THE EVALUATION OF PLANT EXTRACTS AS NATURAL PRESERVATIVES ON THE CHEMICAL, MICROBIAL AND SENSORY QUALITY OF BOEREWORS

By

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DECLARATION

I declare that this thesis hereby submitted for the degree of Master of Science in Agriculture in Food Science at the University of the Free State, is my own independent work, and has not been submitted for degree purposes to any other university. I hereby forfeit any copyright of this thesis to the University of the Free State.

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LIST OF ABBREVIATIONS

*a**	CIE Redness/greenness colour coordinate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
atm	Atmospheres
a_w	Water activity
*b**	CIE Yellowness colour coordinate
B.C.	Before Christ
BPA	Baird-Parker agar
BPW	Buffered peptone water
°C	Degrees Celsius
cfu	Colony forming units
cfu/g	Colony forming units per gram
conc.	Concentration
Cu^{2+}	Copper ion
dH_2O	Distilled water
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
e.g.	Exempli gratia; for example
EPS	Expanded polystyrene
et al.	*Et alia*
etc.	*Et cetera*
Fe^{2+}	Ferrous cation
Fe^{3+}	Ferric ion
FRAP	Ferric Reducing Antioxidant Power
g	Gram
GR050	0.5% w/v green Rooibos extract
GR1	1% w/v green Rooibos extract
GR2	2% w/v green Rooibos extract
GR2 + S100	2% w/v green Rooibos extract with 100 milligram sulphur dioxide per kilogram

GRAS	Generally Regarded as Safe
h	Hours
H	Hydrogen
H025	0.25% w/v honeybush extract
H050	0.50% w/v honeybush extract
H1	1% w/v honeybush extract
H_2O	Water
H050 + S100	0.50% w/v honeybush extract with 100 milligram sulphur dioxide per kilogram
HPLC	High-performance liquid chromatography
i.e.	*Id est*; that is
kg	Kilogram
L^*	CIE Lightness colour coordinate
L. monocytogenes	*Listeria monocytogenes*
LAB	Lactic acid bacteria
log	Log_{10}
M	Molar
MAP	Modified atmosphere packaging
MDA	Malondialdehyde
mEq	Milliequivalents
Mg	Magnesium
mg	Milligram
mg/kg	Milligram per kilogram
min	Minute
ml	Millilitre
mm	Millimetre
MSG	Monosodium glutamate
N	Normality
n	Population size
Na	Sodium

NC	Negative control
nm	Nano meters
No.	Number
NS	Not significant
O_2	Oxygen
ORAC	Oxygen Radical Absorbance Capacity
p	Significance level
PCA	Principle Component Analysis
pH	Potential of hydrogen
ppm	Parts per million
%	Percentage
R	Rooibos
R025	0.25% w/v Rooibos extract
R050	0.5% w/v Rooibos extract
R1	1% w/v Rooibos extract
RBCA	Rose-Bengal Chloramphenicol agar
rH	Relative humidity
rpm	Revolutions per minute
R025 + S100	0.25% w/v Rooibos extract with 100 milligram sulphur dioxide per kilogram
RTE	Ready-to-eat
s	Seconds
S	Sulphur
S. aureus	*Staphylococcus aureus*
S100	100 milligram sulphur dioxide per kilogram
S450	Positive control; 450 milligram sulphur dioxide per kilogram
SA	South Africa
SA DAFF	South African Department of Agriculture, Forestry and Fisheries
SA DoH	South African Department of Health
SANS	South African National Standard
SMSF	Standard meat and spice formulation
SO_2	Sulphur dioxide

SPCA	Standard plate count agar
spp.	Species
ssp.	Subspecies
TBARS	Thiobarbituric acid reactive substances
TBC	Total bacterial counts
TEAC	Trolox equivalent antioxidant capacity
µl	Microlitre
UK	United Kingdom
USA	United States of America
VRBM	Violet red bile agar + 4-methylumbelliferyl-β-D-glucuronide
vs.	Versus
WHC	Water holding capacity
WHO	World Health Organization
w/v	Weight per volume
w/w	Weight per weight
Zn^{2+}	Zinc ion

ABSTRACT

Nowadays, consumers prefer the use of natural preservatives over chemical preservatives. Boerewors, a typical South African fresh sausage, is usually preserved with sulphur dioxide (SO_2), which is associated with negative health effects in humans. When partly replacing a preservative with another preservative, it is essential to maintain the same product quality and safety. The aim of this study was, therefore, to investigate the effect of plant extracts as natural preservatives on the chemical, microbial and sensory quality of Boerewors.

Extracts of green Rooibos, honeybush and Rooibos consist of many beneficial uses, as they all provide antioxidant and antimicrobial activity in food products. The concentrations of use of each preservative should first be determined before it can be included in a food model. In the first part of the study, three concentrations of each of the three plant extracts were evaluated in vitro, using the disc diffusion assay, for microbial activity against five strains each of *Escherichia coli* and *Staphylococcus aureus*. The plant extracts with the most promising inhibition zones, were 0.25% Rooibos (R025), 0.50% honeybush (H050) and 2% green Rooibos (GR2). The GR2 showed the best inhibitory effect against both bacteria.

In the second part of this study, R025, H050, GR2 and combinations of each with low SO_2 (S100 = 100 mg/kg SO_2) were evaluated as preservatives in eight Boerewors models over a period of 6 days at 4 ºC. The treatments included a negative control (NC), which had 0% preservatives and a positive control (S450 = 450 mg/kg SO_2). Physico-chemical analysis was conducted in terms of pH, water activity and lipid stability at 4 ºC on days 1, 3 and 6. Lipid stability, measured by thiobarbituric acid reactive substances (TBARS), were also analysed after 90 days of storage at -18 ºC. Microbial analysis in terms of total bacterial count (TBC), Gram-positive bacteria (lactic acid bacteria and *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*), *Enterobacteriaceae,* coliforms, yeasts and moulds were performed. The sensory quality of these treatments in terms of colour stability, the evaluation of sensory attributes (colour, taste, texture, and overall acceptability) by a sensory panel, as well as thaw-, cooking- and total losses, were analysed.

No significant differences in the pH, water activity and TBARS values of the eight treatments were observed. The H050 + S100 showed the best inhibition against *Staphylococcus aureus* and the total bacterial count in the Boerewors. Lactic acid bacteria and *Staphylococcus aureus* were inhibited more effectively by S450 and the plant extract combinations with S100 near the end of shelf-life (day 6). The GR2 + S100 treatment had the best inhibition against the mould counts over the 6-day storage period at 4 ºC. In terms of CIE colour stability, S450 was the best in preserving the colour of Boerewors. The H050 and R025 treatments had the best colour among the treatments when evaluated by the sensory panel. The GR2 and GR2 + S100

treatments had significantly higher losses during thawing and cooking. In this research, partial replacement of SO_2 with plant extracts such as honeybush and Rooibos have been shown to be possible.

Keywords: Boerewors, Rooibos, honeybush, Green Rooibos, preservative, antimicrobial, antioxidant

Chapter 1

Introduction

1.1. Background

Boerewors is a fresh sausage very popular among consumers and traditional to South Africa (Charimba et al., 2010). It consists of a total meat content that is not below 90% and a fat content of no more than 30% (SA DoH, 2001). This sausage is classified as a ground meat product, it is usually made from beef, pork, mutton, or a mixture of these meat, with fat, contained in a casing that is edible. Flavourants (spices, vinegar, herbs and salt), water, permitted preservatives, starch or cereal products, are the only other ingredients allowed in this fresh sausage (SA DoH, 1990; SA DoH, 2001).

Meat is highly susceptible to spoilage, due to its high nutrient content and water activity (a_w), which serves as a perfect habitat for micro-organisms to proliferate. To protect food against deterioration by micro-organisms, additives such as preservatives are used (Rodford, 1997) Preservatives lengthen the shelf-life of food by inhibiting the growth of some micro-organisms, which is referred to as the preservative's antimicrobial action. Microbial activities can cause undesirable changes in the quality of food (spoilage), but it can also result in human health endangerment if these micro-organisms are pathogens. The use of chemical preservatives is of great significance as it maintains and improves the quality of meat and meat products (Efenberger-Szmechtyk et al., 2020).

Sulphites are some of the meat preservatives that can retain the fresh colour of meat, enhance the flavour and reduce the oxidation of fat in meat (D'Amore et al., 2020; Efenberger-Szmechtyk et al., 2020; Garcia-Fuentes et al., 2015). Boerewors may contain 450 mg/kg sulphur dioxide (SO_2) according to South African regulations (SA DoH, 2001). However, some individuals show allergic reactions towards SO_2. Consumers have also become aware of the adverse effects of chemical preservatives and long for natural preservatives (Yu et al., 2021). Research on natural substances/preservatives derived from plants, animals, and from micro-organisms has, therefore, increased (Hugo & Hugo, 2015; Mathenjwa et al., 2012; van Schalkwyk et al., 2013).

Extracts of green Rooibos, honeybush and Rooibos are typical natural South African products which contain phytochemicals that are beneficial to one's health (McKay & Blumberg, 2007) Rooibos and green Rooibos are known to have anti-inflammatory, and anti-diabetic properties (Ajuwon et al., 2018; Orlando et al., 2019; Windvogel et al., 2020). Honeybush extract is known for its healing properties as it alleviates flu and colds. It is also known for its anti-diabetic and anti-mutagenic properties (Dube et al., 2017).

The antimicrobial activity of Rooibos extracts lies in the flavonoid content and depend on the concentration and structure of the polyphenols present. Rooibos extracts have been shown to inhibit *Staphylococcus aureus*, *Listeria monocytogenes* as well as *Escherichia coli* in a liquid culture (Simpson et al., 2013). According to Makarewicz et al. (2021), flavonoids such as catechin, quercetin and kaempherol, have shown to inhibit *Staphylococcus aureus*. Hesperidin and mangiferin, found in honeybush extracts, were reported to possess antimicrobial activity. The antimicrobial effect of polyphenols may depend on their concentration, as they may be capable of altering the cell membrane of micro-organisms at high concentrations and/or at low concentrations. They may inactivate enzymes involved in energy production of bacterial cells (Kamara et al., 2004).

The antioxidant properties of polyphenols in Rooibos, green Rooibos and honeybush are based on the inhibition of enzymes, such as peroxidase and oxidase, which are responsible for free radical formation, activation of superoxide dismutase and catalase (antioxidant enzymes), binding of metals such as Cu and Fe, scavenging of reactive nitrogen/oxygen species and scavenging of free radicals (Efenberger-Szmechtyk et al., 2020).

Limited research is available on the use of Rooibos, green Rooibos and honeybush extracts in fresh meat products. A few of these studies included the use of different Rooibos concentrations in ostrich and rabbit meat patties (Cullere et al., 2013; Cullere et al., 2019), the use of honeybush in combination with nitrate in salami (Smith, 2020) and the addition of Rooibos to “droëwors’’ made from ostrich, springbok and other animals (Hoffman et al., 2014; Jones et al., 2015). The use of plant extracts in Boerewors have not yet been studied before and the concentrations needed for optimal/sufficient antimicrobial activity in Boerewors are unknown.

1.2. Aim and Objectives

The aim of this study was to determine the effect of green Rooibos, honeybush and Rooibos extracts as natural preservatives to replace, or partially replace, SO_2 (chemical preservative) in Boerewors.

The objectives of this study were:

- To assess three different concentrations of each of green Rooibos, honeybush and Rooibos extracts to be used as natural preservatives. The effect of these preservatives will be evaluated in vitro by means of a disc diffusion assay to evaluate the inhibition effect of these natural preservatives against five strains each of *Escherichia coli* (*E. coli*) (Gram-negative bacterium) and *Staphylococcus aureus* (*S. aureus*) (Gram-

positive bacterium). There two strains were chosen since these bacteria are the most common pathogenic bacteria usually encountered in the food industry. The results will be used to determine which of the different concentrations of these preservatives will perform the best antimicrobial action to be used in Boerewors.

- To manufacture eight Boerewors models containing one of the following: no preservatives (negative control), 450 mg/kg SO_2 (conventional method/positive control), green Rooibos extract (best concentration determined in the previous objective), honeybush extract (best concentration determined in the previous objective), Rooibos extract (best concentration determined in the previous objective), and combinations of each extract with lower levels of SO_2 (100 mg/kg) (Bañón et al., 2007; Roller et al., 2002). The effect of these preservatives will be evaluated by:
  - Physico-chemical analysis (the effect on pH, water activity and lipid stability).
  - Microbial analysis (the effect on the counts of total bacteria, lactic acid bacteria (LAB), *Enterobacteriaceae*, coliforms, *E. coli*, *S. aureus*, *L. monocytogenes*, yeasts and moulds).
  - Sensory analysis (the effect on colour stability, sensory attributes, as well as on moisture loss during thawing and cooking).

Chapter 2

Rooibos and honeybush plant extracts as natural preservatives in Boerewors

2.1. Introduction

Consuming meat as a protein source, will maintain human health as it contributes minerals, proteins, fats and vitamins for bodily support. The composition of meat is, however, an ideal environment for micro-organisms to flourish and promote spoilage. It is important to keep the quality of meat and meat products as high as possible, since a decrease in quality may lead to adverse effects on the colour, odour, flavour and the texture of the meat. Meat is not only degraded by micro-organisms; other factors, such as protein oxidation and lipid oxidation, also contribute to the spoilage of meat (Aminzare et al., 2019; Domínguez et al., 2019; Hadidi et al., 2022).

The use of additives in food is common as it has been used for centuries. They are added to food to achieve certain properties in food products by acting as thickening or emulsifying agents, as well as preserving the products. A food additive can be defined as a substance that is added to food for technological reasons (processing, preparation, transportation) and not for the purpose of being consumed on its own as a nutrient (Surendran Nair et al., 2020; WHO Codex Alimentarius, 2019).

Methods such as preservation are used in meat to maintain the sensorial, biological and physico-chemical properties. Chemical substances are used to stabilize colour, add flavour, maintain texture and prevent microbial growth. Chemical preservatives include organic acids and their derivatives, sulphites, and nitrites (Surendran Nair et al., 2020). Sulphur dioxide (SO_2) is a well-known additive of which the main functions/capabilities are to act as oxygen scavenger, enzyme inhibitor, microbial growth inhibitor, antioxidant and colour enhancer. Sulphur dioxide is currently used in boerewors for preservation, but the use of it in meat has raised concerns among consumers (D'Amore et al., 2020).

New natural preservative techniques are needed to restrict the use of chemical preservatives in meat products. A wide variety of natural preservatives are available which originate from plants (e.g. rooibos and honeybush extracts), animals (e.g., chitosan) or bacteria (bacteriocins) (Efenberger-Szmechtyk et al., 2020; McKay & Blumberg, 2007). Rooibos and honeybush are herbal teas indigenous to South Africa. These tisanes are low in tannins and also free from caffeine. These plant extracts are rich in polyphenols, which contain antioxidant

and antimicrobial properties, which may be used to extend the shelf-life of meat and provide good quality meat for consumption (Simpson et al., 2013).

The aims of this literature review were to give a general background of the composition and manufacturing of boerewors; the microflora to be found in this product; and the intrinsic and extrinsic factors that play a role in the shelf-life of meat. The background and properties of sulphur dioxide; the effect of this chemical preservative on meat and the major concerns that has arisen when using sulphites in meat, were discussed. Rooibos- and honeybush extracts were then discussed as natural plant preservatives in terms of properties, antioxidant activity and antimicrobial activity.

2.2. Boerewors

2.2.1. General background

Sausages have been consumed as a cuisine since 900 B.C. and the Romans preferred it. During the Middle Ages, each country developed its own style of sausage based on its unique taste and climate (Hugo et al., 1992). Boerewors was produced on South African farms since the seventeenth century. Beef and pork meat, as well as speck cubes were used to make Boerewors. Salt, pepper and a variety of spices, including coriander, were used to season the meat mixture (Hugo et al., 1992). In the 1960's, the consumption of South African Boerewors had increased, and new ingredients were used and explored to improve its flavour (van Schalkwyk, 2010).

The latin word "*salsus*" means salted, from which the word "sausage" developed (Jones, 2013). Boerewors is classified as a ground meat product, as the muscle structure has undergone chopping, dicing or mincing. The muscle structure has become particulate in nature and cannot be recognized anymore in its fibrous form. The minced/ground meat is put into casings which are either natural or synthetic. The contents of the sausage are, therefore, protected in the casing against invading micro-organisms and excessive loss of moisture during storage of the sausage (Mathenjwa et al., 2012; van Schalkwyk, 2010). Fresh Boerewors is usually served as breakfast or supper dish by frying the sausage, or it is left to dry, which is known as "droëwors" (Hugo et al., 1992).

2.2.2. Manufacturing and composition of Boerewors

Boerewors is prepared with the use of different ingredients, which include meat, usually a beef and pork mixture, but caprine or ovine meat may also be used, fat for tastiness/flavour and water, to increase the moisture content and to solubilize proteins when combining the meat and fat. Other ingredients added to the product are flavourings and spices, as well as salt for flavour, preservation, protein extraction and inhibition of microbial growth. Antioxidants, such

as ascorbic acid, tocopherols and terpenes are added to the sausage to delay the process of oxidation. In the manufacturing process, the meat is minced in a meat mincer, while the ingredients mentioned above are added. During the mincing of the meat, the temperature needs to be controlled or kept low at -2 ºC for giving a clean meat cut and prevent smearing. Then, the sausage mixture is put into a casing (natural or synthetic) (Falowo et al., 2014; Mathenjwa et al., 2012).

Boerewors should be manufactured according to the South African Department of Health (SA DoH, 1990) Regulations R2718 of 23 November 1990. Boerewors should contain no more than 30% fat content and the total meat content should not be below 90%. Also, no mechanically recovered meat or offal is allowed to be used in this product, unless the offal is used as casing of the boerewors. The only ingredients allowed to be added in the production of boerewors, are the following: water; herbs and spices; vinegar; starch or cereal products; flavourings that are harmless and permitted food additives (SA DoH, 1990).

2.2.3. Types of spoilage and pathogenic micro-organisms

Yeasts, moulds and bacteria are the main micro-organisms found in meat. Various factors may be responsible for the bacterial spoilage of meat and meat products. Post slaughter, the carcass can be contaminated by micro-organisms originating from the faeces, animal skin and intestines. Other factors include water, soil, handling and cross-contamination during the animal slaughter processes (van Schalkwyk et al., 2013). Microbial organisms responsible for spoilage of fresh sausage are identified as mesophiles (grow at mid-range temperature), aerobes and facultative anaerobes (Charimba et al. 2010).

