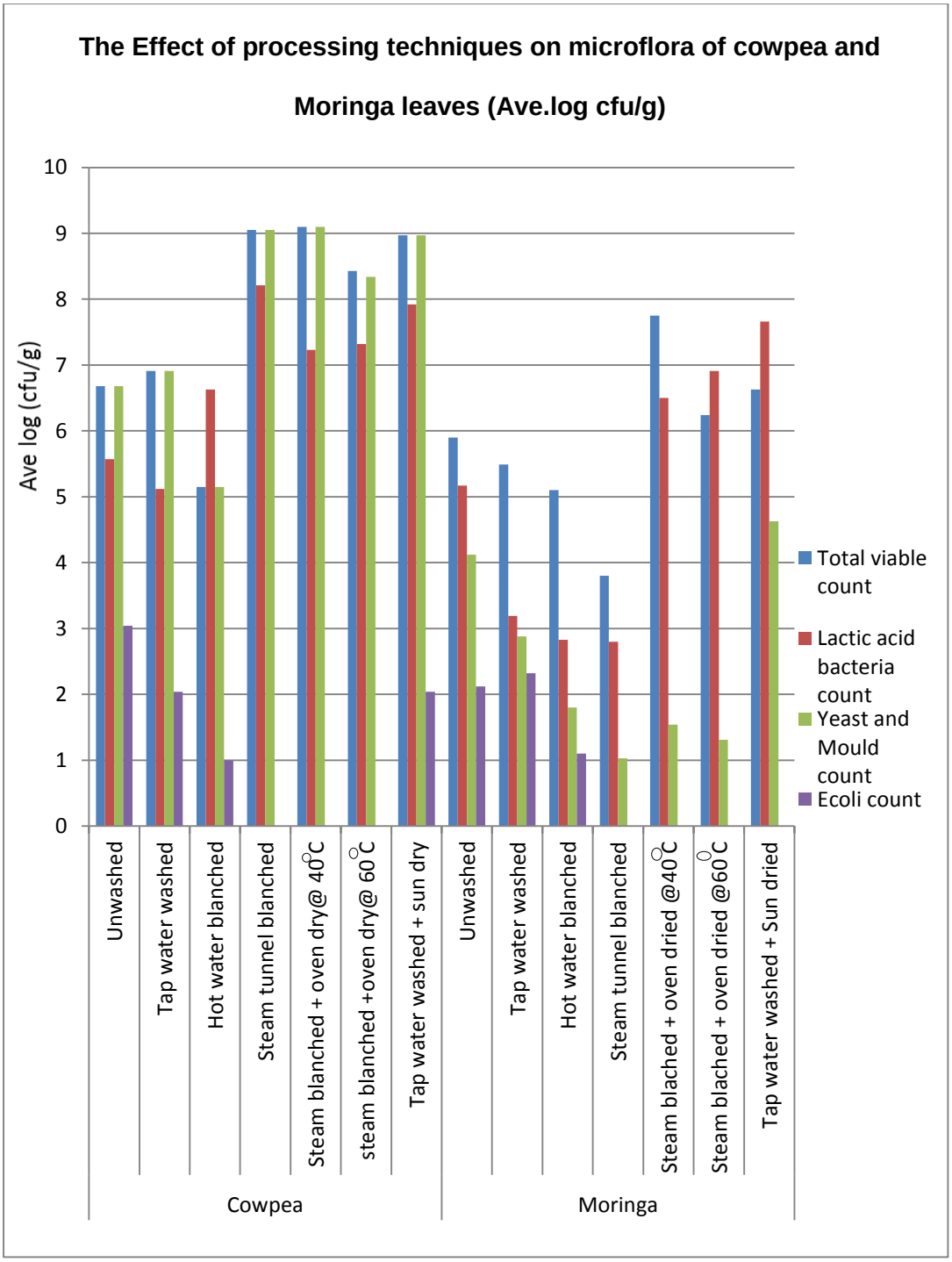
(n = 3)

Figure 13: The effect of drying techniques on the microbial load of moringa leaves

4.3. The effect of processing techniques on the microflora of cowpea and moringa leaves

The results of total viable count, lactic acid bacteria count, yeast and mould count and *E.coli* count for cowpea and moringa leaves (**Figure 14**) show the survival, growth or reduction of the total viable count, total lactic acid bacteria count, total yeast and moulds count and total *E.coli* count under different processing technique conditions.

Steam blanching at 94^{o}C was the most effective technique in terms of reduction of microbial load, and it also eliminated all presence of *E.coli* from the vegetable samples.



(n = 3)

Figure 14: The effect of processing techniques on the total viable count, lactic acid bacteria count, yeast & mould counts, *E.coli* count (Ave.log cfu/g) on cowpea and moringa leaves

4.3.1. Shelf –life study

The shelf–life of tap water washed, steam blanched and milled moringa leaves were studied over a period of time, and the respective microbial load is listed in **Table 7**. The sensory evaluation result on the shelf life of moringa leaves are also listed in **Table 8.** These shelf life studies revealed that there was a significant increase in the microbial load of the washed and blanched leaves after day 4; this could be as a result of its higher moisture content than the unwashed leaves or maybe mechanical damage during washing and handling which could have led to contaminants finding entry into the leaves**.** Dried stored leaves maintained a constant level of microbial load for the first six months only with an insignificant variation (**Table 7**).

Figure 15 shows the level of the microbial load over the period of shelf life study as measured against the acceptable level of South African Bureau of Standards in regards to food safety, acceptable limit of microbial load (https://www.capetown.gov.za/en/CityHealth/Documentation).

This means on day 1 both processed and unprocessed moringa leaves are safe for consumption after which only the dried leaves are safe for consumption.

Table 7: Shelf life study on moringa leaves

	Treatment/ Total viable count (Ave. log cfu/g)			
Day(s)	Unwashed leaves	Tap water washed leaves	Steam blanched leaves	Oven dried leaves @ 60^{o}C
1	5.4±0.04	5.5±0.06	4.1±0.07	5.0±0.03
3	6.4±0.03	6.2±0.03	5.8±0.05	5.1±0.04
5	6.7±0.05	7.1±0.07	6.3±0.09	5.0±0.05
9	6.8±0.08	7.7±0.05	6.8±0.04	5.1±0.04
14	7±0.02	7.2±0.05	6.9±0.06	5.2±0.03
30	>1000	>1000	>1000	5.4±0.01
60	>1000	>1000	>1000	5.5±0.01
90	>1000	>1000	>1000	5.6±0.03
120	>1000	>1000	>1000	5.6±0.02

(n = 3) Mean values ± Standard deviation values ($p < 0.05$).