According to Hugo & Hugo (2015), colony counts of aerobic organisms found on fresh sausages range from $1.5 \times 10^3 – 2.1 \times 10^8$ cfu/g. Aerobic colony counts for frozen sausages, range from 1.4×10^3 to 3.1×10^7 cfu/g. Pathogenic micro-organisms may also be found in beef during the processing stages and include *Listeria*, *Escherichia coli (E. coli)*, *Campylobacter jejuni*, *Staphylococcus aureus* (*S. aureus*) and *Salmonella* (Carballo, 2021). According to the SA Department of Health (2005), the allowable limits for pathogens in items requiring further cooking (with “wors” listed as one of the items) are as follows: *E. coli* <10 cfu/g; *Salmonella* spp. 0 cfu/25 g; *Clostridium perfringens* <100 cfu/g; *Campylobacter* spp. <1000 cfu/g; *Bacillus* spp. 0 cfu/25 g and *Listeria monocytogenes* <100 cfu/g.

Gram-negative bacteria, such as the *Pseudomonas* spp. are one of the main causes of meat spoilage. *Pseudomonas* spp. are also psychrotolerant, hence capable of causing deterioration of meat stored in refrigerators at 8 °C and lower (Mathenjwa, 2010). *Pichia*, *Torulopsis*, *Cryptococcus* and *Candida* are yeasts that have been isolated from ingredients used to produce fresh sausages (van Schalkwyk et al., 2013). Meat cuts can contain moulds such as

Penicillium, *Cladosporium*, *Mucor*, *Sporotrichum, Rhizopus* and *Thamnidium* (Carballo, 2021). A fresh sausage with a yeast and mould count of less than or equal to 10^2 cfu/g and a coliform count of less than or equal to 10^4 cfu/g, are regarded as having a good microbial quality (Shapton & Shapton, 1991).

2.2.4. Factors affecting shelf-life

There are a variety of factors (extrinsic and intrinsic) that may play a role in the shelf-life of food, but this study will only focus on temperature, pH, water activity and the lipid and protein degradation, since these are the main factors affecting the shelf-life of fresh sausages like Boerewors.

2.2.4.1. Temperature

There are many factors that may determine whether micro-organisms will flourish or not. An important factor is temperature, since micro-organisms can be divided into four groups: thermophiles, hyper-thermophiles, mesophiles and psychrophiles/psychrotolerant according to their ideal temperature for growth. Pathogens may be found in all four groups: *Listeria monocytogenes* which are psychrotolerant; *Clostridium botulinum*, which are thermophilic (grow at high temperatures) and *Staphylococcus aureus*, which are mesophilic (grow at average/mid-range temperatures). Other bacteria such as *Pseudomonas* also cause spoilage as it survives and grows at low temperatures. Since Boerewors is usually stored at a temperature of 4 °C and is subsequently cooked, all these pathogens and spoilage bacteria, such as *Pseudomonas*, may be present in the Boerewors mixture (Mathenjwa et al., 2012; Murali, 2011).

2.2.4.2. pH

The pH of meat is in the range of 5.5 to 6.5, according to the study of Efenberger-Szmechtyk et al. (2020). Fresh sausages also fall in this range with a pH of 5.5 and above. Spoilage bacteria, such as *Brochothrix thermosphacta* and *Pseudomonas*, grow best at a pH of 5.5 to 7 (Van Schalkwyk, 2010). *Salmonella* is one of the pathogens that is responsible for food poisoning in a wide variety of food products with an optimum pH that is neutral (pH 7). However, growth is also observed at around pH 6.6 to 8.2 (Gouws et al., 2014).

2.2.4.3. Water activity (a_w)

Boerewors has a water activity of 0.97 or higher, whereas that of fresh meat is 0.99 (van Schalkwyk et al., 2013). Water activity is one of the factors that can make Boerewors highly susceptible to microbial spoilage. A water activity of 0.96 to 0.97 is conducive for the growth of pathogens and spoilage micro-organisms such as *E. coli*. and *Pseudomonas* spp.

Staphylococcus aureus is a pathogen that can tolerate a water activity of 0.86 (Fontana, 2000). Other pathogens, such as *Salmonella,* is not able to grow at a water activity beneath 0.94 and cannot survive in high salt content conditions (Gouws et al., 2014). The minimum water activity level for growth of micro-organisms, is indicated in Table 2.1.

Table 2.1

Micro-organisms and the minimum level of water activity for growth at 25 °C (Kim, 2006; Mathenjwa et al., 2012).

Type of micro-organism	Minimum water activity level
Most yeasts	0.88
Osmophilic yeasts	0.62-0.60
Most bacteria	0.91-0.88
Halophilic bacteria	0.75
Moulds	0.80

2.2.4.4. Lipid and protein oxidation of meat

Oxidation can be activated by reactive oxygen species or metallic ions. Hydroperoxides, which form during the lipid oxidation process, can be oxidised further to products such as malonaldehyde and ketones, which can lead to health problems in humans and affect the quality of meat (Hadidi et al., 2022; Pateiro et al., 2021). Free radicals, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), bring about lipid oxidation of meat, due to chain reactions. There are three stages in which these chain reactions of lipid oxidation take place, namely initiation, propagation and completion. The production of free radicals is promoted in the first two stages by factors such as exposure to gamma irradiation and oxygen, the presence of metal ions and the disturbance of the integrity of the cells. The radicals are transformed into hydroperoxides and conjugated dienes. Secondary products, like alcohols, ketones, carbonyl compounds and aldehydes, are formed during the last oxidation stage, when the primary products get decomposed. Aldehydes are some of the secondary products which react rapidly with proteins, causing changes in the nutritional and organoleptic properties of meat which are unpleasant and unwanted (Manessis et al., 2020).

Another factor responsible for the decreasing quality of meat properties, is protein oxidation (Jones, 2013; Manessis et al., 2020). Protein oxidation leads to three major changes in meat: the development of protein crosslinks, the development of carbonyl derivatives that affect flavour and the loss of sulphydryl groups (Jones, 2013). This process occurs when oxidized lipids, a metallic stimulus or myoglobin reacts with the side chains of amino acids, which form protein radicals (Manessis et al., 2020).

The basic methods used to assess lipid oxidation, are known as thiobarbituric acid reactive substances (TBARS) analysis, chromatography and peroxide value analysis (Manessis et al., 2020). The TBARS value is expressed as mg malonaldehyde (MDA)/kg of meat. A TBARS value of 2 mg MDA/kg of meat and less are considered acceptable, as values higher than 2 mg MDA/kg are regarded as rancid meat. However, in a study by Boles & Parrish (1990), rancid off-flavours in pork become noticeable by a taste panel when the TBARS values were greater than 1 mg MDA/kg. Therefore, 1 mg MDA/kg meat and less are regarded as good lipid stability (van Schalkwyk et al., 2013).

Peroxide value is another parameter used to identify the degree of oxidation, which is determined by iodometric titration (Domínguez et al., 2019). In the article of Chizzolini et al. (1998), the oxidation of dry-fermented sausages and dry-salted hams were evaluated. Peroxide values of 2–4 milliequivalents (meq) oxygen per kg fat were considered as an acceptable level.

Peroxide value or gas chromatography can be used to evaluate the oxidation process in meat products, but the TBARS analysis is the most common method used (Domínguez et al., 2019).

2.3. Sulphur dioxide

Sulphur dioxide (SO_2) is the main preservative in the production of Boerewors. In the next sections, some background will be given on SO_2 and the properties of SO_2 will be discussed. Sulphur dioxide's antimicrobial and antioxidant properties will be discussed in more detail and then the disadvantages of using SO_2 in food products will also be highlighted.

2.3.1. Background

Sulphites are some of the most widely used additives in the food industry. The use of sulphur dioxide started back in 1664, to prevent spoilage of cider in casks. Sulphur dioxide, bisulphite, metabisulphite, potassium sulphite and sodium sulphite are all sulphiting agents with a variety of uses in the food industry (Vally et al., 2009). Sulphites are used in food products such as sugar, beverages, meat, vinegar, seafood, dried- and processed fruits, as well as processed vegetables (Garcia-Fuentes et al., 2015).

In South Africa, Boerewors is preserved with SO_2. It is added to the product in a sulphite salt form, mostly as sodium metabisulphite. The sulphite salts all share the ability to generate molecular SO_2, which correlate to its preservative properties. The SO_2 inclusion levels in foods are quoted as parts per million (ppm) or mg/kg SO_2 (Gould & Russell, 2003). The SA DoH (2001) stipulates that Boerewors may contain SO_2 in a concentration that does not exceed 450 mg/kg or 450 ppm.

2.3.2. Properties

Sulphites are used for their basic functions: to serve as antioxidants and antimicrobials in food products. They inhibit the growth of many micro-organisms, which reduces the spoilage of food. Sulphites can act as bleaching agents, anti-browning agents, colour enhancers and inhibit the activity of enzymes. Sulphites also absorb oxygen and serve as antioxidants (D'Amore et al., 2020). Sulphites are used as active ingredient preservatives in the cosmetics and pharmaceutical industries (D'Amore et al., 2020; Garcia-Fuentes et al., 2015).

2.3.3. Antimicrobial activity

The quantity of sulphites used in food determines the degree of its effectiveness. It may lead to the death of the microbial cell or may be bacteriostatic (D'Amore et al., 2020). When sulphites move through the microbial cell membrane, thiol and S-sulphonates are formed by the reaction with disulphide bonds, resulting in many protein conformational changes. Therefore, the proteins and enzymes found on the cell surface - responsible for the microbial life cycle - are inactivated (D'Amore et al., 2020; Hugo & Hugo, 2015).

Spoilage organisms, such as lactic acid bacteria, yeasts and moulds, are also reported to be sensitive to the antimicrobial activity of sulphite. This includes yeast species, such as *Cryptococcus* spp., *Saccharomyces* spp., *Candida* spp. and moulds, such as *Aspergillus*, *Cladosporium*, *Penicillium*, *Geotrichum* and *Sporotrichum* (D'Amore et al., 2020; Surendran Nair et al., 2020).

Sulphites are effective against many Gram-positive bacteria and most Gram-negative bacteria. A minimum concentration of 1.5 mg/L of free sulphur dioxide is capable of inhibiting lactic acid bacteria (*Oenococcus*, *Pediococcus, Leuconostoc* and *Lactobacillus viridescens*), acetic acid producing bacteria (*Acetobacter* spp.) and bacteria belonging to the families of *Campylobacteraceae*, *Vibrionaceae, Pseudomonadaceae* and *Listeriaceae.* Free sulphite at the concentration range of 10–240 µg/ml inhibits *Hafnia alvei*, *Serratia marcescens*, *Enterobacter agglomerans*, *Yersinia enterocolitica*, *Citrobacter freundii, Salmonella* and *Escherichia coli* (D'Amore et al., 2020).

2.3.4. Antioxidant activity

Sulphites can inhibit or minimise several steps of both enzymatic and non-enzymatic browning processes (Gould & Russell, 2003). Enzymatic browning is associated with the enzymatic degradation of proteins and lipids in meat. Sulphites prevent the proteolytic breakdown of meat by linking proteases like tyrosinase. Therefore, the reactions of lipid peroxidation are stopped, which inactivates key enzymes like lipase and peroxidase. Radicals and oxygen are

scavenged by sulphites, which also prevents meat from discolouration and assists in retention of meat flavour (D'Amore et al., 2020).

2.3.5. Toxicity and negative effects

Health problems may arise, as the ingestion of sulphites are toxic to some individuals that show sensitivity towards these preservatives. Before the uprising of many cases regarding the negative effect, sulphites were considered to have GRAS status. The FDA cancelled the GRAS status for sulphite usage in food products in 1986 after continuous research has shown increasing cases of harmful reactions when ingesting sulphites (D'Amore et al., 2020).

The sensitivity towards sulphites varies and mainly depends on consumers, the source, and amount of exposure. The symptoms can range from mild to more serious symptoms, like anaphylaxis. Symptoms may include vomiting, diarrhoea, nausea, abdominal pain, headache, urticaria, and dermatitis. Individuals that suffer from asthma commonly show more sensitivity towards sulphur dioxide in food than others (Vally et al., 2009). According to D'Amore et al. (2020) and Garcia-Fuentes et al. (2015), the addition of sulphites in meat and other food products may cause the degradation and loss of vitamins such as vitamin B_1 (also known as thiamine) and vitamin B_{12}. Due to the risk of harmful outcomes, the use of sulphur dioxide and other sulphites as additives in food are restricted by the WHO Codex Alimentarius (2019).

2.4. Plant extracts as natural preservatives

Chemical preservatives can be replaced with natural preservatives. These can either be from microbial, plant or animal origin. The compounds that are associated with preservative action in these three sources, are illustrated in Table 2.2.

Table 2.2

Natural preservatives divided into three main groups of origin which display antimicrobial properties (Efenberger-Szmechtyk et al., 2020).

Bacterial origin	Plant origin	Animal origin
Lacticin	Polyphenols	Chitosan
Nisin	Essential oils	Lactoferrin
Pediocin	Iridoids	Peptides
Reuterin	Saponins	Lysozyme

The benefit of natural preservatives, above chemical or synthetic ones, is that they are more quickly accepted by consumers since they are considered safe. They get regulatory clearance more easily, because no safety testing is required if the substance has GRAS (Generally Recognised as Safe) status already (Jones, 2013).

The following sections of this study will only focus on preservatives originating from plants, since plant extracts will be the focus of this study.

Sausages like raw Boerewors, are known to have a short shelf-life as they can be easily spoiled by micro-organisms. The use of plant extracts as natural preservatives can prolong the shelf-life of fresh sausages and improve the sensory quality and nutritional value of these products (Ajourloo et al., 2021; Jones, 2013; Pfukwa et al., 2019). The main natural sources of bioactive compounds for extension of the shelf-life of meat products are essential oils and plant polyphenols (Aminzare et al., 2019). The redox characteristics of phenolic compounds allow them to operate as hydrogen donors, singlet oxygen quenchers and reducing agents (Jones, 2013).

Essential oils are abundant in extracts from vegetables, fruits, herbs and spices. Plants' antioxidant and antimicrobial activities are attributed to the essential oils found in their leaves (basil, thyme, rosemary), seeds (fennel, parsley), fruits (pepper), rhizomes (asafoetida) and/or bulbs (garlic). Essential oils in food products have the potential to kill microbial cells or inhibit the development of secondary metabolites, like mycotoxins (Hugo & Hugo, 2015). Owing to the existence of a single layer cell wall, Gram-positive bacteria are more susceptible to different groups of phytochemicals, while Gram-negative bacteria have a double membrane, making them more resistant to plant extracts (Mulat et al., 2020). Although this study will focus only on rooibos (fermented and unfermented/green) and honeybush as natural preservatives, various plant extracts have been investigated in past years. Plant extracts that have been used in studies as an alternative preserving agent are given in Table 2.3.

2.4.1. Rooibos

2.4.1.1. Background

Rooibos (*Aspalathus linearis*) is a plant (Fig. 2.1) found in areas of the Cederberg Mountains in the Western Cape. There are more than 200 species of the genus *Aspalathus*, which are only found in South Africa. The needle-like leaves of this shrub are harvested, fermented and dried to produce Rooibos tea (South African red tea) (Gouws et al., 2014; McKay & Blumberg, 2007; Simpson et al., 2013). Khoi people have been consuming rooibos as a beverage since 1772 (Cullere et al., 2013). Rooibos from South Africa has been registered as a Protected Designations of Origin (PDO) product (European Commission, 2021).

Table 2.3

Plant extracts used as natural preservatives in food.

Preservative	Property	Bioactive compounds	Effective/activity against	Reference
Rooibos tea	Antioxidant and antimicrobial	Flavonoids (orientin, iso-orientin, vitexin, isovitexin, quercetin, isoquercetin, luteolin, rutin, nothofagin, aspalathin), ferulic acid, *p*-coumaric acid, vanillic acid, caffeic acid, protocatechuic acid, *p*-hydroxybenzoic acid	*Bacillus cereus* *Escherichia coli* *Streptococcus mutans* *Listeria monocytogenes* *Saccharomyces cerevisiae* *Staphylococcus aureus* *Pseudomonas putida* *Pseudomonas aeruginosa* *Salmonella enteritidis* *Campylobacter jejuni* *Candida albicans*	Dube (2015) Krafczyk & Glomb (2008) Simpson et al. (2013)
Honeybush tea	Antioxidant, antimicrobial and antifungal	Flavonoids (hesperetin, hesperidin, eriocitrin, narirutin epigallocatechin gallate), xanthones (mangiferin, isomangiferin), flavones (scolymoside, orobol and luteolin)	*Escherichia coli* *Botrytis cineria* *Candida albicans* *Staphylococcus aureus*	McKay & Blumberg (2007) de Beer et al. (2012) Dube (2015) Dube et al. (2017) Smith (2020) Smith et al. (2020)
Rosemary extract	Antioxidant and antimicrobial	Rosmaridiphenolic, rosmanariquinone, rosmanol, carnosol, carnosic acid, coumaric acid, chlorogenic acid vanillic acid, ferulic acid, caffeic acid, phenolic diterpenes, flavonones and flavones (apigenin-7-*O*-glucoside, rutin, hesperetin, luteolin, naringin,	*Listeria monocytogenes* *Salmonella enteritidis* *Salmonella* Typhi	Al-Hijazeen & Al-Rawashdeh (2019) Mathenjwa (2010) Mena et al. (2016) Schilling et al. (2018) Senanayake (2018)

		apigenin, quercetin, hispidulin and genkwanin)		
Bidens pilosa extract	Antioxidant and antimicrobial	Flavonoids (rutin)	*Escherichia coli* *Shigella flexinerii*	Falowo et al. (2016) Falowo et al. (2019)
Moringa oleifera extract	Antioxidant and antimicrobial	Flavonoids, saponins, alkaloids, catechol-type tannins and gallic acid	*Shigella flexinerii* *Escherichia coli* *Proteus mirabilis* *Klebsiella pneumoniae* *Listeria monocytogenes* *Pseudomonas aeruginosa* Methicillin-resistant *Staphylococcus aureus* *Enterococcus faecalis*	Falowo et al. (2016) Mulat et al. (2020) Garba et al. (2021)
Artemisia afra extract	Antioxidant and antimicrobial	Flavonoids (rutin), phenolic compounds, alkaloids, terpenoids, saponins and glycosides	*Bacillus subtillis* *Staphylococcus aureus* *Shigella* spp. *Escherichia coli*	Keshebo et al. (2016) Falowo et al. (2019)
Mint extract	Antioxidant, weak/no antimicrobial properties	Sinapic acid, ellagic acid, hesperidin, gallic acid, p-coumaric acid, caffeic acid, chloregenic acid and trans-ferulic acid	*Escherichia coli* *Bacillus cereus* *Staphylococcus aureus*	Kanatt et al. (2008) Mahdavikia et al. (2017)
Grape seed extract	Antioxidant and antifungal	Dimeric, trimeric and tetrameric procyanidins; epicatechin-3-*O*-gallate, epicatechin and catechin	Coliforms, yeasts and moulds	Mathenjwa (2010) Nikmaram et al. (2018)

Camellia sinensis tea	Antioxidant and antimicrobial	Catechins (epigallocatechin-3-gallate, epicatechin-3-gallate, epicatechin and epigallocatechin)	*Streptococcus mutans* *Micrococcus luteus* *Pseudomonas aeruginosa* *Pseudomonas fluorescens* *Staphylococcus epidermis* *Listeria monocytogenes*	Dube (2015) Rahardiyan (2018)
Green and white tea	Antibacterial agent	Catechins, epidogalloctechin, epicatechin gallate, epicatechin, teaflavin digallate	*Staphylococcus aureus* *Streptococcus* spp. *Penicillium chrysogenum* *Saccharomyces cerevisiae*	Murali (2011)
Grapefruit seed extract	Antimicrobial effect	Proanthocyanidins, anthocyanins, catechins, phenolic acids and flavonols	*Listeria monocytogenes* *Pseudomonas* spp. *Campylobacter jejuni*	Chibane et al. (2018)
Olive leaf extract	Antimicrobial effect	Flavonoids and phenolics	*Candida albicans* *Escherichia coli* *Salmonella enteritidis* *Listeria monocytogenes*	Chibane et al. (2018)
Vernonia cinerea leaves extract	Strong antioxidant	Phenolic compounds, flavonoids, sesquiterpenes terpenoids, stigmasterols and amyrin	Not applicable	Alara et al. (2018)

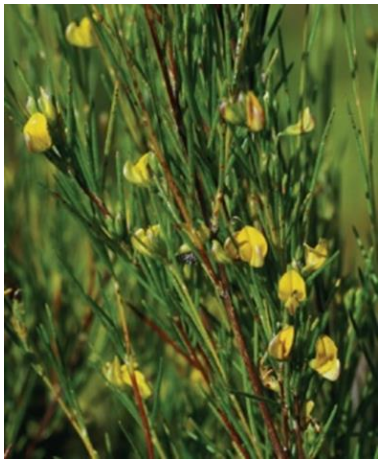

Fig. 2.1. Rooibos plant with flowers (Adapted from Rooibos+bigtreehealth.com.jpg (250×304) (bp.blogspot.com).