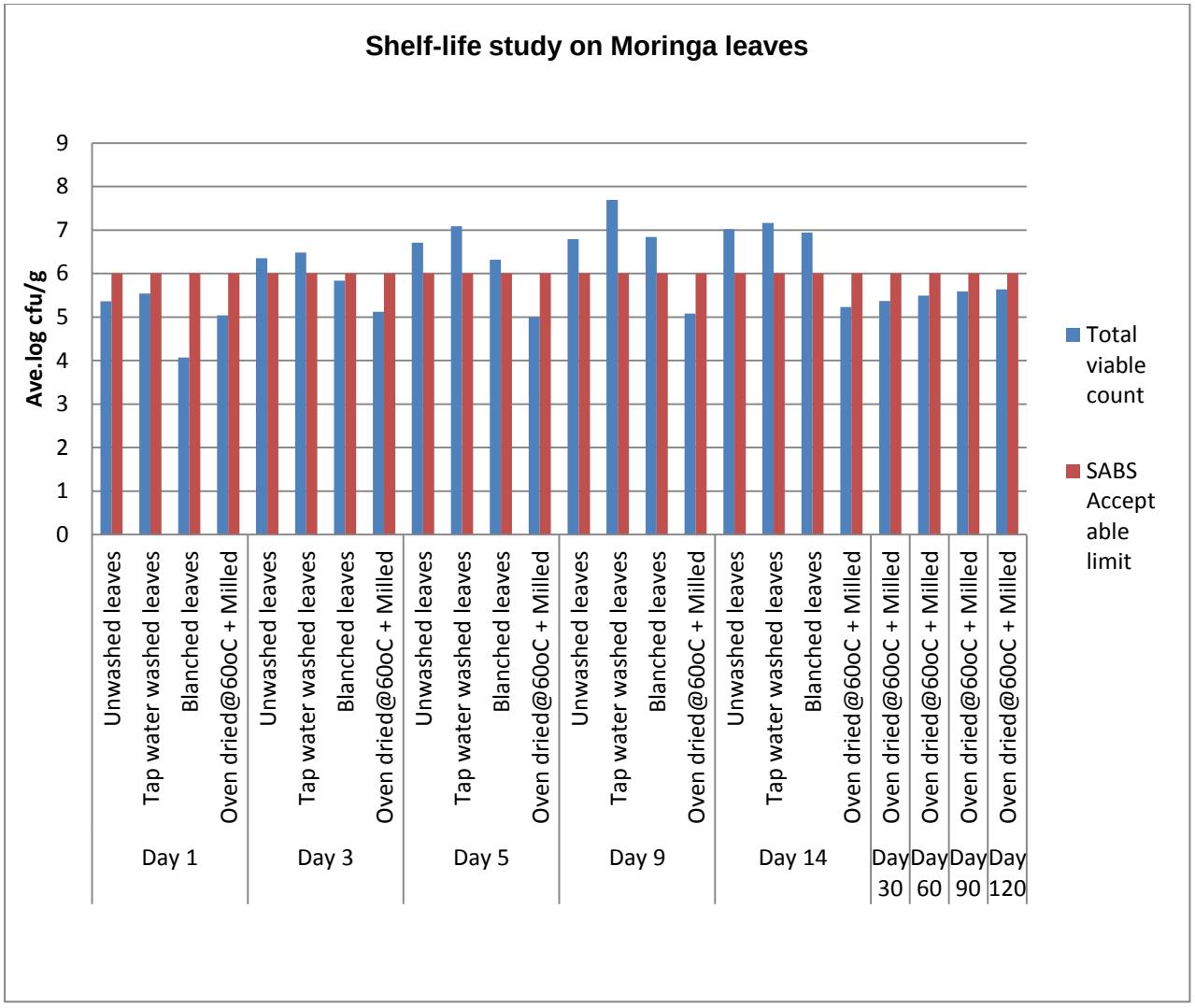


Figure 15: Shelf life study of moringa leaves as compared to SABS acceptable limit

4.3.2. Sensory evaluation

Table 8: Sensory evaluation of moringa leaves tested over 15 days of shelf life

Shelf life	Sample	General appearance	Colour	Texture	Odour
Day 1	Unwashed	4	4	5	3
	Tap water washed	5	5	5	4
	Hot water blanched	3	3	3	4
	Steam Blanched	4	3	3	5
	Milled	3	2	4	2
Day 3	Unwashed	4	4	5	3
	Tap water washed	4	4	5	4
	Hot water blanched	3	3	3	4
	Steam Blanched	4	3	3	5
	Milled	3	2	4	2
Day 5	Unwashed	4	4	5	3
	Tap water washed	3	4	3	4
	Hot water blanched	2	3	2	3
	Steam Blanched	3	3	2	3
	Milled	3	2	4	2
Day 9	Unwashed	3	2	2	3
	Tap water washed	2	3	3	2
	Hot water blanched	2	2	2	2
	Steam Blanched	3	2	3	3
	Milled	3	2	4	2
Day 15	Unwashed	2	2	2	1
	Tap water washed	1	5	5	1
	Hot water blanched	1	3	3	1
	Steam Blanched	2	2	3	2
	Milled	3	2	4	2

(no of panellists = 10)

Values represent means ± standard deviation ($p < 0.05$).of triplicate determinants based on a 5 point scale, where 1= extremely unacceptable and 5= extremely acceptable.

Key

5	Extremely acceptable
4	Acceptable
3	Neutral
2	Not acceptable
1	Extremely Unacceptable

4.3.3. Proximate quality of cowpea and moringa leaves

The proximate composition revealed that both cowpea and moringa leaves are very nutritious, containing protein, carbohydrates, fiber, etc. **(Table 9)** and this agrees with the reports by Arun and Sarita (2011) and Okonya *et al* (2010). Tap water washing and blanching resulted in a net uptake of water into the leaves. Drying of the leaves resulted in a mean moisture loss of 85% with an increase in other proximate compositions. Steam blanching further increased the moisture contents for both vegetables. Cowpea's ash content remained constant (1.1%) during all treatment except after drying (2.4%), and for moringa leaves its ash content after drying was increased from 2.37%to 9.70%.The carbohydrate content in cowpea was found to be lower than that of moringa leaves and the total energy calculated from moringa leaves is also higher than that of cowpea leaves.