Rooibos is harvested and gathered for further processing, usually during the warm months (January to April). The plant moves through machines where they are cut into lengths of three to four millimetres. Water is added to the tea to increase the moisture content and bruising of the tea follows. Oxidative enzymes and polyphenols are released during the bruising step. These enzymes are necessary to accelerate the process of fermentation. The tea is left to ferment in heaps for 12–14 h at ambient temperature. Temperatures of 38 ºC to 42 ºC are reached and the fermented heap is turned over continuously to ensure that enough aeration, required for oxidation, occurs. This is where the tea obtains its characteristic taste and colour (red/brown). The fermented tea leaves are then placed on tea courts for six hours of sun-drying. Before the tea packaging step, the tea is pasteurised by steaming (92 ºC) the leaves to reduce any contamination by micro-organisms and to remove moisture that is in excess (Gouws et al., 2014).

2.4.1.2. Properties

Rooibos tea can either be unfermented or fermented when produced and contains polyphenols with antioxidant properties. During fermentation, a red colour is achieved due to the oxidation of polyphenols, while unfermented Rooibos, often called green Rooibos, remains green in colour. The fermented Rooibos with its red/brown colour, taste sweet and is consumed in more than 37 countries. Green Rooibos is known to have a mild taste with a less sharp flavour than Rooibos (Koch et al., 2012; McKay & Blumberg, 2007; Simpson et al., 2013). Rooibos contains no caffeine and has a very low content of tannin in the leaves (3.2 to 4.4%) (Ajuwon et al., 2015; Coetzee et al., 2016).

According to Hübsch et al. (2014), Rooibos tea has benefits that go far beyond the means of only providing a pleasant cup of tea. Individuals are relieved from asthma, allergies, heartburn, nausea, mild depression, headache, infantile colic and dermatological- and skin problems.

Rooibos contains flavonoids which provide health-promoting effects in humans. It is also responsible for the prevention/inhibition of oxidative stress in other biological activities (Ajuwon et al., 2015). Fermented Rooibos extracts/infusions are reported to have anti-obesity as well as anti-diabetic activities. The consumption of the tea leads to lowered levels of free fatty acids, triglycerides and cholesterol, which has the potential in reducing the chance of becoming overweight (Marnewick et al., 2011). A study done by Orlando et al. (2019), showed that green Rooibos extracts displayed anti-diabetic activity, as it lowered the LDL-cholesterol levels in high-fat diet induced vervet monkeys. This anti-diabetic effect of Rooibos is attributed to the ability of aspalathin to increase GLUT 4 translocation to the plasma membrane via adenosine 50 monophosphate-activated protein kinase and activation of Akt in skeletal muscle, as well as to reduce the gene expression of hepatic enzymes involved in lipogenesis and glucose production. According to Joubert & De Beer (2012), a human study was done, where adults at risk for cardiovascular disease who drank six cups of fermented Rooibos daily for six weeks saw improvements in their lipid profiles and redox status. Rooibos (unfermented) extracts displays anti-cancer activity. The growth of tumours and multiplying of cancer cells in the skin of rats was inhibited (Abdul & Marnewick, 2023; Marnewick et al., 2005).

2.4.1.3. Antioxidant and antimicrobial activity

The main flavonoids present in the leaves of Rooibos are rutin, quercetin, isoquercetin, iso-vitexin, vitexin, iso-orientin, orientin, nothofagin and aspalathin (Stander et al., 2017). Nothofagin, rutin, luteolin, iso-vitexin, isoquercetin and quercetin are flavonoids also present in Rooibos, which have displayed antioxidant activity (Cullere et al., 2013; Simpson et al., 2013). Factors on which antioxidant activity is dependent, include: the presence of other antioxidants, oxygen presence, temperature, lipid composition and the concentration of the antioxidant (Jones, 2013). The antioxidant activity of Rooibos extracts is mainly dependent on the total content of polyphenols, which is determined by the processing method of the plant (Simpson et al., 2013). According to Jones (2013), Rooibos tea extracts can be marketed as a natural antioxidant in “droëwors”, but when determining the level of Rooibos tea extract to be used, the composition of the extract, particularly the levels of quercetin and aspalathin, should be considered.

Rooibos extracts could also perform as a pro-oxidant, depending on the concentration of total polyphenols and/or total flavonoids present (Joubert et al*.,* 2005). The addition of quercetin and aspalathin in high concentrations possess pro-oxidant properties and, therefore could clarify the increased lipid oxidation values when higher amounts of Rooibos tea extracts are added. The use of 0.25% of Rooibos tea extract has given low values of lipid oxidation and heme-iron in “droëwors”. At 0.50% and 1% usage, oxidation increased, as the binding sites of

heme-iron have been saturated. As a result, a 0.25% Rooibos tea extract inclusion level could be advocated as the threshold for using Rooibos tea extract as an antioxidant in “droëwors” (Jones, 2013).

The total polyphenols in green, unfermented Rooibos are much higher than those of fermented Rooibos, due to the loss of some antioxidants in the fermentation process of Rooibos. Changes in the phenolic composition of the Rooibos’ shrub material can also be found when fermentation has taken place. Fermentation can cause the oxidation of aspalathin to orientin and iso-orientin (Abdul & Marnewick, 2023; Jones, 2013; Joubert & de Beer, 2012; Mulaudzi et al., 2022). According to Jones (2013) and Joubert & de Beer (2012), a hot water extract of fermented Rooibos (1.3 g) is commonly used as a food component and contains on average 0.58% aspalathin, 0.80% iso-orientin and 0.84% orientin of the plant’s main flavonoids, respectively. Nothofagin and aspalathin (dihydrochalcone glucoside) are two of the flavonoids in unfermented Rooibos that are present in the largest amounts, and they also have properties that are anti-mutagenic (Erickson, 2003; Snijman et al., 2007). The major flavonoids (with chemical structures) found in Rooibos, are shown in Fig. 2.2.

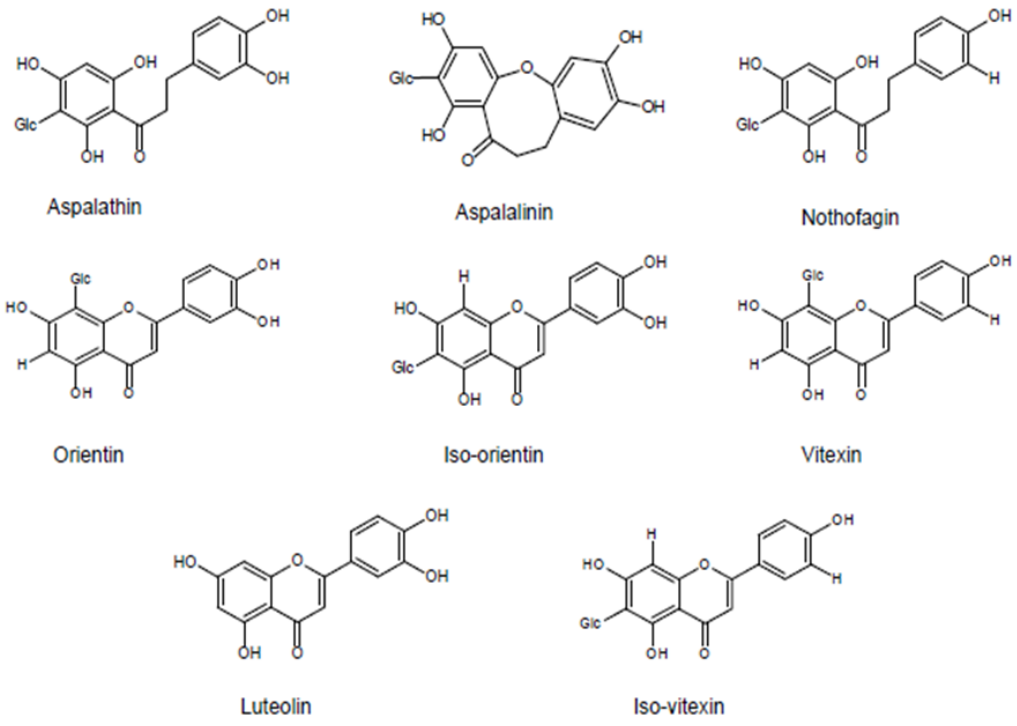

Fig. 2.2. Chemical structures of the major flavonoids present in Rooibos (Ajuwon et al., 2015).

Antioxidants are important as they bind to free radicals. Thus, theyprevent free radicals from destroying DNA in cells, which could be the cause of strokes, heart-attacks and cancer

(Erickson, 2003; Shah et al., 2014). Previous investigators have used Rooibos in processed meat products to extend the shelf-life by inhibiting oxidation and scavenging radicals. These included: “droëwors” (Jones, 2013), rabbit patties (Cullere et al., 2019) and ostrich salami (Cullere et al., 2013). *In vitro* and *in vivo* evidence has shown the effectiveness of antioxidants present in Rooibos (Marnewick et al., 2011). In rats, oxidative stress was partly reduced after consumption of the tea extracts (Ulična et al., 2006). Studies on humans have also proven that Rooibos extracts provided dietary antioxidants (Coetzee et al., 2016; McKay & Blumberg, 2007).

The antimicrobial activity of phenolic substances depends on their concentration and chemical structure (Kalogianni et al., 2020). It has been reported, according to Simpson et al. (2013), that the extracts of Rooibos have antimicrobial activity against *Micrococcus luteus* that grows on a solid matrix, *Bacillus cereus*, *Escherichia coli*, *Streptococcus mutans*, *Listeria monocytogenes*, *Saccharomyces cerevisiae* and *Staphylococcus aureus* that grow in culture (liquid form). In the study by Murali et al. (2011), it was found that a phosphate buffer saline extract of Rooibos displays effectiveness in antimicrobial activity against *Pseudomonas putida*, *Salmonella enteritidis*, *Pseudomonas aeruginosa* and *Campylobacter jejuni* after incubation of a few hours.

According to Dube et al. (2017), studies were done by two different sources and displayed results that are completely different from one another. The one study showed that the relationship between the antimicrobial and antioxidant activity in medicinal plants are working against each other. Thus, when a plant such as *Camelia sinensis* contains a high antioxidant activity, then a weak antimicrobial activity is expected (Yildirim et al., 2000). While the other study concluded that a higher antioxidant activity present in a plant or tea made from *Camellia sinensis* will also have a stronger antimicrobial activity (Chan et al., 2011).

2.4.2. Honeybush

2.4.2.1. Background

Honeybush is found in the fynbos regions of South Africa. There are 24 *Cyclopia* species, of which two, *Cyclopia subternata* and *Cyclopia intermedia*, are used to produce the honeybush tisane. The tea contains no caffeine and only a small amount of tannin, but its taste and aroma are very popular among same individuals (Dube et al., 2017; Kamara et al., 2004). The flowers of the honeybush are bright yellow with a sweet honey-like smell (du Toit et al., 1998) (Fig. 2.3). The production and marketing of honeybush was almost stopped, as it was not as popular as Rooibos, but new interest in the properties of the tea have started in the 1990’s (Dube et al., 2017; Joubert et al., 2011).

Fig. 2.3. Honeybush plant with flowers (Adapted from https://i.pinimg.com/originals/54/17/fa/5417faf9acb7fc404dc7abb67de81a23.jpg)

2.4.2.2. Properties

Honeybush was originally used to treat various digestive disorders and stimulate the production of milk in lactating women. The tea is a good cure for calves with digestive problems and beneficial for patients and children with heart problems who should avoid tannins. The absence of caffeine can contribute to the tea's calming effect and aid in the treatment of insomnia (Dube et al., 2017; du Toit et al., 1998). According to du Toit et al. (1998), there is anecdotal proof that one's appetite is increased by the consumption of the honeybush infusion. The tea, especially from *Cyclopia subternata,* displays antifungal and antiviral activities (Smith, 2020). Honeybush is commercialized in 25 countries, of which the Netherlands and Germany are the big marketing countries (Joubert et al., 2011).

2.4.2.3. Antioxidant activity

Polyphenols are the compounds in plants that carry antioxidants. Isokuranetin, hesperetin, hesperidin and mangiferin are the principal polyphenols present in honeybush (McKay & Blumberg, 2007). The phenolic compounds found in one of the species (*Cyclopia intermedia*) are revealed as xanthones, cinnamic acid, isoflavones, flavones, flavonones, flavonols and coumestans (Kamara et al., 2004). Another specie, namely, *Cyclopia subternata,* along with other species of *Cyclopia,* is abundant in polyphenols that includes: eriocitrin; narirutin; epigallocatechin gallate; hesperidin; orobol; luteolin; scolymoside; iosmangifern and mangiferin (de Beer et al., 2012; Smith, 2020).

The use of oxygen radical absorbance capacity (ORAC), lipid peroxidation (LPO) and 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assays, have proven to give the antioxidant capacity of both unfermented and fermented honeybush (Smith, 2020). The

compounds responsible for the antioxidant activity of honeybush are not well known, as they have not yet been researched to a great extent. Some compounds in *Cyclopia subternata* that should contribute to its antioxidant capacity by looking at its chemical structure, are isorhoifolin (a flavone), phloretin-3',5'-di-C-β-glucoside (a dihydrochalcone) and iriflophenone-3-C-β-glucoside (a benzophenone) (de Beer et al., 2012; Smith, 2020). There are a few studies (*in vitro*) that have documented that mangiferin possesses antioxidant properties, as it has been proven to be a strong oxygen scavenger (Smith, 2020). Mangiferin contains properties that promotes the health of consumers, properties such as anti-diabetic, anti-inflammatory, antioxidant and improvement in recognition memory (de Beer et al., 2012).

A study by Dube (2015), evaluated the antioxidant capacity of the whole plant extracts of honeybush and Rooibos, by means of three assays. It was found that the free radical scavenging potential (TEAC) and ORAC capacity of honeybush (extract) was much greater than those of Rooibos. While the ferric reducing ability (FRAP) of the extracts was higher for Rooibos than honeybush (Dube, 2015). An HPLC (high-performance liquid chromatography) method was used to identify the quantity of each phenolic compound present in honeybush (de Beer et al., 2012).

2.4.2.4. Antimicrobial activity

According to Márquez-Rodríguez et al. (2020), polyphenols that have shown to possess antimicrobial activity, are epicatechin, catechin, hydroxycinnamic acids, protocatechuic acid, chlorogenic acid and gallic acid. According to Coetzee et al. (2016), honeybush has a bacteriostatic effect on *Escherichia coli* and reduces the spore germination of *Botrytis cineria.* There are two compounds in honeybush that provide antimicrobial activity, namely mangiferin and hesperidin (Joubert et al., 2008; Kamara et al., 2004). According to a study by Dube et al. (2017), solvent extracts from both green and fermented honeybush contained a high antioxidant activity, but the antimicrobial activities were rather weak.

On bacterial strains, both honeybush and Rooibos extracts, show a bacteriostatic effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Propionibacterium acnes* and *Escherichia coli*, while a fungistatic effect against *Candida albicans* was established. The growth of this fungus was only suppressed for six hours, then the growth of the yeast continued after recovery. In the presence of 10 to 50 mg/ml of honeybush or Rooibos extracts, the growth of *Listeria monocytogenes*, *Salmonella enterica*, *Streptococcus mutans*, *Enterococcus faecalis*, *Proponibacterium acnes*, a *Vibrio* sp., *Staphylococcus aureus* and *Escherichia coli,* were suppressed by 50 to 100% after 6–24 hours by using a spectrophotometric method based on cell density

(https://www.innovus.co.za/assets/files/Technologies%20Afrikaans/A%20 natural %20preservative%20afr.pdf, Retrieved on 29 April 2021).

2.5. Conclusion

The hygienic manufacturing of Boerewors is important as many steps in the process, such as the handling of fresh meat and the incorporation of ingredients into the meat, can contribute to the distribution of bacteria. Therefore, many factors capable of spoiling Boerewors need to be controlled. The most widely known way to control the microflora found in meat, are the use of a common preservative, namelySO_2. According to above-mentioned studies, this chemical substance is no longer an ideal and healthy manner of preserving meat; as it causes unwanted allergic reactions in certain individuals. The use of natural substances, however, may be an excellent replacement for controlling microbial activities in meat. Plant extracts can be used as they act as preserving agents, which also have anticancer, anti-diabetic and antimutagenic properties.

Bioactive phytochemicals from the various plant materials contribute to food quality preservation by preserving the colour, flavour, texture and nutrients of meat. The antioxidant and antimicrobial properties of these compounds are needed to control the spoilage of fresh meat by scavenging radicals, to inhibit protein- and lipid oxidation and to prolong the shelf-life of meat. The use of Rooibos and honeybush extracts as natural preservatives in fresh sausages to replace chemical preservatives (sulphur dioxide) partially or perhaps fully, can hold tremendous health benefits.