Table 9: Proximate composition of cowpea and moringa leaves

	Proximate composition	Unwashed	Tap water washed	Steam blanched	Oven dried
Cowpea	Moisture content (%)	85.2 ±0.04	87.1±0.03	89±0.06	5.6±0.03
	Ash (g)	1.1±0.04	1.1±0.04	1.0±0.03	2.4±0.01
	Total Fat (g)	0.2±0.06	0.2±0.07	0.2±0.05	2.7±0.06
	Total Dietary Fibre (g)	7.5±0.03	7.5±0.07	7.0±0.05	45.5±0.05
	Protein (Nx6.25) (g)	3.6±0.07	3.6±0.04	3.6±0.04	26.1±0.03
	Available Carbohydrates (g)	0	0	0	3±0.03
	Energy (KJ)	70±0.06	70±0.03	70±0.06	590±0.08
Moringa	Moisture content (%)	74.5±0.05	80.7±0.02	84.48±0.04	5.30±0.04
	Ash (g)	2.37±0.02	2.4±0.04	1.21±0.03	9.70±0.05
	Total Fat (g)	0.41±0.06	0.41±0.05	0.41±0.05	2.3±0.08
	Total Dietary Fibre (g)	0.9±0.07	4,3±0.03	4.0±0.01	9.68±0.05
	Protein (N x 6.25) (g)	6.9±0.04	12±0.03	11±0.05	27.1±0.02
	Available Carbohydrates (g)	12.5±0.04	11.2±0.03	11.9±0.05	38±0.02
	Energy (KJ /100g)	305.2±0.03	340±0.06	345.5±0.08	1320±0.01

(n = 3) All nutrient composition are expressed as mean of triplicate analysis of fresh weight

Mean values ± Standard deviation values ($p < 0.05$).

4.3.4. Identification of bacteria and fungi contaminant found on the leaves of treated and untreated moringa and cowpea leaves

Figure 16 represents the morphology of the yeast cells obtained in leaves of *Moringa oleifera.* Morphological and biochemical tests such as the catalase, oxidase and indole tests were used to identify the bacteria derived from the moringa leaves as indicated in **Table 10**.

Table 10: The morphological and biochemical characterization of bacteria found on moringa leaves

Colony Description	Gram stain	Cell Shape	Indole test	Catalase test	Suspected Bacteria
Smooth pink	-ve	Short Rod	+ve	+ve	*E.coli*
Mucoid pink	-ve	Short Rod	-ve	+ve	*Klebsiellia pneumonia*
Mucoid pink	-ve	Rod	-ve	+ve	*Enterobacter* spp.
Red pink	-ve	Short Rod	-ve	+ve	*Serratia marcescens*
Pale colored	-ve	Rod	+ve	+ve	*Salmonella spp.*
Pale colored	-ve	Rod	-ve	+ve	*Shigella dysenteriae*
Mucoid pale	+ve	Rod	-ve	+ve	*Bacillus*
Yellow	+ve	Round	-ve	-ve	*Staphylococcus*
Joined together in a chain	+ve	Round	-ve	-ve	*Streptococcus*
Cream/ yellow	-ve	Rod	-ve	+ve	*Pseudomonas*

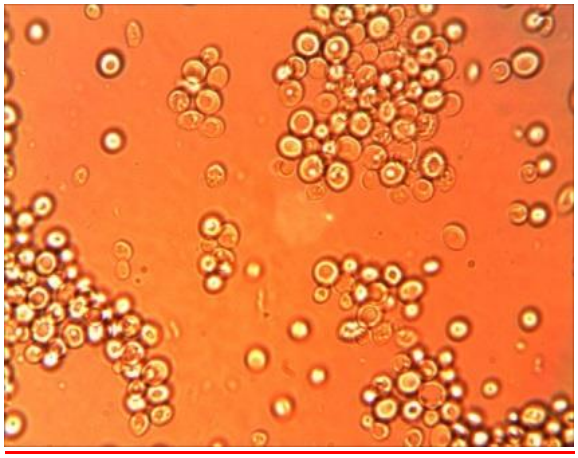


Figure 16: Photograph of yeast cells from moringa leaves under oil immersion (x100)

Source: Photograph taken by author

4.3.5. Identification of predominant coliform bacteria using polymerase chain reaction (PCR).

Results of the PCR were positive regarding the amplification of the 16S ribosomal RNA gene in 11 wells out of a total of 24 wells. The results of the PCR test are shown in **Figure 17**. The band related to the 264 bp genomic segment was clearly visible in the agar gel under UV in the PCR positive samples. This 264 bp amplicon is noted in lanes 1,2,3,4,5,8,9,10,11,12,19 and 23 in Figure 18 below. In addition, a similar band was also seen for DNA of *E coli* used as positive control (lane 26). As expected, no band was seen in the negative control in lane 16 where sterile distilled water was included instead of bacterial DNA.

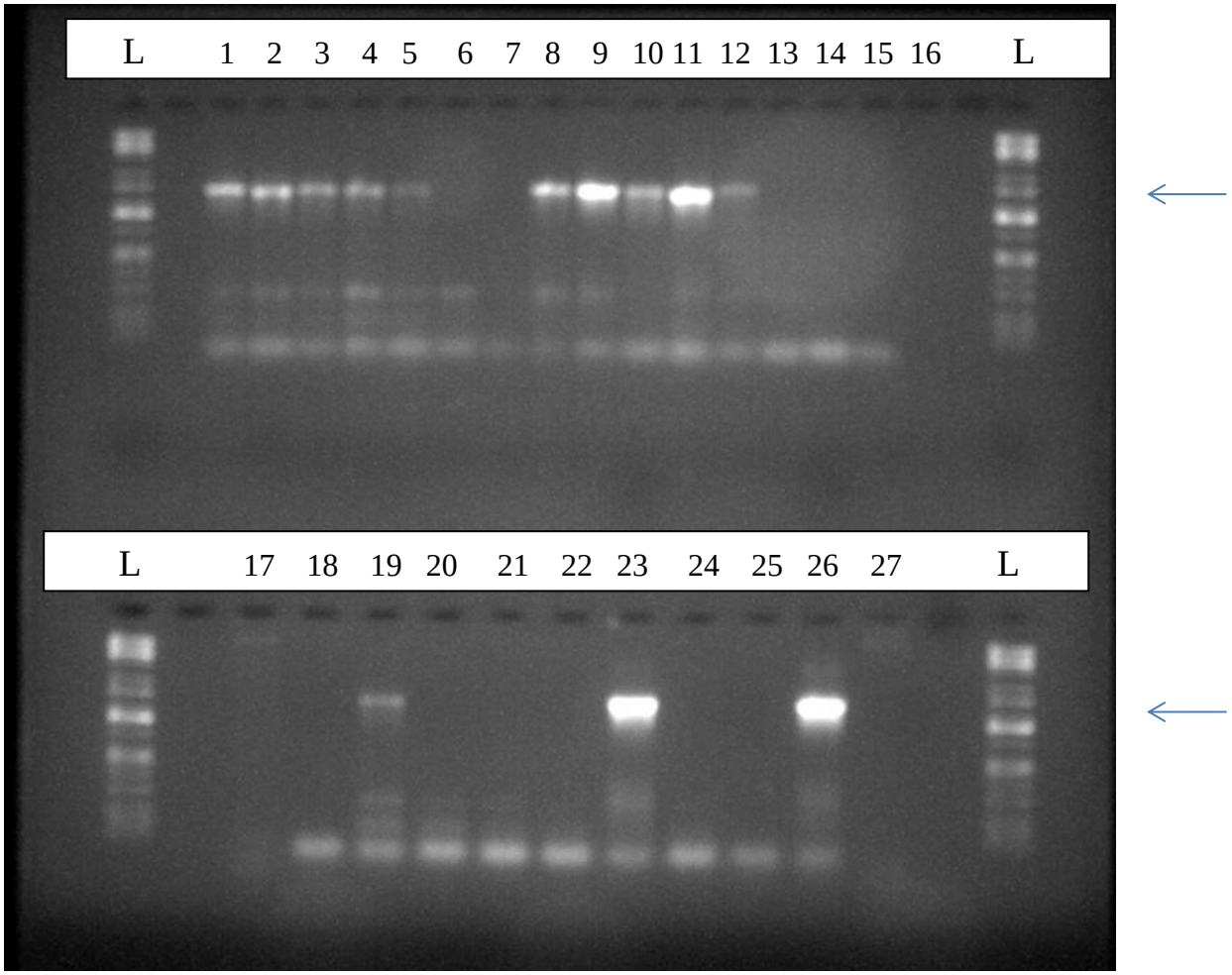


Figure 17: Genomic Bacterial DNA isolated from moringa leaves subjected to PCR with primers pAF and pHR. The horizontal arrows indicate the position of the 264 bp amplicon. Lanes 1 to 15 and 18 to 26 show the results of amplification of bacterial DNA. Samples in lanes 1 to 5, 8 to 12, 19, 23 and 26 were successfully amplified with this primer pair and thermal conditions. Lanes marked L contains 50 bp molecular weight ladder DNA. Lane 16 is a negative control reaction and lane 26 the positive control.