Both Rooibos and honeybush contains flavonoids and other phenolic compounds, which have antioxidant and antimicrobial properties. According to several studies mentioned above, both honeybush and Rooibos extracts are effective against *Escherichia coli*, *Staphylococcus aureus* and a few other organisms. Almost all organisms that may be present in fresh sausages can be reduced and growth delayed by these extracts according to this literature study.

Chapter 3

The in vitro inhibitory effect of Rooibos, green Rooibos and honeybush extracts against strains of *Escherichia coli* and *Staphylococcus aureus*

Abstract

In this study, the inhibition effect of different concentrations (R025, R050, R1, GR050, GR1, GR2, H025, H050, and H1) of three plant extracts (Rooibos, green Rooibos and honeybush) and two controls: 100 mg/kg sulphur dioxide (S100) and 450 mg/kg sulphur dioxide (S450) against five strains each of Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) were evaluated by means of a disc diffusion assay. The results were evaluated by calculating the Z-scores of each treatment, on days 1, 3 and 6. The use of especially GR2, H050 and R025 in this trial, were more effective than the other concentrations of the plant extracts and both concentrations of the sulphur dioxide against the strains of E. coli and S. aureus. The results from this chapter will be used to evaluate the use of these plant extract concentrations as replacers of sulphur dioxide as preservative in Boerewors in the next chapter.

3.1. Introduction

Plant extracts have been of interest lately, as studies have shown that they contain phenolic acids and flavonoids with antioxidant, as well as, to some extent, antimicrobial properties (Cullere et al., 2019; Hadidi et al., 2022). Rooibos tea (*Aspalathus linearis*) has been well-known for its anti-diabetic, anti-mutagenic, and anti-inflammatory properties, as well as its pleasantness as a beverage high in antioxidants, thus reducing the risks of chronic disease (Cullere et al., 2013; Erickson, 2003; Joubert & de Beer, 2012; McKay & Blumberg, 2007). Aspalathin is the major flavonoid found in Rooibos with potent antioxidant activity, which is also a scavenger of free radicals and inhibits the peroxidation of lipids (Cullere et al., 2019). Honeybush tea is traditionally produced from *Cyclopia genistoides*, but nowadays, other species (*Cyclopia subternata* and *Cyclopia intermedia*) are also used. It is traditionally used for stimulating the production of milk during breast-feeding, to improve digestion and increase one's appetite (Mulaudzi et al., 2022). The xanthones, mangiferin, isomangiferin and hesperidin (flavonone) make up the main phenolic content found in all *Cyclopia* species. The fermentation process during tea production reduces the polyphenol content in honeybush - especially the xanthone content - the same way as those of Rooibos (McKay & Blumberg, 2007; Mulaudzi et al., 2022).

When chemical preservatives are replaced by natural preservatives in food products, it is very important that the natural preservative has the same, or better, safety and preservative characteristics as the chemical preservative. The natural product's antimicrobial effect against possible pathogens in food, such as *Escherichia coli* (*E. coli)* (Gram-negative bacterium) and *Staphylococcus aureus* (*S. aureus*) (Gram-positive bacterium), should, therefore, be evaluated. The coliform group of bacteria, which includes *E. coli* strains, is a common part of the normal facultative anaerobic microflora found in the intestinal tract of most mammals, including humans. The great majority of serotypes of *E. coli* are not harmful to people or warm-blooded animals. There are, however, some serotypes that can be harmful when ingested (Charimba et al., 2010). Due to its extensive variety of virulence factors and antibiotic resistance, *S. aureus* can cause a broad range of illnesses, e.g. food poisoning, soft tissue infections and septicemia (Gaupp et al., 2012).

According to Sun et al. (2011) and Hertz & Benington (2020), the cell wall of Gram-negative bacteria consists of a thin layer of peptidoglycan and contains a protective outer layer membrane (containing lipopolysaccharides), whereas those of Gram-positive bacteria, consist of a thick peptidoglycan layer, but they do not possess an outer membrane. The outer polysaccharide-rich membrane of Gram-negative bacteria may limit the diffusion of hydrophobic compounds. The absence of an outer membrane may result in Gram-positive bacteria being more easily influenced by environmental changes e.g. temperature, natural extracts and pH (Ahn et al., 2004; Hugo & Hugo, 2015; Human et al., 2020).

Studies on Rooibos and honeybush extracts as preservatives in meat products are limited. Previous studies that were done using plant extracts, have been used in different meat products, e.g., the use of different concentrations of green Rooibos or fermented Rooibos in ostrich meat patties and salami (Cullere et al., 2013); rabbit meat patties (Cullere et al., 2019); "droëwors" made from ostrich or other animals (Hoffman et al., 2014; Jones et al., 2015). Honeybush extracts were used in salami (Smith et al., 2020). However, no studies using these plant extracts as preservatives have yet been performed on Boerewors, which is one the most consumed fresh sausages in South Africa (Prinsloo, 2021).

The demand of using natural products, rather than chemicals, in meat products, have increased as consumers increasingly experienced health problems (Carballo, 2021). In Boerewors, sulphur dioxide (SO_2) is used as a preservative at a concentration of 450 mg/kg (SA DoH, 1990; SA DoH, 2001). The concentration at which natural preservatives are included in a food product, should be determined in vitro first before it can be evaluated in a food model such as Boerewors (Ahn et al., 2004; Miklasińska-Majdanik et al., 2018; Rodford, 1997).

In this study, three types of plant extracts were investigated, namely Rooibos, green Rooibos and honeybush. The aims of this chapter were, therefore, to:

- Evaluate the inhibitory effect/antimicrobial activity of three concentrations of all three types of extracts against five different strains each of *E. coli* and *S. aureus*.

- Evaluate the antimicrobial effect of two concentrations of SO_2 as a control in the form of sodium metabisulphite against five different strains each of *E. coli* and *S. aureus*.

- Determine which concentration, as well as which type of extract, will perform the best for acting as a preservative in Boerewors.

3.2. Materials and Methods

3.2.1. Strains used and reactivation of strains

Five strains each of *Escherichia coli* and *Staphylococcus aureus* were used in this study (Table 3.1). All the strains used were chosen based on availability and were obtained as freeze-dried cultures from the University of the Free State Bacterial Culture Collection (UFSBC) in the Food Microbiology section of the Department of Microbiology and Biochemistry.

Table 3.1

Different strains of *Escherichia coli* and *Staphylococcus aureus* used in this study.

Strains used	Culture collection number
Escherichia coli strain 1 (Ec1)	UFSBC 490 (Serogroup 018)
Escherichia coli strain 2 (Ec2)	UFSBC 712 (ATCC 25922)
Escherichia coli strain 3 (Ec3)	UFSBC 286 (ATCC 10418)
Escherichia coli strain 4 (Ec4)	UFSBC 287^T (ATCC 11775^T)
Escherichia coli strain 5 (Ec5)	UFSBC 290 (Beecham 705)
Staphylococcus aureus strain 1 (Sa1)	UFSBC 398 (Beecham 6612)
Staphylococcus aureus strain 2 (Sa2)	UFSBC 396 (Beecham 5637)
Staphylococcus aureus strain 3 (Sa3)	UFSBC 678^T (ATCC 6538^T)
Staphylococcus aureus strain 4 (Sa4)	UFSBC 395 (Beecham 171)
Staphylococcus aureus strain 5 (Sa5)	UFSBC 499 (ATCC 25923)

The strains were revived by inoculating the freeze-dried culture in 10 ml of nutrient broth (Oxoid CM0001) and were incubated at 37 °C for 48 h. Purity of the strains were checked by streaking on nutrient agar (Oxoid CM0003) and by incubating at 37 °C for 24 h. Afterwards, pure single colonies, confirmed by Gram-staining, were streaked onto nutrient agar slants,

incubated at 37 °C for 24 h and kept at 4 °C as working cultures. Every 7–8 weeks, the working cultures underwent sub-culturing (Mwanza et al., 2022).

3.2.2. Plant extracts and concentrations used

The plant extracts and concentrations evaluated in this study are given in Table 3.2. These plant extracts were obtained from Rooibos Limited (Clanwilliam, South Africa) and the colour of the three plant extracts are shown in Fig. 3.1. The concentrations in Table 3.2 were chosen for evaluation based on the findings of previous studies which have shown effectiveness in meat products such as ostrich patties, salami, “droëwors” from springbok, blesbok and ostrich meat (Cullere et al., 2013; Hoffman et al., 2014; Jones et al., 2015).

A total of nine treatments were used to analyse the different concentrations, to investigate which concentration and which extract will provide the best antimicrobial action to be used in the Boerewors. Analysis involving a method to obtain zone (Z) scores were performed. This analysis included only the plant extract, water medium and the organism strain, therefore, the effect of solely the Rooibos, green Rooibos and honeybush extracts on the strain, were evaluated.

3.2.3. Sulphur dioxide (control) used

Sulphur dioxide in the form of sodium metabisulphite was analysed as a control, therefore, the use of 100 mg/kg SO_2 (0.16 g sodium metabisulphite) and 450 mg/kg SO_2 (0.7 g sodium metabisulphite) were investigated on all the bacterial strains. The 450 mg/kg SO_2 concentration is used as the major preservative in Boerewors according to the South African regulation (SA DoH, 2001). The 100 mg/kg SO_2 concentration was chosen based on the findings of previous studies (Roller et al., 2002; Bañón et al., 2007; Charimba et al., 2012) using 100 mg/kg in combination with other preservatives/extracts, which showed effectivity in inhibiting microbial growth in meat.

Table 3.2

The plant extracts and concentrations evaluated and abbreviations used in this study.

Treatment	**Rooibos Ltd Code**	**Concentrations (%, w/v)**	**Abbreviation**
Rooibos (R)	E1CCJ, cold-water soluble	0.25; 0.50; 1.00	R025; R050; R1
Green Rooibos (GR)	E2CCJ, cold-water soluble	0.50; 1.00; 2.00	GR050; GR1; GR2
Honeybush (H)	E4CCJ, cold water soluble	0.25; 0.50; 1.00	H025; H050; H1

Fig. 3.1. Rooibos (chestnut reddish-brown colour when dissolved), green Rooibos (light orange colour when dissolved) and honeybush (light brownish colour when dissolved) powder used (from left to right) in this study (Adapted from rooibosltd.co.za).

3.2.4. Antimicrobial activity

The antimicrobial activity patterns of the test organisms were determined using the Kirby-Bauer Disc Diffusion method as prescribed by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2007). The concentrations of each plant extract as indicated in Table 3.2, were prepared in sterile distilled water. A 24 h nutrient broth culture of the test strain was streaked with sterile cotton swabs (Lasec, Bloemfontein) on two nutrient agar (Oxoid CM0003) plates per strain. Antibiotic assay discs (FLAS321260, Lasec, Bloemfontein South Africa) were placed in triplicate, with a sterile pinsette onto the inoculated plates, resulting in six data points per strain (see Fig. 3.2 as an example). The discs were then infused with the concentrations of the plant extracts by pipetting 40 µl of each plant extract onto the discs. The plates were incubated for 24 h at 37 °C. Observation and measurement of the zones were done after 24 h, as well as on days three and six. The diameters of the zones of clearance around each disc were measured with a caliper. The size of zone inhibition was measured to evaluate the effectiveness of all nine treatments against strains of *E. coli* and *S. aureus.* The treatment with a Z-score of low value correlated with the biggest zone of inhibition, while the highest value resulted in the smallest zone of inhibition.

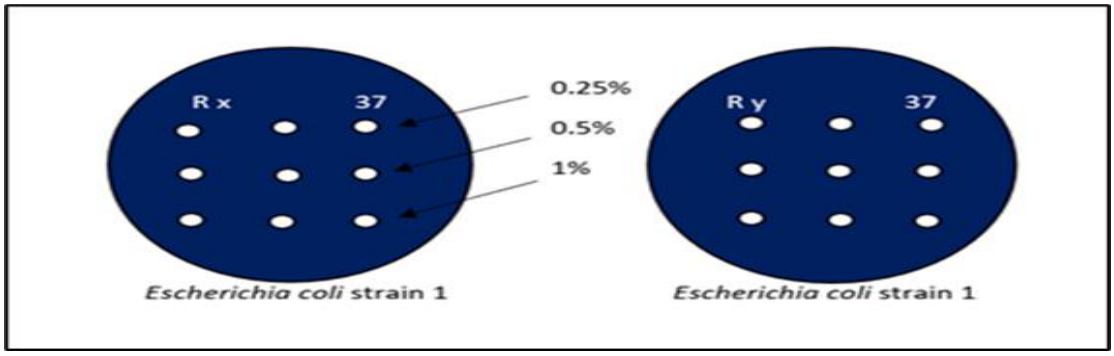

Fig. 3.2. Example of an *Escherichia coli* strain tested in duplicate by means of the disc diffusion method with three concentrations (0.25%; 0.50% and 1%) of Rooibos (R) at 37 °C.

3.2.5. Statistical analysis

The data used to obtain the antimicrobial sensitivity of the strains of *E. coli* and *S. aureus* was standardised using Z-scores (Mwanza et al., 2022). The Z-scores were calculated as:

Zone score = colony diameter (mm) divided by (colony diameter (mm) + zone size (mm)).

The primary analysis employed a repeated measures ANOVA to evaluate the effect of the preservative, day, and their interaction (Agresti, 2017). This will give an idea of the effect of day on the Z-score, and account for the repeated measurement nature of the data. The follow-up analysis involved doing an ANOVA for each day to break down the effect. This allowed for evaluation of the simple effect at each time point. A Post-Hoc Tukey-HSD test was then conducted to indicate specific differences (Agresti, 2017). The analyses were conducted using the XLSTAT statistical and data analysis solution from Lumivero (2023). All testing was done at a predetermined significance level of $\alpha = 0.05$.

It is important to note that the multiple testing that was done by testing each day for each organism, will increase the Type I error rate (the probability of rejecting the null hypothesis when it is true). This might lead to identifying differences where none exist. Due to the exploratory nature of the data, this approach was followed. Findings deemed significant in this context, should be considered preliminary and warrant further investigation in subsequent confirmatory studies.

3.3. Results and Discussions

3.3.1. The antimicrobial effect on Escherichia coli strains

The results obtained from the disc diffusion assay evaluating the effect of SO_2 and the different plant extracts on the strains of *E. coli,* are shown in Tables 3.3 to 3.7.

3.3.1.1. Escherichia coli strain 1 (Ec1)

On days 1, 3 and 6, green Rooibos 2% (GR2) gave the significant ($p < 0.001$) lowest Z-score (0.360), which correlated with the largest zone of inhibition against Ec1 (Table 3.3). This might be due to the higher quantity of polyphenols present in the unfermented extract rather than in the fermented extract, because of the structural changes that takes place during the process of fermentation (McKay & Blumberg, 2007). Therefore, the action of inhibiting the strain increased, as a higher concentration of polyphenolic compounds were present to denature the proteins of the bacterial cell (Tiwari et al., 2009). Phenolic acids in Rooibos may also play a role in preventing the formation of biofilms and inhibiting bacterial movement (Makarewicz et al., 2021). Other studies have shown that phenolic acids, i.e., ferulic acid, inhibited Gram-negative bacteria by changing the negative surface charge and hydrophobicity of the cell membrane (Almasaudi, 2021; Makarewicz et al., 2021).

The two controls used in the study, S100 and S450, differed significantly ($p < 0.001$) from all other treatments, showing the highest Z-score (0.500) on days 1, 3 and 6, which represented no inhibition. Sulphur dioxide was, therefore, ineffective as an antimicrobial against Ec1. The reasons for the ineffectiveness of the SO_2, might be ascribed to the Gram-negative impermeable cell wall, therefore, making the strain more resistant towards antimicrobials. Another reason might be that the two concentrations (S100 and S450) were too low, to have an inhibition effect on the bacterial strain. Sulphur dioxide is more well known for its antioxidant properties, such as controlling the oxidative reactions that causes spoilage in food (D'Amore et al., 2020; Walker, 1985).

Table 3.3

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Escherichia coli* strain 1 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{g} \pm 0.01$	$0.500^{g} \pm 0.01$	$0.500^{f} \pm 0.01$
S450	$0.500^{g} \pm 0.01$	$0.500^{g} \pm 0.01$	$0.500^{f} \pm 0.01$
R025	$0.400^{c} \pm 0.01$	$0.400^{c} \pm 0.01$	$0.400^{c} \pm 0.01$
R050	$0.435^{e} \pm 0.02$	$0.435^{e} \pm 0.02$	$0.440^{e} \pm 0.02$
R1	$0.462^{f} \pm 0.01$	$0.462^{f} \pm 0.01$	$0.500^{f} \pm 0.01$
GR050	$0.410^{cd} \pm 0.02$	$0.410^{cd} \pm 0.02$	$0.410^{cd} \pm 0.02$
GR1	$0.392^{bc} \pm 0.01$	$0.392^{bc} \pm 0.01$	$0.392^{bc} \pm 0.01$
GR2	$0.360^{a} \pm 0.01$	$0.360^{a} \pm 0.01$	$0.360^{a} \pm 0.01$
H025	$0.429^{de} \pm 0.01$	$0.429^{de} \pm 0.01$	$0.429^{de} \pm 0.01$
H050	$0.375^{ab} \pm 0.01$	$0.375^{ab} \pm 0.01$	$0.375^{ab} \pm 0.01$
H1	$0.400^{c} \pm 0.01$	$0.400^{c} \pm 0.01$	$0.400^{c} \pm 0.01$
p-value	<0.001	< 0.001	< 0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

3.3.1.2. Escherichia coli strain 2 (Ec2)

In the case of Ec2, H050, H1, GR1 and GR2 had significantly ($p < 0.001$) lower Z-scores than the rest of the treatments evaluated on days 1, 3 and 6 (Table 3.4). Although these treatments did not differ significantly from each other, H050 had the lowest Z-score (0.379) over the six days of evaluation. This may be contributed to flavonoids, such as hesperidin (a flavanone) and other compounds found in honeybush. High affinity for protein binding of phenolic hydroxyl groups may both boost affinity for cytoplasmic membranes and block microbial enzymes, hence increasing the antibacterial effect (Miklasińska-Majdanik et al., 2018).

None of the Rooibos extract concentrations evaluated had antimicrobial activity against Ec2 over the 6 days. This strain may be more resistant towards the phenolic compounds in Rooibos, as the polysaccharide (outer membrane) may prevent phytochemicals from entering the cell wall (Mantzourani et al., 2022).

The S100 (0.500) did not show any antimicrobial activity against Ec2, but S450 showed a small zone of inhibition. Because of the higher concentration, S450 was more effective than S100. The Z-score of S450 (0.442) and H025 (0.429) did not differ significantly from each other, but they did differ significantly ($p < 0.001$) from the rest of the treatments on days 1 and 6. Thus, Ec2 was more sensitive to the plant extract treatments than to the S450. This might show that SO_2 is dependent on other components for its antimicrobial action, and use on its own, is not efficient against all bacteria (Bañón et al., 2007).