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DISCUSSION

Cowpea and moringa leaves have been reported to be highly nutritious and to have medicinal properties. However, they are still prone to microbial contamination resulting from pre-harvest or post-harvest contamination. Possible sources of contamination are manure-contaminated soil, irrigation water, water used to apply fungicides and insecticides, dust, insects, inadequately composted manure, human handling, harvesting equipment, transport containers, transport vehicles, processing equipment. The practice of animal grazing may result in the introduction into the soil of enteric bacteria such as *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* (Barth *et al*, 2010; Ibrahim and Jude 2009).

To reduce microbial contamination of the vegetables, good agricultural practices, high levels of worker hygiene and proper human waste management at production sites should be enforced, but most importantly, effective processing techniques must be utilized in eliminating these microbial contaminants from the vegetables (Ibrahim and Jude, 2009).

Microbiological analysis

As part of the objectives of this research, the effect of processing techniques on microflora and nutrient contents of cowpea and moringa leaves were investigated. The high average log cfu/g of viable counts from the unwashed cowpea leaves could be attributed to unhygienic farm conditions, contamination from the soil, and contamination from harvesting, packaging and transportation (Nielsen *et al*, 2000). The high moisture content of the fresh leaves also provided a favourable environment for the growth of microorganisms (Sanjukta *et al*, 2013). The relatively lower microbial load on unwashed moringa leaves as compared to cowpea leaves could be because the moringa leaves grow well above the soil as compared to the cowpea leaves that grow very close to the soil. The reduced bacterial count may also be due to its reported anti-microbial properties (Abalaka *et al*, 2012). All the processing techniques showed a reduction in the average log of microbial density. Washing with detergent was the

most effective of the washing modes, but it made the leaves soapy and slimy, an undesirable characteristic. Thus, tap water washing was the preferred method, while the use of the sanitizer wash had the lowest effect on the microbial load of the vegetables. Steam blanching was more effective than hot water blanching, and oven drying at 60^{o}C was the most effective preservation technique when compared to other drying techniques.

The effect of drying techniques on the microflora of cowpea and moringa were also studied. High moisture content provide a favourable environment for the growth of microorganisms, thus bacterial growth was reduced during drying. Sun-drying in direct sunshine and under shade is the common practices used in most parts of the world to preserve vegetables for dry season consumption (Nielson *et al*, 200). However, vegetable processing and preservation techniques may significantly affect the concentration and availability of nutrients and other essential compounds in food.

Different numbers and types of microorganisms were found to be present on the surface of cowpea and moringa leaves. Bacterial and fungal colonies were found when it was plated on Tryptose soy agar media and acidified Potato Dextrose media, respectively. The results obtained for bacterial colonies and fungal cells are shown in **Table 10** and **Figure 16.** Spore formers were abundant in cowpea (Average log cfu/g <9) and moringa leaves (Average log,cfu/g <7), several species of Bacillus were isolated and this could be as a result of the soil flora associated with these vegetables during growth. This agrees with the findings of Mpuchane and Gashe (1998) who reported several species of bacillus (<10$^{7)}$ from traditional leafy vegetables in Botswana.

Coliform bacteria counts are used for monitoring the bacteriological safety of food, based on the fact that the presence of coliform bacteria in food, water or vegetables is an indicator of level of hygiene and potential human fecal contamination and therefore the possible presence of enteric pathogens (Asim *et al*, 1989).The presence of pathogenic organism

which belong to this family has been reported in many fresh leafy vegetables (Mpuchane and Gashe, 1998), and this is mostly associated with the use of faeces-contaminated water and soil and crops contaminated by faeces from wild and farm animals and birds.

Proximate Analysis

The proximate composition revealed that both cowpea and moringa leaves are very nutritious **(Table 9)** and this agrees with the reports by Arun and Sarita (2011) and Okonya *et al* (2010).

Table 9 reveals values for moisture content and shows that cowpea has the highest value (87.1%) (tap water washed cowpea leaves) making cowpea leaves more prone to spoilage since foods with high moisture content are more prone to perishability (Sanjukta *et al*, 2013). The ash content of moringa leaves was higher than that of the cowpea leaves. The high ash content of the moringa leaves is a reflection of the mineral contents preserved in the food materials. The results, therefore, suggest a high deposit of mineral elements in the leaves.
The total fat content of water-treated cowpea leaves (0.2g) was lower than that of the moringa leaves (0.41g). A diet including moringa leaves may be more palatable than that of cowpea leaves because dietary fats function to increase food palatability by absorbing and retaining flavours (Sanjukta *et al*, 2013). A diet providing 1 - 2% of its caloric energy as fat is said to be sufficient for human beings, as excess fat consumption contributes to certain cardiovascular disorders such as atherosclerosis, cancer and aging (Ogbe and John, 2011). These vegetables are low-fat foods.

In comparison, moringa leaves had a higher protein content than the cowpea leaves. This makes moringa leaves a good source of protein. The protein content of moringa samples varied and are lower than those described by Ogbe *et al,* (2012) and Oduro *et al* (2008). These differences could be attributed to geographical locations of growth and stage of maturity of the plant (Ogbe *et al*, 2012).

The crude fibre content of moringa leaves was higher than that of cowpea leaves, and this makes it a more favourable vegetable since high fibre content of foods help in digestion and prevention of colon cancer (Mepha *et al*, 2007). Non-starchy vegetables are the richest sources of dietary fibre (Sanjukta *et al*, 2013) and are employed in the treatment of diseases such as obesity, diabetes and gastrointestinal disorders. The caloric value obtained showed fresh *M. oleifera* as having the lowest value (305.62 cal/g). The caloric value of these leaves make them a good source of energy for all.

Shelf life studies revealed that there was a significant increase in the microbial load of the washed and blanched leaves after day 4. This could result from its high moisture contents compared to the unwashed leaves **(Figure 15).** Dried stored leaves maintained a constant level of microbial load for the first six months only with an insignificant variation ($\pm$0.02).

The sensory evaluation revealed that on the 15th day, both the processed and unwashed moringa leaves were spoilt and unacceptable for consumption. This means that the shelf life of fresh moringa leaves whether processed or not is 9 days, except for the steam blanched that has a longer shelf life of 15 days. However, from the 3rd day, it is no longer safe microbiologically to consume the processed vegetable with the exception of the milled sample that had a constant microbial level and sensory test over a period of 120 days.

Conclusion

According to the South African regulations governing microbiological standards for foodstuffs and related matters in 2002 that state that “in the food industry, a product shall be deemed to be contaminated, impure, decayed or harmful or injurious to human health if any such product contains more than 10^6cfu per gram for total viable count, 10^4cfu per gram of yeasts and moulds and 10^3cfu per gram of coliforms (**https://www.capetown.gov.za/e/CityHealth**).

Although limitations exist, the results of this research revealed that cowpea and moringa leaves are good sources of nutrients, but that they harbour microbial contaminants and are, therefore, unsafe to eat without using proper processing techniques. Most South Africans prefer to utilise fresh vegetables even though the nutritional quality of dry vegetables is better, as this concentrates the nutrients and has a longer shelf-life. Hence, the quality of fresh produce of the local market must be maintained in hygienic conditions and proper handling, transport, storage must be ensured so that risk of contaminants decreases and chances of food borne disease outbreaks can be minimized. This can be achieved by pre-treatment of fresh vegetables by tap water washing twice and steam blanching so as to decrease the density of microbial contaminants on the surface of the vegetables, while oven drying at 60^oC, followed by milling and packaging in an air tight container will prolong the product shelf life. The consumption of these processed vegetables should be encouraged and promoted as vegetables in South Africa and other developing countries, due to their high nutrient content and other nutraceutical benefits.

RESEARCH DATA ANALYSES

Four groups of microorganisms were evaluated at five different processing points (fresh leaves, washing, drying, milling and storage (shelf-life). The experiment was repeated three times, once per month consecutively for four months. The results are presented with their average log values, means, standard deviation and standard error.

The data were analysed using the Microsoft Excel package and compared by ANOVA using the GLM procedure.