Table 3.4

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Escherichia coli* strain 2 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
S450	$0.442^{bc} \pm 0.04$	$0.441^{bc} \pm 0.04$	$0.475^{bc} \pm 0.03$
R025	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
R050	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
R1	$0.500^{d} \pm 0.01$	$0.488^{cd} \pm 0.03$	$0.500^{c} \pm 0.01$
GR050	$0.456^{c} \pm 0.01$	$0.451^{cd} \pm 0.03$	$o.456^{b} \pm 0.01$
GR1	$0.400^{a} \pm 0.01$	$0.400^{ab} \pm 0.01$	$0.400^{a} \pm 0.01$
GR2	$0.388^{a} \pm 0.01$	$0.400^{ab} \pm 0.05$	$0.400^{a} \pm 0.05$
H025	$0.429^{b} \pm 0.01$	$0.483^{cd} \pm 0.04$	$0.483^{bc} \pm 0.04$
H050	$0.379^{a} \pm 0.01$	$0.379^{a} \pm 0.01$	$0.379^{a} \pm 0.01$
H1	$0.396^{a} \pm 0.01$	$0.396^{ab} \pm 0.01$	$0.396^{a} \pm 0.01$
p-value	< 0.001	< 0.001	< 0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

3.3.1.3. Escherichia coli strain 3 (Ec3)

R025 differed significantly ($p < 0.001$) from all the other treatments on days 1, 3 and 6, as it gave the lowest Z-score (0.400), having the largest zone of inhibition (Table 3.5). This *E. coli* strain was not sensitive towards any of the green Rooibos treatments with Z-scores of 0.500 on days 1, 3 and 6. This may be ascribed to the susceptibility of micro-organisms to the plant

extract and depends on the molecular structure of the phenolic compounds of the extract, as well as on the species and the strain (Efenberger-Szmechtyk et al., 2020). High concentrations of Rooibos may not be effective against Ec3, but low concentrations may act as a potent antioxidant as it contains aspalathin (flavonoid) (Jones et al., 2015). R025 had the best inhibition effect against Ec3 and thus, may be used for further investigation as a preservative.

Table 3.5

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Escherichia coli* strain 3 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z -score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
S450	$0.462^{c} \pm 0.02$	$0.462^{c} \pm 0.02$	$0.500^{c} \pm 0.01$
R025	$0.400^{a} \pm 0.01$	$0.400^{a} \pm 0.01$	$0.400^{a} \pm 0.01$
R050	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
R1	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
GR050	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
GR1	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
GR2	$0.500^{d} \pm 0.01$	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$
H025	$0.445^{bc} \pm 0.02$	$0.445^{bc} \pm 0.02$	$0.445^{b} \pm 0.02$
H050	$0.436^{b} \pm 0.03$	$0.436^{b} \pm 0.03$	$0.436^{b} \pm 0.03$
H1	$0.445^{bc} \pm 0.02$	$0.445^{bc} \pm 0.02$	$0.445^{b} \pm 0.02$
p-value	< 0.001	< 0.001	< 0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

All the treatments remained the same on days 1, 3 and 6, without the strain becoming more sensitive or less sensitive towards the treatments, except for S450, that showed a higher Z-score on day 6. S100 had a higher Z-score than S450 on days 1 and 3, but there were no significant differences between the treatments on day 6. This may be explained by the higher concentration of SO_2 used, therefore, S450 was more effective than S100 on days 1 and 3 against strain Ec3.

3.3.1.4. Escherichia coli strain 4 (Ec4)

The significant ($p < 0.001$) largest inhibition zones against Ec4 were obtained from GR2, H050, GR1 and R025. Although these treatments did not differ significantly from each other, treatment GR2 had the lowest Z-score (0.353) (Table 3.6). The Z-score also remained constant over the 6 days. The high polyphenol content of the GR2 treatment might have contributed to the inhibition of Ec4 and the destruction of its cell wall. Rooibos contains aspalathin, orientin, catechin and many other phenolic compounds that may destroy or cause disruption of the cell wall (Efenberger-Szmechtyk et al., 2020; Joubert et al., 2005).

S100 and S450 had the highest Z-score (0.500) and differed significantly ($p < 0.001$) from all other treatments, except from R1 (0.474). R1, S100, and S450 remained ineffective against Ec4, as the Z-scores were constant from day 1 to day 6. This might be due to the concentration of SO_2, which was not able to have an inhibitory effect on the strain.

Table 3.6

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Escherichia coli* strain 4 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$	$0.500^{d} \pm 0.01$
S450	$0.500^{d} \pm 0.01$	$0.500^{c} \pm 0.01$	$0.500^{d} \pm 0.01$
R025	$0.374^{ab} \pm 0.04$	$0.374^{ab} \pm 0.04$	$0.374^{ab} \pm 0.04$
R050	$0.396^{bc} \pm 0.01$	$0.396^{b} \pm 0.01$	$0.396^{b} \pm 0.01$
R1	$0.474^{d} \pm 0.02$	$0.474^{c} \pm 0.02$	$0.474^{d} \pm 0.02$
GR050	$0.419^{c} \pm 0.02$	$0.400^{b} \pm 0.01$	$0.400^{b} \pm 0.01$
GR1	$0.379^{ab} \pm 0.01$	$0.379^{ab} \pm 0.01$	$0.379^{ab} \pm 0.01$
GR2	$0.353^{a} \pm 0.01$	$0.353^{a} \pm 0.01$	$0.353^{a} \pm 0.01$
H025	$0.401^{bc} \pm 0.02$	$0.401^{b} \pm 0.02$	$0.401^{bc} \pm 0.02$
H050	$0.357^{a} \pm 0.01$	$0.357^{a} \pm 0.01$	$0.357^{a} \pm 0.01$
H1	$0.400^{bc} \pm 0.01$	$0.400^{b} \pm 0.01$	$0.429^{c} \pm 0.01$
p-value	< 0.001	< 0.001	< 0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

3.3.1.5. Escherichia coli strain 5 (Ec5)

The results showed that H050 (0.360) had the largest zone of inhibition (lowest Z-score) and differed significantly ($p < 0.001$) from all other treatments, except from GR2 and R025, on days 1, 3 and 6 (Table 3.7). Honeybush contains a high number of polyphenols that may increase the antimicrobial activity. According to Xie et al. (2017), hesperetin (a flavonone) has shown substantial antimicrobial activity against Gram-negative bacteria. Thus, this might explain the large inhibition zone obtained from H050 as honeybush contains hesperetin (McKay & Blumberg, 2007).

Table 3.7

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Escherichia coli* strain 5 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{f} \pm 0.01$	$0.500^{g} \pm 0.01$	$0.500^{g} \pm 0.01$
S450	$0.500^{f} \pm 0.01$	$0.500^{g} \pm 0.01$	$0.500^{g} \pm 0.01$
R025	$0.379^{abc} \pm 0.01$	$0.379^{bc} \pm 0.01$	$0.379^{bc} \pm 0.01$
R050	$0.405^{d} \pm 0.01$	$0.400^{de} \pm 0.01$	$0.400^{de} \pm 0.01$
R1	$0.462^{e} \pm 0.01$	$0.462^{f} \pm 0.01$	$0.462^{f} \pm 0.01$
GR050	$0.410^{d} \pm 0.02$	$0.414^{e} \pm 0.02$	$0.414^{e} \pm 0.02$
GR1	$0.392^{bcd} \pm 0.01$	$0.392^{cd} \pm 0.01$	$0.392^{cd} \pm 0.01$
GR2	$0.375^{ab} \pm 0.01$	$0.375^{ab} \pm 0.01$	$0.375^{ab} \pm 0.01$
H025	$0.400^{d} \pm 0.01$	$0.400^{de} \pm 0.01$	$0.400^{de} \pm 0.01$
H050	$0.360^{a} \pm 0.01$	$0.360^{a} \pm 0.01$	$0.360^{a} \pm 0.01$
H1	$0.396^{cd} \pm 0.01$	$0.396^{d} \pm 0.01$	$0.396^{d} \pm 0.01$
p-value	< 0.001	< 0.001	< 0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

All the other plant extract treatments showed some inhibition against the strain. The phenolic acids of these plant extracts were able to disrupt the enzyme activity and denature the proteins of Ec5. The outer membrane of Gram-negative bacteria contains porins, which are permeable to water, food, etc., allowing the plant extract, dissolved in the water, to reach the proteins and disrupt the structure of the cell wall (Mantzourani et al., 2022).

Treatments S100 and S450, however, showed no inhibition of Ec5 and differed significantly ($p < 0.001$) from all the other treatments. This could be attributed to the amount of SO_2 not being enough to inhibit Ec5 or due to volatilization (Walker, 1985).

3.3.2. The antimicrobial effect on Staphylococcus aureus strains

The results obtained from the disc diffusion assay evaluating the effect of SO_2 and different plant extracts on the strains of *S. aureus,* are shown in Tables 3.8 to 3.12. In this study, not one of the five strains evaluated were inhibited by either of the concentrations of SO_2 (S100 and S450). This might be explained by the inhibitory/antimicrobial activity of sulphiting agents that are known to inhibit Gram-negative bacteria more than Gram-positive bacteria (Walker, 1985).

3.3.2.1. Staphylococcus aureus strain 1 (Sa1)

No significant differences ($p > 0.05$) were observed between the Z-scores of the 11 treatments against Sa1 on days 1, 3 and 6 (Table 3.8). The Z-score values also remained constant on days 1, 3 and 6. The Z-scores ranged between 0.400 and 0.500, indicating very low levels of inhibition by all the treatments in this study. Although not significant, R025 showed the lowest Z-score (0.400), which indicated that it was the most effective against strain Sa1. R050, GR1 and GR2 showed some inhibition (0.462, 0.462 and 0.429, respectively), but the honeybush concentrations did not have any inhibition effect on Sa1. According to Efenberger-Szmechtyk et al. (2020), sensitivity towards plant extracts is dependent on the concentration and the molecular structure of the polyphenols, which might be a reason why there was no inhibition effect from any of the honeybush concentrations.

Table 3.8

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Staphylococcus aureus* strain 1 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
S450	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
R025	0.400 ± 0.01	0.400 ± 0.01	0.400 ± 0.01
R050	0.462 ± 0.01	0.462 ± 0.01	0.462 ± 0.01
R1	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
GR050	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
GR1	0.462 ± 0.01	0.462 ± 0.01	0.462 ± 0.01
GR2	0.429 ± 0.01	0.429 ± 0.01	0.429 ± 0.01
H025	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
H050	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
H1	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
p-value	NS	NS	NS

Means with different superscripts in the same column differed significantly ($p < 0.05$). NS = not significant.

3.3.2.2. Staphylococcus aureus strain 2 (Sa2)

On days 1, 3 and 6, GR2 had the lowest Z-score (0.450) against Sa2, which differed significantly ($p = 0.009$) from all the treatments, except from R025 (0.487), R050 (0.474), GR050 (0.587) and H05 (0.488), as shown in Table 3.9. This may be due to type of strain present and the concentration of certain polyphenols present in Rooibos and honeybush, which is effective in inhibiting Sa2. The efficiency of the flavonoids may depend largely on the concentration and the type of strain (Makarewicz et al., 2021). A Z-score of 0.500 was obtained for H025 and H1, which indicated that these treatments had no inhibition against Sa2. Each of the treatments remained constant over time, as the Z-score did not change on days 3 or 6.

Table 3.9.

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Staphylococcus aureus* strain 2 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$
S450	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$
R025	$0.487^{ab} \pm 0.02$	$0.487^{ab} \pm 0.02$	$0.487^{ab} \pm 0.02$
R050	$0.474^{ab} \pm 0.02$	$0.474^{ab} \pm 0.02$	$0.474^{ab} \pm 0.02$
R1	$0.494^{b} \pm 0.02$	$0.494^{b} \pm 0.02$	$0.494^{b} \pm 0.02$
GR050	$0.487^{ab} \pm 0.02$	$0.487^{ab} \pm 0.02$	$0.487^{ab} \pm 0.02$
GR1	$0.494^{b} \pm 0.02$	$0.494^{b} \pm 0.02$	$0.494^{b} \pm 0.02$
GR2	$0.450^{a} \pm 0.06$	$0.450^{a} \pm 0.06$	$0.450^{a} \pm 0.06$
H025	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$
H050	$0.488^{ab} \pm 0.03$	$0.488^{ab} \pm 0.03$	$0.488^{ab} \pm 0.03$
H1	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$	$0.500^{b} \pm 0.01$
p-value	0.009	0.009	0.009

Means with different superscripts in the same column differed significantly ($p < 0.05$).

3.3.2.3. Staphylococcus aureus strain 3 (Sa3)

There were no significant differences ($p > 0.05$) between any of the treatments on days 1, 3 and 6 against Sa3 (Table 3.10). Very low Z-scores, ranging between 0.429 and 0.500 were observed with R025 having the lowest Z-score (0.429) on day 1. However, on days 3 and 6, R025 showed no inhibition against Sa3 with a Z-score of 0.500. The GR2 treatment, however, had a Z-score of 0.462 on days 1, 3 and 6. GR2 and R025 can be seen to have a small inhibition effect against Sa3 and may be further explored as natural preservatives in Boerewors.

Table 3.10

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Staphylococcus aureus* strain 3 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Z-score value		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
S450	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
R025	0.429 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
R050	0.462 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
R1	0.500 ± 0.01	0.500 ± 0.01	0.500± 0.01
GR050	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
GR1	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
GR2	0.462 ± 0.01	0.462 ± 0.01	0.462 ± 0.01
H025	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
H050	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
H1	0.500 ± 0.01	0.500 ± 0.01	0.500 ± 0.01
p – value	NS	NS	NS

Means with different superscripts in the same column differed significantly ($p < 0.05$). NS = Not significant.

3.3.2.4. Staphylococcus aureus strain 4 (Sa4)

GR2, with the lowest Z-score of 0.481 against Sa4, differed significantly ($p = 0.002$) on day 1 from all the treatments except for H050 (Table 3.11). The antimicrobial action of GR2 may be attributed to the phenolic acids of green Rooibos. A study by Vattem et al. (2005), suggested that the dissociation of phenolic acids is one of the mechanisms that may be responsible for the hyper-acidification that takes place at the interphase of the plasma membrane of Gram-positive bacteria (Makarewicz et al., 2021).

On days 3 and 6, all the treatments reached a Z-score of 0.500, which indicated that there was no inhibition of Sa4 after day 1 with any of the treatments. This lack of inhibition by all the

treatments may be due to the species of the bacterial strain being more resistant, or the lack of flavonoids present in the plant extracts (Efenberger-Szmechtyk et al., 2020).

Table 3.11

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Staphylococcus aureus* strain 4 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR050 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
S450	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
R025	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
R 050	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
R1	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
GR050	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
GR1	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
GR2	$0.481^{a} \pm 0.02$	0.500 ± 0.01	0.500 ± 0.01
H025	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
H050	$0.494^{ab} \pm 0.02$	0.500 ± 0.01	0.500 ± 0.01
H1	$0.500^{b} \pm 0.01$	0.500 ± 0.01	0.500 ± 0.01
p-value	0.002	NS	NS

Means with different superscripts in the same column differed significantly ($p < 0.05$). NS = Not significant.

3.3.2.5. Staphylococcus aureus strain 5 (Sa5)

On days 1 and 3, GR2 had the lowest Z-score (0.377), as it inhibited Sa5 and differed significantly ($p < 0.001$) from the rest of the treatments, except from R025 (0.421). This may be explained by the absence of the outer membrane in the cell membrane of Sa5, which causes the strain to be more susceptible towards external environmental changes, such as plant extracts (Ahn et al., 2004). All the treatments on day 1 had the same Z-score on day 3, with changes only occurring on day 6.

On day 6, GR2 did not inhibit Sa5 anymore, while more sensitivity towards H025 (0.445) and H050 (0.445) were observed. The flavonoids in honeybush might change the functions of the microbial cell membrane of Sa5, thus increasing the sensitivity of the strain towards these compounds with the inactivation of membrane-bound enzymes (Ahn et al., 2004). Treatments R050, R1, GR050, GR1 and GR2 did not have any inhibition (Z-score of 0.500) activity by day 6.

Table 3.12

The inhibitory effect (mean Z-score ± standard deviation, n = 6) of 11 different treatments on *Staphylococcus aureus* strain 5 on days 1, 3 and 6 at 37 °C. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR0.50 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract. The lowest (best) Z-score is indicated in red.

	Mean Z-score value ± standard deviation		
Treatment	**Day 1**	**Day 3**	**Day 6**
S100	$0.500^{c} \pm 0.01$	$0.500^{c} \pm 0.01$	$0.500^{c} \pm 0.01$
S450	$0.500^{c} \pm 0.01$	$0.500^{c} \pm 0.01$	$0.500^{c} \pm 0.01$
R025	$0.421^{ab} \pm 0.04$	$0.421^{ab} \pm 0.04$	$0.475^{b} \pm 0.03$
R050	$0.438^{b} \pm 0.07$	$0.438^{b} \pm 0.07$	$0.500^{c} \pm 0.01$
R1	$0.445^{b} \pm 0.02$	$0.445^{b} \pm 0.02$	$0.500^{c} \pm 0.01$
GR050	$0.462^{bc} \pm 0.01$	$0.462^{bc} \pm 0.01$	$0.500^{c} \pm 0.01$
GR1	$0.462^{bc} \pm 0.01$	$0.462^{bc} \pm 0.01$	$0.500^{c} \pm 0.01$
GR2	$0.377^{a} \pm 0.03$	$0.377^{a} \pm 0.03$	$0.500^{c} \pm 0.01$
H025	$0.451^{bc} \pm 0.02$	$0.451^{bc} \pm 0.02$	$0.445^{a} \pm 0.02$
H050	$0.445^{b} \pm 0.02$	$0.445^{b} \pm 0.02$	$0.445^{a} \pm 0.02$
H1	$0.462^{bc} \pm 0.01$	$0.462^{bc} \pm 0.01$	$0.462^{bc} \pm 0.01$
p-value	<0.001	<0.001	<0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

3.3.3. Determination of the best treatment

To determine which of the 11 preservative treatments used in this study, showed the best inhibition against the five *E. coli* and five *S. aureus* strains, a score was assigned to each strain and treatment according to the best Z-score results obtained and indicated in red in Tables 3.3 to 3.12. A zero was assigned to the preservative when it showed no/weak inhibition against the *E. coli* or *S. aureus* strains. The percentage of strains showing the best inhibition, was then indicated in Table 3.13. The results showed that both the S100 and S450 treatments gave the highest Z-scores against all 10 strains used in this study, indicating that both concentrations of SO_2 were the least effective in the in vitro inhibition of the 10 strains (Table 3.13). Sulphur dioxide is well-known for its antioxidant properties, but is also known to be more effective against Gram-negative bacteria than Gram-positive bacteria (D'Amore et al., 2020; Hugo & Hugo, 2015; Walker, 1985).

With the R050, R1, GR050, GR1, H025 and H1, none (0%) of the strains showed best inhibition scores (Table 3.13). Only three preservatives, R025, GR2 and H050, with 20%, 50% and 30% of the 10 strains, respectively, showed best inhibition, with the lowest Z-scores, in Tables 3.3 – 3.12.

The R025 treatment showed only one strain of *E. coli* and one strain of *S. aureus* that had a best inhibition score (Table 3.13). This was in accordance with the study by Jones et al. (2015), who found the lowest amount of Rooibos (R025) to give the best preservative action based on reduced oxidation of fat and an extension of shelf-life. Rooibos can act as a pro-oxidant with increasing concentrations. The oxidation of compounds might cause structural changes in Rooibos, which lowers the antimicrobial activity (McKay & Blumberg, 2007).

For the GR2 treatment, 50% of the strains showed the best Z-scores with three *S. aureus* strains and two *E. coli* strains being inhibited the best by this treatment (Table 3.13). A study by Human et al. (2020) showed that high concentrations of green Rooibos extracts were more effective against Gram-positive strains than towards Gram-negative strains, such as *E. coli*. This may be due to the acidification by phenolic acids in Rooibos, which causes alteration of the cell membrane potential, therefore, increases permeability of Gram-positive bacteria (Makarewicz et al. 2021). The higher the concentration of green Rooibos used, the greater the effect of inhibition was (Erickson, 2003). This could be due to the high polyphenol content (especially aspalathin), present in green Rooibos (Erickson, 2003; McKay & Blumberg, 2007).

The H050 scored 30%, with two *E. coli* and one *S. aureus* strain showing the best Z-scores (Table 3.13). According to a study by Mudenda et al. (2023), honeybush had stronger antibacterial activity against *E. coli* than against *S. aureus.*

Table 3.13

The score of the number of *Escherichia coli* and *Staphylococcus aureus* strains showing inhibition by the 11 preservative treatments evaluated in this study. S100 = 100 mg/kg SO_2; S450 = 450 mg/kg SO_2; R025 = 0.25% Rooibos extract; R050 = 0.50% Rooibos extract; R1 = 1% Rooibos extract; GR0.50 = 0.50% green Rooibos extract; GR1 = 1% green Rooibos extract; GR2 = 2% green Rooibos extract; H025 = 0.25% honeybush extract; H050 = 0.50% honeybush extract and H1 = 1% honeybush extract.

Preservative	*Escherichia coli*	*Staphylococcus aureus*	**Total**	**Percentage (%)**
S100	0	0	0	0
S450	0	0	0	0
R025	1	1	2	20
R050	0	0	0	0
R1	0	0	0	0
GR050	0	0	0	0
GR1	0	0	0	0
GR2	2	3	5	50
H025	0	0	0	0
H050	2	1	3	30
H1	0	0	0	0
Total	**5**	**5**	**10**	**100**

3.4. Conclusions

In this in vitro study, the disc diffusion assay was used to evaluate which concentration of Rooibos, green Rooibos and honeybush showed the best inhibition activity against five strains each of *E. coli* and *S. aureus*. The use of R025, GR2 and H050 showed the best inhibition against most strains of *E. coli* and *S. aureus,* while the treatment with the best inhibiting effect overall, against all the strains, was GR2. The inhibitory effect by green Rooibos was attributed to the high polyphenol content (especially aspalathin), of this plant extract. The pro-oxidant effect of Rooibos caused structural changes to the Rooibos extract or to the antioxidant itself, e.g. polyphenol, which lowered its antimicrobial effect at higher concentrations.

In general, the days did not have any effect on the efficiency of the preservative evaluated since most Z-scores stayed constant on days 1, 3 and 6. *Staphylococcus aureus* was more

resistant towards SO_2 compared to *E. coli*. The different strains of *S. aureus* and *E. coli* sometimes displayed different reactions towards the same preservatives.

The three plant extract concentrations, GR2, R025 and H050, will be further explored as possible replacers for SO_2 in a Boerewors model in the next Chapter.

Chapter 4

The physico-chemical, microbial and sensory effect of Rooibos and honeybush extracts as preservative replacers of sulphur dioxide in Boerewors

Abstract

In this study, the addition of plant extracts to Boerewors models, for the purpose of replacing SO_2 as a preservative, was investigated. Different Boerewors treatments were formulated containing plant extracts (2% green Rooibos, GR2; 0.50% honeybush, H050 and 0.25% Rooibos, R025) and combinations of each of the three plant extracts with 100 mg/kg SO_2 (GR2 + S100, H050 + S100 and R025 + S100), as well as two controls (NC and S450) for comparison means. The Boerewors treatments were stored at 4 ^oC and evaluated on days 1, 3 and 6 by using physico-chemical, microbial and colour analyses. Sensory analysis was performed after the Boerewors was cooked, and thaw-, cooking- and total losses were also determined. The pH, water activity and lipid stability of all the plant extract treatments were comparable to the S450 with no significant differences. SO_2 still inhibited Gram-negative bacteria (E. coli, coliforms, Enterobacteriaceae) and Gram-positive bacteria (lactic acid bacteria) more effectively than the plant extracts. However, S. aureus was inhibited more effectively by the plant extract combinations with low SO_2 (100 mg/kg). The H050, R025 and S450 treatments were preferred by the sensory panel in terms of taste, texture, colour and overall acceptability. The GR2 treatment was not preferred by the sensory panel in terms of colour, and the low L-value of this treatment confirmed a significantly darker colour. The thaw-, cooking- and total losses of all the plant extract treatments with their combination with S100, were comparable to the S450 treatment. However, the GR2 and GR2 + S100 treatment resulted in significantly high losses. This study indicated that the H050 and R025 treatments, with their combination with S100, may be suitable replacers of SO_2.*

4.1. Introduction

The use of chemicals as preservatives in foods has started to decline and has become unwanted, as consumers have become more interested in safer food and the use of natural products (Yu et al., 2021). Boerewors is a typical South African fresh sausage. The main preservative in Boerewors is sulphur dioxide (SO_2) included at a concentration of 450 mg/kg (SA DoH, 2001). Many consumers struggle with allergies, due to the presence of SO_2, since it may cause nausea, headaches, and other health problems (Bañón et al., 2007; D'Amore et al., 2020; Garicia-Fuentes et al., 2015; Hugo & Hugo, 2015).

The phytochemicals found in plant extracts have been investigated as an alternative/replacement for chemical preservatives in meat products, as they contain antioxidant and antimicrobial properties (Hadidi et al., 2022; Hugo & Hugo, 2015; Mathenjwa et al., 2012; Shah et al., 2014). Studies at the University of the Free State have been exploring the use of natural products, such as rosemary extract and chitosan (Mathenjwa et al., 2012), Citrox® (van Schalkwyk et al., 2013) and commercial plant extracts and protective cultures (Freitag, 2023) as replacers of SO_2 in Boerewors. Although many of these natural preservatives did show excellent antimicrobial characteristics, the antioxidant effect of SO_2 has not yet been matched.

Rooibos and honeybush are well-known South African plants of which the extracts show antimicrobial and antioxidant characteristics. According to previous studies by McKay & Blumberg (2007) and Cullere et al. (2013), a larger percentage of polyphenols are found in green Rooibos than of fermented Rooibos since the fermentation process reduces the total polyphenols and changes the chemical composition of Rooibos. The plant extracts that were used in this study showed that the green Rooibos cold-water soluble extract contains a minimum of 30% polyphenols, while that of the Rooibos cold-water soluble extract contains a minimum of 24% polyphenols, anda minimum of 15% polyphenols was found in honeybush cold-water soluble extract (Rooibos Limited).

In the previous chapter, different concentrations of Rooibos, green Rooibos and honeybush extracts were evaluated in vitro, to determine which concentration of each of these plant extracts had the best antimicrobial effect on five strains each of *Escherichia coli* (*E. coli*), a Gram-negative bacterium, and *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium. It was found that 0.25% Rooibos, 2% green Rooibos and 0.5% honeybush showed the best antimicrobial activities against these strains.

In this chapter, therefore, the aim was to determine the preservative effect of these three plant extracts, at these concentrations, either on their own or in combination with lowered levels (100 mg/kg) of SO_2 in a Boerewors model in terms of their physico-chemical, microbial and sensory characteristics.

4.2. Materials and methods

4.2.1. Preservatives used in this study

Sulphur dioxide (SO_2) in the form of sodium metabisulphite was obtained from Crown National (Bloemfontein, South Africa). Rooibos extract (E1CCJ, cold-water soluble), green Rooibos extract (E2CCJ, cold-water soluble) and honeybush extract (E4CCJ, cold-water soluble), were obtained from Rooibos Limited (Clanwilliam, South Africa) in powder form.

4.2.2. Boerewors preparation and formulation

Fresh meat was bought at a butchery in the Bloemfontein District. Preparation of the meat was done in the meat laboratory at the University of the Free State according to standard procedures for Boerewors production as proposed by the South African Department of Health (SA DoH, 1990). The standard meat and spice formulation (SMSF) for all the Boerewors models in this study was as follows: a total meat content of 90% (w/w), which consisted of 60% (w/w) lean meat (beef 90/10), 15% (w/w) pork 90/10 and 15% (w/w) pork backfat; white spirit vinegar (1.50% w/w); water (3.40% w/w); Worcestershire sauce (0.38% w/w); ground coriander (0.5538% w/w), ground black pepper (0.1620% w/w), monosodium glutamate (MSG) (0.1755% w/w), ground nutmeg (0.1350% w/w), ground cloves (0.0400% w/w), rubbed thyme (0.0270% w/w), dextrose (0.1004% w/w), ascorbic acid (0.0062% w/w) and sodium chloride (1.5001% w/w). The preservatives and cereal binder (Addpro fine cereal binder, B.T. Enterprises) which were added to the SMSF to obtain the different treatments in this study, are indicated in Table 4.1. Sulphur dioxide (450 mg/kg) was used as the positive control. The green Rooibos extract (2% w/w), honeybush extract (0.50% w/w), Rooibos extract (0.25% w/w) and combinations of each of these plant extracts individually, with lowered levels (100 mg/kg) SO_2, were used as the test treatments.

Table 4.1

The eight preservative treatments used in this study.

Treatment	Ingredients and Preservatives
NC	SMSF + cereal binder (2.0175% w/w) (negative control)
S450	SMSF + cereal binder (1.9475% w/w) + SO_2 (0.07% w/w) (positive control)
GR2	SMSF + cereal binder (0.0175% w/w) + Green Rooibos (2.00% w/w)
H050	SMSF + cereal binder (1.5175% w/w) + Honeybush (0.50% w/w)
R025	SMSF + cereal binder (1.7657% w/w) + Rooibos (0.25% w/w)
GR2 + S100	SMSF + cereal binder (0.0000% w/w) + Green Rooibos (2.00% w/w) + SO_2 (0.0175% w/w)
H050 + S100	SMSF + cereal binder (1.5000% w/w) + Honeybush (0.50% w/w) + SO_2 (0.0175% w/w)
R025 + S100	SMSF + cereal binder (1.7500% w/w) + Rooibos (0.25% w/w) + SO_2 (0.0175% w/w)

SMSF, Standard meat and spice formulation

The procedure for production of the different Boerewors treatment sausages, entailed mixing the Worcester sauce, vinegar and spice mixture containing the cereal binder and respective preservatives with ice water and leaving it to stand for 5 min, allowing for hydration. This mixture was then added to each 2 kg batch of meat, mixed and minced through a 4.5 mm mincing plate (number 32 Okto mincer, Crown National). The minced meat was then filled into hog casings (Gold Crown, Crown National) with a manual sausage filler (Tre-Spade filler, Crown National,). This resulted in a roll of sausage that was cut into 100 g pieces. Each of these 100 g sausages was put onto polyester trays containing an absorbent pad, which were then overwrapped with a polyvinyl chloride film. For each treatment, six trays were prepared for each treatment and stored under fluorescent light at 4 °C (refrigeration conditions) for six days and an additional two trays were frozen at -18 °C for product lipid stability analysis after 90 days of storage.

The experimental design is indicated in Fig. 4.1. For the microbial and chemical analysis, three replicates of Boerewors were manufactured, with different batches of meat bought within different months. This was done to compensate for microbial variations in the raw materials. One replicate of Boerewors consisted of 2 kg batches of meat for each of the eight treatments. Four samples per treatment were used and were sampled on days 1, 3 and 6. Two sausages of each treatment were also sampled for analysis after being frozen for 90 days. A fourth replicate was manufactured for sensory evaluation and for determining thaw-, cooking- and total losses, however, 3 kg batches of meat were prepared for each treatment group.

4.2.3. Physico-chemical analysis

4.2.3.1. pH measurement

The pH was measured directly using a direct pH measurement probe (Model MA920, Milwaukee Instruments, Rock Mount, USA), coupled to a pH meter (Thermo Scientific, Orion 3-Star Plus Model, Labotec, Midrand, SA) to record quadruple pH measurements, per treatment group, per replicate, at room temperature. Each day before use, the pH meter was calibrated with standardised buffers (Merck, Johannesburg, SA) with pH values of 4.01 and 7.00, respectively.

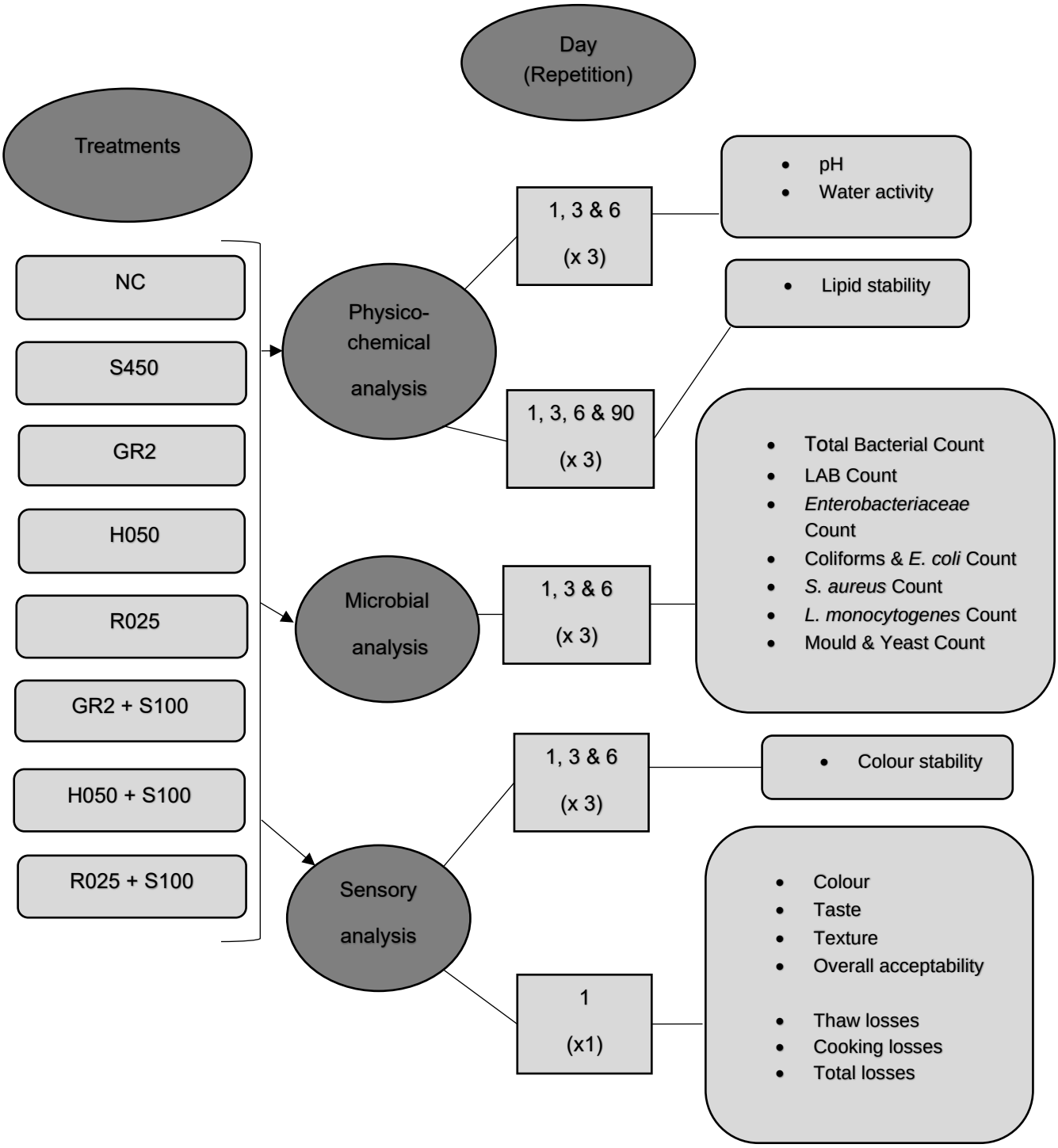

Fig. 4.4. Experimental design of the Boerewors sampling procedure. Microbial analysis = 3 replicates which were done in duplicate; Chemical analysis = 3 replicates which were done in duplicate; sensory analysis = colour (3 replicates were done in duplicate), sensory evaluation of attributes were done once, as well as moisture loss of the treatments.

4.2.3.2. Water activity (a_w)

A homogenously mixed sample was filled into a a_w container (height of 5 mm and diameter of 39 mm) to the appropriate level. The a_w was determined, using a Novasina Thermoconstanter TH 200 (Labotec, Midrand, SA) water activity meter. After equilibrium was reached with deionised distilled water, duplicate measurements per treatment group, per replicate, were taken at a temperature of 25 °C. The results were reported as percentage relative humidity (% rH) and converted to a_w values, by dividing each value by a factor of a 100.

4.2.3.3. Lipid stability

Five grams of Boerewors sample from each 100 g portion of sausage from each treatment were used in the analysis of thiobarbituric acid reactive substances (TBARS) using the aqueous extraction method of Raharjo, Sofos, & Schmidt (1992) to determine the effect of the preservative on the lipid stability of these meat products. TBARS were measured on days 0, 3 and 6 of storage at 4 °C. TBARS were also measured after 90 days of storage at -18°C. Moisture content (%) was determined by oven drying overnight at 121 °C and used as a second establishing parameter (AOAC, 2005) as it was required in the calculation of TBARS.

4.2.4. Microbial analysis

A 10 g Boerewors sample (area chosen randomly)from each 100 g product was aseptically weighed and placed into a WhirlPak™ bag (Lasec, Bloemfontein, SA), 90 ml of a sterile 0.1 M phosphate buffer was added and stomached (AME Stomacher Lab-Blender 400, Labotec. Johannesburg) for 1 min. For each of these samples, a dilution series (10^{-2} to 10^{-7}) was prepared using McCartney bottles containing 9 ml sterile 0.1 M phosphate buffer (Harrigan, 1998). These dilutions were plated on various media as follows using the pour plate method unless otherwise specified.