ETHICAL CONSIDERATIONS

Research work was carried out in the CSIR Food Analysis Laboratory. This is a government- and UNISA-approved institution that may execute all necessary procedures required by the study, under supervision of Ms Annali Jacobs (CSIR Senior Researcher) and by Dr Khayaletu Ntushelo at the UNISA molecular laboratory. Ethics approval was obtained from the UNISA Ethics Committee under the number 2013/CAES/060. The laboratory code of conduct and safety regulations was strictly adhered to, so as to prevent any harm or injuries to personnel.

Plagiarism was avoided and all citations were done in the form of interpretation and reconstruction of published works. Any references to uses of moringa by communities acknowledge and respect the present indigenous knowledge and dietary behaviour.

All research work in the CSIR would be kept strictly confidential until published as a completed MSc dissertation.

Finally, the study plants (*Vigna unguiculata* and *Moringa oleifera*) needed for the research was supplied by CSIR from cultivated crops. This ensured sustainable use of the plant without damaging effects on the environment or biodiversity.

QUALITY ASSURANCE:

All methods used in this research project are CSIR approved methods which have been tested and proven to be the most reliable analytical methods. These methods are also accredited by SABS: South African Bureau of Standards, ISO: International Standards Organisation and SANAS.

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http://www.CaribbeanPot.com

APPENDICES

APPENDIX 1: COMPLETE LIST OF PRODUCTS AND USES ASSOCIATED WITH THE MORINGA TREE

i. Alley cropping (biomass production),

ii. Animal forage (leaves and treated seed-cake)

iii. Biogas (from leaves)

iv. Domestic cleaning agent (crushed leaves)

v. Blue dye (wood)

vi. Fencing (living trees)

vii. Fertilizer (seed-cake),

viii. Foliar nutrient (juice expressed from the leaves)

ix. Green manure (from leaves)

x. Gum (from tree trunks)

xi. Honey- and sugar cane juice-clarifier (powdered seeds)

xii. Honey (flower nectar)

xiii. Medicine (all plant parts),

xiv. Ornamental plants

xv. Biopesticide (integration of leaves into the soil to avoid seedling damping off)

xvi. Pulp (wood)

xvii. Rope (bark)

xviii. Tannin for tanning hides (bark and gum),

xix. Water purification (powdered seeds).

xx. Moringa seed oil used in salads,

xxi. Machine lubrication (Seed oil)

xxii. Manufacture of perfume and hair care products (Seed oil).

xxiii. The seeds are also eaten raw, roasted

Adapted from Fahey, (2005) and Agyepong, (2009)

APPENDIX 2: REPORTED THERAPEUTIC USES OF *MORINGA OLEIFERA*

Adapted from*:* Fuglie (1999, 2000), Fahey (2005) and Awanish *et al* (2012).

Ailment	**Moringa plant part used for treatment**
Dental Caries/Toothache	RBG
Syphilis	G
Typhoid	G
Common cold	F
Helminths	LFP
Other / Not Attributed to a Specific Pathogen	
Bronchitis	L
Fever	LRGS
Throat Infection	F
Cancer Therapy / Protection	
Prostate	L
Anti-tumour	LFSB
Circulatory/Endocrine Disorders	
Anti-anaemic	L
Cardiotonic	R
Digestive Disorders	
Colitis	LB
Diarrhoea	LR
Dysentery	LG
Inflammation	

Joint Pain	P
Arthritis	S
Edema	R
Nervous Disorders	
Epilepsy	RB
Headache	LRBG
Reproductive Health	
Lactation Enhancer	L
Aphrodisiac	RB
Prostate function	O
Skin Problems	
Antiseptic	L
Astringent	R
General Disorders/Conditions	
Catarrh	LF
Scurvy	LSRBO
HIV/AIDS	L
KEY:	
Bark	B
Roots	R
Gum	G
Oil (from seeds)	O
Leaves	L
Flower	F

APPENDIX 3: SENSORY EVALUATION OF THE ACCEPTABILITY OF MORINGA LEAVES WITH RESPECT TO VARIOUS CRITERIA.

Kindly, evaluate the acceptability of the leafy vegetable products with respect to the given criteria by marking the appropriate square with a X

Criteria	Sample codes	Extremely acceptable	Acceptable	Neutral	Not acceptable	Extremely Unacceptable
General appearance	1					
	2					
	3					
	4					
	5					
Colour	1					
	2					
	3					
	4					
	5					
Texture	1					
	2					
	3					
	4					
	5					
Odour	1					
	2					
	3					
	4					
	5					