4.2.4.1. Total bacterial count

Standard plate count agar (SPCA, Oxoid 0463) was used for total bacteria count and incubated at 32 °C for 48 h. All colonies were enumerated using a colony counter (J-3 colonymeter; CJ Labs, Bredell, South Africa).

4.2.4.2. Lactic acid bacteria (LAB) count

De man, Rogosa, Sharpe broth (M.R.S. Broth; Oxoid CM0359) with 1.5% (w/v) agar (Oxoid LP0011) was used for lactic acid bacteria enumeration and the plates were incubated at 32 °C for 48 h (Harrigan, 1998). All colonies were enumerated using a colony counter (J-3 colonymeter; CJ Labs, Bredell, South Africa).

4.2.4.3. Enterobacteriaceae count

Violet red bile glucose agar (Oxoid; CM0485) was used for *Enterobacteriaceae* enumeration. A double layer of agar was poured and was incubated at 37 °C for 24 h (Harrigan, 1998). All colonies were enumerated using a colony counter (J-3 colonymeter; CJ Labs, Bredell, South Africa).

4.2.4.4. Coliform count and Escherichia coli

Violet red bile lactose agar + 4-methylumbelliferyl-β-D-glucuronide (VRBA + MUG; Oxoid CM0978) was used for total coliform and *Escherichia coli* (*E. coli*) counts and the plates were incubated at 37 °C for 24 h. All colonies were enumerated using a colony counter (J-3 colonymeter; CJ Labs, Bredell, South Africa). The presence of *E. coli* on VRBA plates was confirmed by fluorescence under ultraviolet light (366 nm; CAMAG Universal UV Lamp) (Harrigan, 1998).

4.2.4.5. Staphylococcus aureus count

Baird-Parker agar (BPA; Oxoid CM0275) was used for *Staphylococcus aureus* enumeration and the plates were incubated at 37 °C for 24 – 48 h. *Staphylococcus aureus* typically forms colonies that are 1.0–1.5 mm in diameter, black, shiny, convex with a narrow white entire margin and surrounded by clear zones extending 2–5 mm into the opaque medium (Harrigan, 1998).

4.2.4.6. Listeria monocytogenes count

RAPID'L. *mono* detection agar (Biorad 356-4293; AEC-Amersham; Johannesburg, South Africa) was used with two supplements, namely reconstituted RAPID'L. *mono* Supplement 1 (Biorad 3564294) and RAPID' *Listeria* spp. Supplement 2 (Biorad 3564746) for the *Listeria monocytogenes* (*L. monocytogenes*) enumeration and the plates were incubated at 37 °C for 24 h. Light blue colonies were identified as *Listeria monocytogenes* colonies.

4.2.4.7. Yeast and Mould counts

Rose-bengal chloramphenicol agar (RBCA; Oxoid CM0549) with chloramphenicol supplement (SR0078) was used for yeast and mould enumeration and the plates were incubated at 25 °C for 4 days. All colonies were enumerated using a colony counter (J-3 colonymeter; CJ Labs, Bredell, South Africa).

All these counts were used as common potential microbial, pathogenic, and spoilage parameters associated with this type of product (Harrigan, 1998).

4.2.5. Sensory analysis

4.2.5.1. Colour stability

For colour analysis, on days 1, 3 and 6, the sausage samples were sliced through after a sample for microbial analysis was aseptically removed. The sausage sample was left to bloom for 30 min before taking of the measurements. The colour of each sample was measured six times using a Minolta CR 400 chromometer (8 mm measuring area). The CIE *L*a*b** colour scale was used for comparison, where *L** represents lightness, *a** represents redness/greenness and *b** represents yellowness/blueness (Ripoll et al., 2001).

4.2.5.2. Sensory quality

For sensory analysis, packets of Boerewors from the eight treatments were removed from the freezer and defrosted in a refrigerator at 4 °C, one day before it was to be evaluated. Boerewors from a specific treatment was pan-fried (Sunbeam, Model SPES-3038A, Catro Foodservice Solutions) to an internal temperature of above 72 °C, removed from the frying pan, cut into 25 mm bite-sized samples and kept warm in glass bowls in a pie warmer (ANVIL, Model PWK0004, Catro Foodservice Solutions). The approximate temperature of each Boerewors sample at the time of tasting was 60 °C.

A 73-member consumer panel, made up of students and staff from the Agricultural Building of the University of the Free State, ages ranging from 19 to 60 years, both men and women, was used to taste/evaluate and give an acceptability opinion on the cooked Boerewors samples from the eight treatments. A nine-point hedonic scale ranging from 1, for dislike extremely, up to 9, for like extremely, was used to score colour, taste, texture and overall liking of these cooked Boerewors treatments (Table 4.2). Respondents were asked to respond to the question “How much do you like or dislike the sample?”

Table 4.2

Nine-point hedonic scale used in this study for sensory analysis (Lawless & Heymann, 1998; Stone & Sidel, 2004).

OVERALL LIKING								
1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

The Boerewors samples were coded with randomized, three-digit codes and rotated to prevent bias. The tasting of the eight treatments was done in two sessions (five treatments for each session, with controls included) over a period of two days. At each session, each respondent received a 25 mm piece of Boerewors per treatment, from five treatments. The following session included the last three treatments, as well as the positive and negative control samples. Testing was done in individual booths and at an ambient temperature of 20–22 °C. Chilled water was provided as a palette cleanser.

4.2.5.3. Thaw and cooking losses

Twelve sausages of each treatment group were kept frozen at −18 °C for 3 days. After 3 days, the samples were kept at 4 °C for 24 h, to allow it to thaw gradually, after which the samples were removed from their packaging and weighed. The sausages were then cooked in a frying pan with a litre of water, pre-heated to 150 °C. During cooking, the temperature was reduced to 120 °C after 10 minutes. The sausage was turned every 5 min. The sausages were cooked until an internal temperature of 72 °C was reached. The sausages were removed from the frying pan and air-cooled to room temperature before being weighed again.

Thaw loss was calculated with the formula:
Thaw loss (%) = [(initial weight – weight after thawing) / initial weight] x 100

Cooking loss was calculated with the formula:
Cooking loss (%) = [(weight after thawing – weight after cooking) / weight after thawing] x 100

The total loss percentage was calculated as the sum of the thaw and cooking losses.

4.2.6. Statistical analysis

All data were collected in Excel spreadsheets. Statistical analyses were conducted using XLSTAT (2023) and SPSS (2022). A significance level of $\alpha = 0.05$ was used. To check the assumptions of normality and homoscedasticity, QQ-plots, Shapiro-Wilk's test (for normality), and Levene's test (for homoscedasticity) were employed (Agresti, 2017). Given the exploratory nature of the data analysis, the assumptions underpinning specific methods, namely ANOVA (Agresti, 2017) and the General Linear Model, were handled with a more lenient approach.

The microbial counts obtained of total bacteria, lactic acid bacteria, *Enterobacteriaceae*, yeasts and moulds, coliforms, *E. coli* and *S. aureus* for each treatment, were transformed to log cfu/g. A General Linear Model was fitted to the transformed values.

The results for pH, water activity and colour measurements were subjected to analysis of GLM (linear regression) to model the effect of day, treatment, and the day with treatment interaction. The follow up analysis was an ANOVA for each day to break down the effect. This allowed the evaluation of the simple effect at each time point. A Post-Hoc Tukey-HSD test was then conducted to determine specific differences (Agresti, 2017).

General Linear Model (Linear regression) was also used for the analysis of the effects in TBARS. Analysis was performed on day, treatment, and day with treatment interaction. For further analysis of simple effects, ANOVA statistical analysis was conducted per treatment over day and then per day over treatment (Agresti, 2017).

The sensory analysis data was collected using Compusense Inc (2023). The goal of the sensory analysis was to determine liking of the product with the use of hedonic scales (9-point scale of liking). After the data was collected, an ANOVA statistical analysis was conducted, followed by a Tukey-HSD test.

For thaw-, cooking- and total losses, an analysis of variance (ANOVA) or non-parametric Kruskal-Wallis was used for comparison between the treatments, with their respective post-hoc tests (Agresti, 2017).

4.2.7. Ethical clearance

Ethical clearance was obtained from the General Human Research Ethics Committee of the University of the Free State, Bloemfontein, with clearance number UFS-HSD2020/0539/0809/21/22.

4.3. Results and discussions

4.3.1. Physico-chemical evaluation

4.3.1.1. Effect of treatment on pH

The results obtained for the pH values of the eight Boerewors treatments are presented in Table 4.3. In this study, no significant differences were observed in the pH between all the treatments on days 1, 3 and 6. The pH of all the treatments ranged from 5.432 on day 1 (GR2 + 100) to 5.280 on day 6 (R025). Hence, all the treatments on all the days were in the range of 5.2 to 5.7, which is the normal pH of meat (Hugo & Hugo, 2015). The findings, therefore,

indicated that the eight different treatments did not have an influence on the pH of the Boerewors samples which could have had an effect on the microbial results found in this study.

Table 4.3

The pH values (mean pH ± Standard deviation, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

	Mean pH ± Standard deviation (n = 12)		
Treatment	**Day 1**	**Day 3**	**Day 6**
NC	5.531 ± 0.093	5.400 ± 0.099	5.278 ± 0.166
S450	5.517 ± 0.097	5.413 ± 0.142	5.406 ± 0.130
GR2	5.439 ± 0.112	5.360 ± 0.120	5.303 ± 0.100
H050	5.502 ± 0.058	5.395 ± 0.105	5.326 ± 0.118
R025	5.498 ± 0.104	5.383 ± 0.118	5.280 ± 0.130
GR2 + S100	5.432 ± 0.091	5.318 ± 0.103	5.325 ± 0.071
H050 + S100	5.472 ± 0.082	5.373 ± 0.125	5.380 ± 0.082
R025 + S100	5.488 ± 0.073	5.391 ± 0.130	5.413 ± 0.100
p-value	0.092	0.634	0.160

Means with different superscripts in the same column differed significantly ($p < 0.05$).

4.3.1.2. Effect of treatment on water activity (a_w)

The a_w of the eight treatments on days 1, 3 and 6, are indicated in Table 4.4. On days 1 and 6, there were no significant differences ($p = 0.279$ and $p = 0.313$, respectively) between the treatments. All the treatments had an a_w between 0.94 and 0.95, which correlated with the a_w results for Boerewors in another study (Mathenjwa et al., 2012). The a_w of all the treatments were above 0.91, which is the minimum water activity needed for most bacteria to survive in meat products (Mathenjwa et al., 2012).

On day 3, however, the S450 and GR2 treatments had the significantly ($p = 0.024$) lowest a_w when compared to NC. Certain flavonoids in green Rooibos, contain many hydroxyl groups in the ring of their structure (Makarewicz et al., 2021). The availability of many hydroxyl groups for hydrogen binding reduces water activity (Damodaran et al., 2008). The low a_w of GR2 may be attributed to the availability of hydroxyl groups in the flavonoids for hydrogen binding in the Boerewors.

Table 4.4

The water activity (mean a_w ± Standard deviation, n = 12) of the eight Boerewors treatments on days 1, 3 and 6 stored at 4 °C. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

	Mean a_w ± Standard deviation (n = 12)		
Treatment	**Day 1**	**Day 3**	**Day 6**
NC	0.952 ± 0.007	0.952^{b} ± 0.008	0.952 ± 0.011
S450	0.941 ± 0.007	0.936^{a} ± 0.015	0.937 ± 0.016
GR2	0.944 ± 0.007	0.938^{a} ± 0.014	0.943 ± 0.013
H050	0.944 ± 0.004	0.941^{ab} ± 0.010	0.946 ± 0.010
R025	0.943 ± 0.009	0.944^{ab} ± 0.010	0.945 ± 0.014
GR2 + S100	0.944 ± 0.013	0.944^{ab} ± 0.012	0.939 ± 0.016
H050 + S100	0.944 ± 0.013	0.944^{ab} ± 0.009	0.941 ± 0.0018
R025 + S100	0.944 ± 0.014	0.946^{ab} ± 0.009	0.941 ± 0.020
p-value	0.279	0.024	0.313

Means in the same column with different superscripts, differed significantly ($p < 0.05$).

4.3.1.3. Lipid stability

TBARS analysis was used for the measurement of the degree of lipid oxidation and was expressed as mg MDA per kg meat (Boles & Parrish, 1990). The results of the TBARS of the treatments on days 1, 3 and 6 with storage at 4 °C, are indicated in Fig. 4.2, while Fig. 4.3 indicates the lipid stability of the Boerewors treatments on days 1 and 90 after storage at -18 °C.

There were no significant differences ($p = 0.985$) in the TBARS between all the treatments on day 1 ($p = 0.985$), 3 ($p = 0.965$) and day 6 ($p = 0.991$) with values ranging between 0.261 mg MDA/kg (day 1) and 0.726 mg MDA/kg (day 6) (Fig. 4.2). According to a study by Boles & Parrish (1990), rancid-off flavours become noticeable with TBARS-values greater than 1 mg MDA/kg of meat. According to a study by van Schalkwyk (2013) on Boerewors, a value < 1 mg MDA/kg of meat, showed good lipid stability. Therefore, all the treatments in this study showed good lipid stability with storage at 4 °C with TBARS values < 1 mg MDA/kg on days 1, 3 and 6. Although there were no significant differences between the treatments and the days, it was interesting to note that TBARS were increased in all the treatments that contained

SO_2, while the plant extract treatments showed better antioxidant activity. The presence of polyphenols, which is high in antioxidants, may be the reason for the lower values. Flavonoids and phenolic acids scavenge free radicals by donating a hydrogen atom to free radicals and by chelation of metal ions like Zn^{2+}, Fe^{3+}, Fe^{2+} and Cu^{2+} (Efenberger-Szmechtyk et al., 2012; Hadidi et al., 2022; Kumar et al., 2015). The antioxidant activity of polyphenols increases when multiple – OH groups are present (Hadidi et al., 2022; Makarewicz et al., 2021). According to McKay & Blumberg (2007), the aspalathin in green Rooibos is more effective in the scavenging of oxygen and radicals, than α- tocopherol, BHA and BHT. This may explain the lower TBARS of GR2, although insignificant.

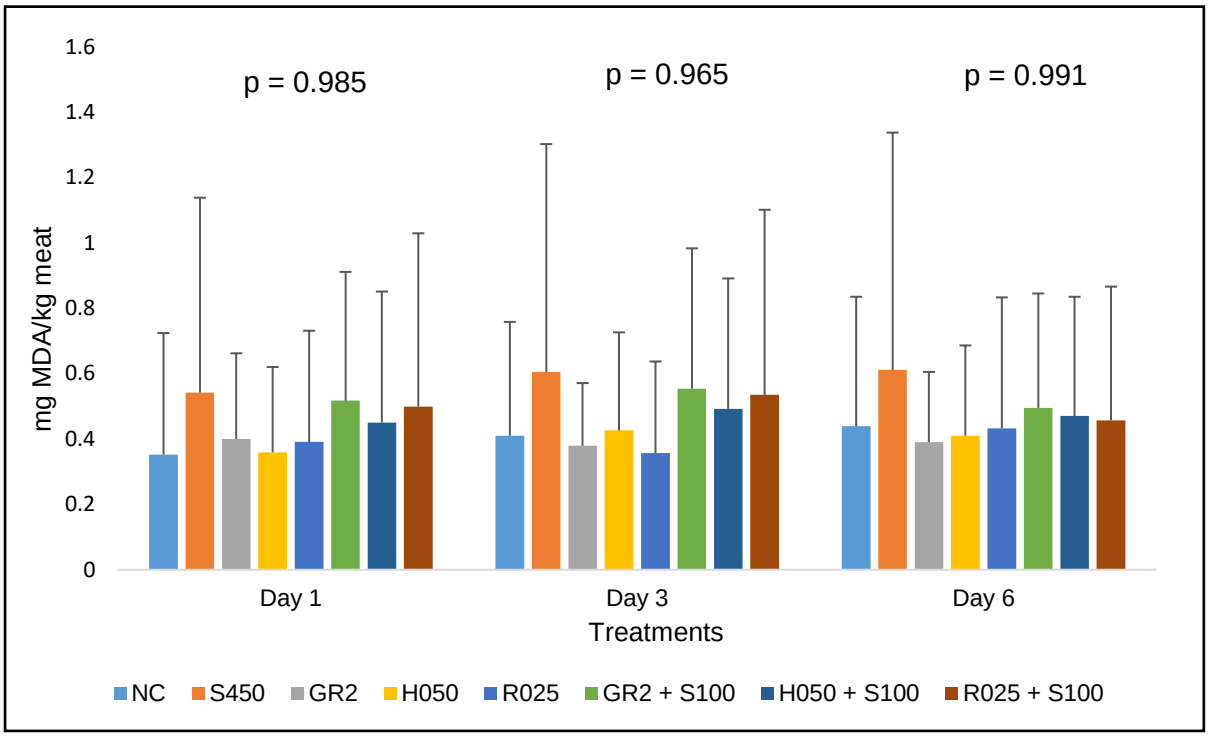

Fig. 4.2. TBARS values (mean mg MDA/kg meat; n = 6) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

No significant differences (p = 0.582) between the treatments were also observed after 90 days of storage at -18 ºC (Fig. 4.3). All the TBARS values were below the < 1 mg MDA/kg limit for good lipid stability (van Schalkwyk et al., 2013), except for the NC treatment, that had the

highest TBARS (1.016 mg MDA/kg meat). Since the NC contained no preservative, the oxidation of meat lipids is expected to increase with time.

Although there was no significant difference between the treatments (Fig. 4.3), GR2 had the lowest TBARS on day 1 (0.400 mg MDA/kg meat) and day 90 (0.494 mg MDA/kg meat). According to Snijman et al. (2009), lipid peroxidation was found to be inhibited the most by quercetin and aspalathin, which are found in green Rooibos. Other studies also reported aspalathin as an effective oxygen and free radical scavenger found in green Rooibos (McKay & Blumberg, 2007). This may be the reason for the lowest value obtained by GR2.

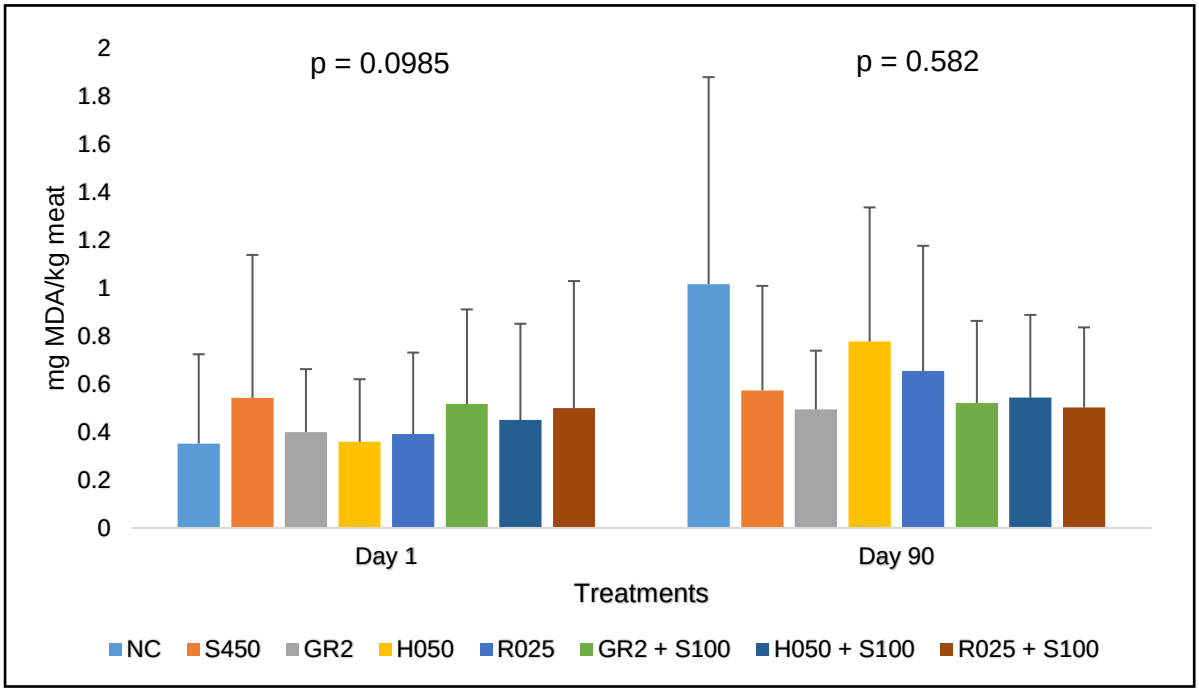

Fig. 4.3. TBARS values (mean mg MDA/kg meat; n = 6) of eight Boerewors treatments on days 1 and 90 stored at -18 °C. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

The results obtained for TBARS showed that the use of plant extract treatments had lower lipid oxidation compared to the NC on day 90, although there were no significant differences. Thus, the oxidation of lipids in meat have indeed been reduced by only the presence of plant extracts. The antioxidant activity may be attributed to the presence of polyphenols in Rooibos (for e.g. quercetin, orientin, catechin, vanillic acid, ferulic acid, caffeic acid, luteolin, p-Hodroxybenzoic acid) and honeybush (kaempherol, hesperetin etc.), depending on its

structure (Markarewicz et al., 2021). The only known polyphenols that were demonstrated to be in both Rooibos and honeybush, were luteolin and eriodictyol (McKay & Blumberg, 2007).

4.3.2. Microbial analysis

4.3.2.1. Total bacterial count (TBC)

The total bacterial count of the Boerewors treatments on days 1, 3 and 6 are given in Fig. 4.4. There was no significant difference ($p = 0.139$) in the total bacterial count between all the treatments on day 1, with counts ranging between 6.023 and 6.543 log cfu/g. The NC treatment, which had no preservatives, had the highest count (6.543 log cfu/g ± 0.384), as can be expected as no preservative was present to inhibit the microbial growth.

On day 3, treatment S450 had the lowest total bacterial count (6.046 ± 0.549 log cfu/g) which differed significantly ($p < 0.001$) from treatment NC having the highest count (6.899 ± 0.296 log cfu/g). The combination of lowered levels of SO_2 (100 mg/kg) with GR2 and H050 were, similarlyto S450, significantly ($p < 0.001$) lower than NC. Sulphur dioxide is known for its antimicrobial activity, as it causes cell death of many micro-organisms (Garcia-Fuentes et al., 2015; Walker, 1985). The plant extract treatments also showed some antimicrobial activity, as the TBC was lower than NC on day 3. However, there were no significant differences when compared to the NC and S450 (Fig. 4.4).

Polyphenols compounds such as flavonoids (rutin, luteolin, quercetin, isoquercetin), flavones (vitexin, isovitexin, orientin, isoorientin) are found in Rooibos, and xanthones (mangiferin), flavonones (hesperidin, and hesperetin) are found in honeybush, may reduce the TBC in a few ways. According to Markarewicz et al. (2021), antimicrobial activity of polyphenols against micro-organism can differ, as it depends on the species of the bacteria as well as the type of polyphenol present. Phenolic acids are either able to interact with the cell wall of most bacteria, suppress the formation of the cell biofilm, damage the cytoplasmic membrane by altering its function or by the reaction with proteins (Markarewicz et al., 2021).

GR2 + S100 and H050 + S100 had the significantly ($p < 0.001$) lowest TBC on day 6 when compared to NC (Fig. 4.6). However, it did not differ significantly from the S450, GR2 and the R025 + S100. Green Rooibos and honeybush extracts have antioxidant activity, as well as antimicrobial activity, because of the presence of flavonoids and other phenolic compounds (Kamara et al., 2003). Hence, it will reduce the microbial growth and with combinations with low levels of SO_2, the antimicrobial action increases. On day 6, NC had the highest TBC and differed significantly ($p < 0.001$) from all the other treatments, as it had a count of log 7.450 cfu/g ± 0.768.

According to Shapton & Shapton (1991), the microbial limit for TBC of raw sausages, is 7 log cfu/g. None of the treatments exceeded this limit, except for NC on day 6.

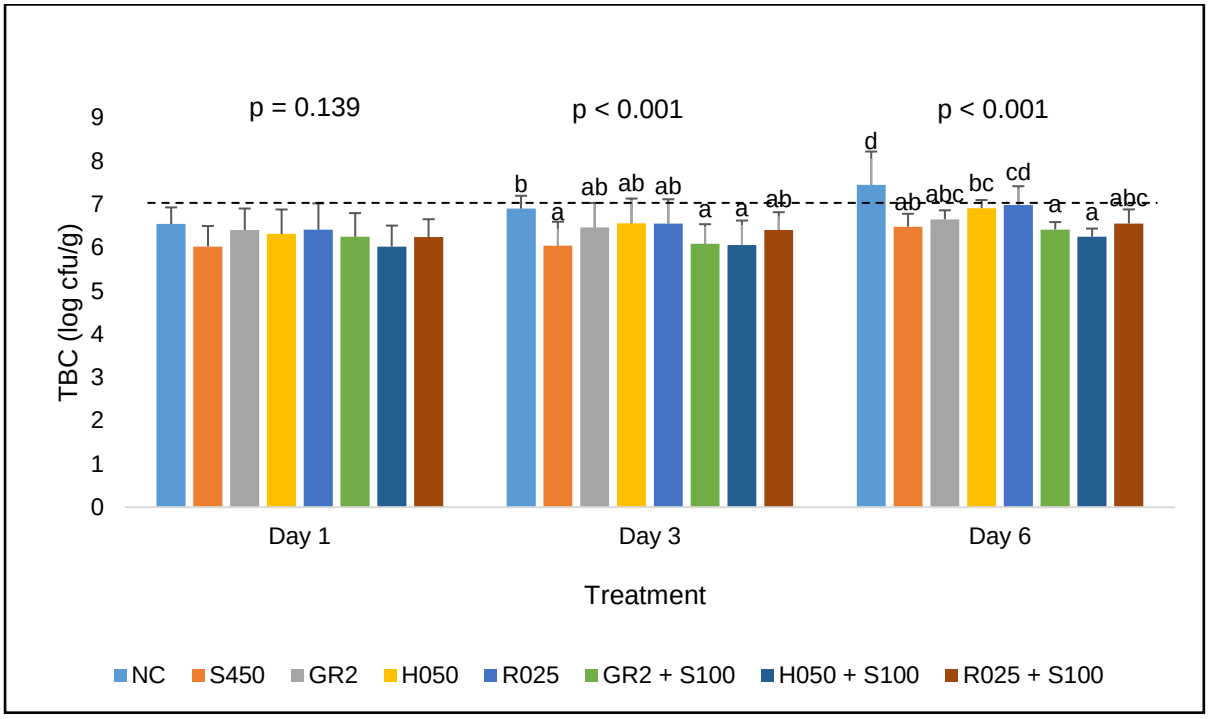

Fig. 4.4. Total bacteria count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly ($p < 0.05$). The dotted line represents the microbial limit of < 7 log cfu/g for TBC (Shapton & Shapton, 1991).

4.3.2.2. Lactic acid bacteria (LAB)

Lactic acid bacteria are Gram-positive bacteria that contribute to the spoilage of fresh meat or, with certain species, serve as a protective agent by inhibiting other microbial spoilage micro-organisms (Pothakos et al., 2015). On day 1, no significant ($p = 0.051$) differences were observed between any of the treatments (Fig. 4.5). The LAB counts ranged between 4.226 log cfu/g and 4.752 log cfu/g. According to Carballo et al. (2019), when LAB counts reached more than 7 to 8 log cfu/g, off-flavours were detected in fresh pork sausages. The results obtained in this study did not reach 7 log cfu/g.

On day 3, treatment S450 had the significantly ($p < 0.001$) lowest LAB count of all the treatments, but S450 did not differ significantly from treatments GR2 + S100 or H050 + S100 (Fig. 4.5). The natural preservative, GR2, also gave comparable results to GR2 + S100 and H050 + S100. These findings on day 3 were proof of the antimicrobial action of SO_2 on Gram-positive bacteria. Sulphiting agents are known to cause several changes in the protein conformation by the formation of S-sulphonates, which leads to the inactivation of the organisms' life cycle (D'Amore et al., 2020). Also, the good preservative action of GR2 in this study against LAB could possibly be associated with the amount of hydroxyl groups in the B-ring in flavones and flavonols. Rooibos contain quercetin with more hydroxyl groups (Makarewicz et al., 2021).

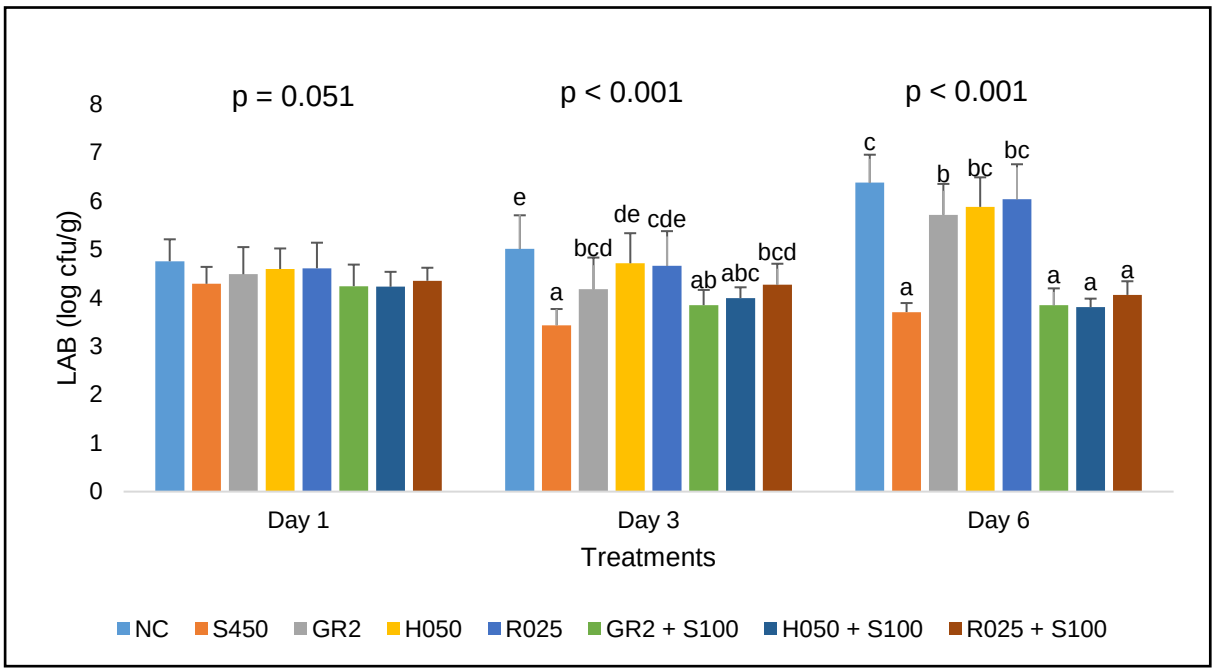

Fig. 4.5. Lactic acid bacteria count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 °C. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly ($p < 0.05$).

On day 6, treatments S450, GR2 + S100, H050 + S100 and R025 + S100 differed significantly ($p < 0.001$) from the rest of the treatments. This may be due to the presence of SO_2, which inhibits LAB more effectively (D'Amore et al., 2020). There was an increase from day 1 to day

6 in the LAB count of the plant extract treatments. This may be expected, as LAB growth tends to increase at a lower pH. Plant extracts may aid in the increase of LAB counts in meat, as phenolic acids may reduce the pH of meat as well, which provides a tolerable environment for LAB to grow (Makarewicz et al., 2021). Treatment NC differed significantly ($p < 0.001$) from all the treatments, by having the highest LAB count (6.372 log cfu/g ± 0.578).

4.3.2.3. Enterobacteriaceae

Enterobacteriaceae is used as indicator for good hygienic practices (Mladenović et al., 2021). The *Enterobacteriaceae* count of the eight Boerewors treatments on days 1, 3 and 6, are represented in Fig. 4.6. There were no significant differences on days 1 ($p = 0.751$) and 3 ($p = 0.199$) between any of the eight treatments. However, there was a significant difference ($p < 0.001$) between the treatments on day 6. The *Enterobacteriaceae* counts of the S450 and H050 + S100 were significantly ($p < 0.001$) lower than the NC.

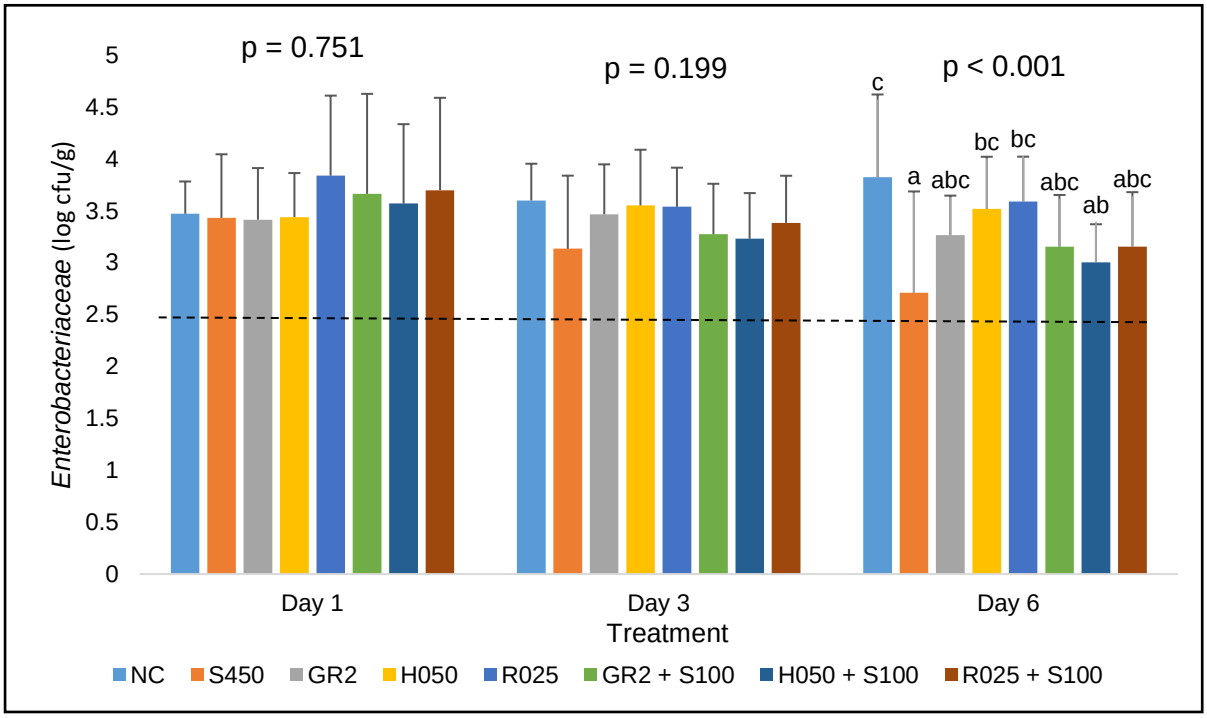

Fig. 4.6. *Enterobacteriaceae* count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ^{o}C. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly ($p < 0.05$). The dotted line represents the microbial limit of 2.5 log cfu/g for *Enterobacteriaceae* in fresh sausages (SA DAFF, 2018).

Sulphites have demonstrated the ability to reduce microbial growth, due to the reaction of disulfide bonds with the thiol groups of microbial enzymes/proteins, which causes the formation of S-sulphonates, responsible for the conformational changes of bacteria that leads to cell death (D'Amore et al., 2020). Honeybush contains kaempherol, while Rooibos contains catechin, which are known as antioxidants (Makarewicz et al, 2021). According to D'Amore et al. (2020), sulphite gets depleted when formation of organic sulfonates might occur in meat, but with the co-addition of chelating agents (scavengers or antioxidants), the loss of sulphites was prevented. This might explain the lower counts of the plant extract treatments combined with low SO_2, as the antioxidant containing compounds in these two plant extracts may prevent the loss of sulphur dioxide, which will increase the antimicrobial action.

According to SA DAFF (2018), the count for *Enterobacteriaceae* should not exceed 2.5 log cfu/g in meat. All the treatments exceeded this limit. This may be due to the initial microbial load that was present on the meat when bought at the butchery.

4.3.2.4. Coliforms

No significant differences were observed in the coliform counts of the eight treatments on days 1 ($p = 0.866$) and 3 ($p = 0.375$) (Fig 4.7). All the treatments were just below the maximum allowable microbial limit of 4 log cfu/g (SA DAFF, 2018; Shapton & Shapton, 1991).

On day 6, S450 had significant ($p < 0.001$) lower counts than the NC, but the counts did not differ from GR2 + S100, H050 + S100 and R025 + S100. This suggests that the treatments containing SO_2, were more effective in decreasing the coliform count than the treatments containing only plant extracts. The NC treatment had the highest coliform count of all the treatments and exceeded the coliform count limit of < 4 log cfu/g on day 6. This suggested that the plant extract treatments did show inhibition towards microbial growth. Coliforms are an indication of whether proper hygienic practices occurred in all stages of handling the meat (Shapton & Shapton, 1991).

4.3.2.5. Escherichia coli

Escherichia coli is used as an indicator organism of faecal contamination of food (Rock et al., 2019). This organism may be harmless commensals of humans and animals, but some species may be pathogenic. It should normally be absent in meat products, or the presence thereof should be no more than 2 log cfu/g (100 cfu/g) in meat (SA DAFF, 2018).

All the treatments over the 6-day storage period at 4 ^{o}C, showed *E. coli* counts (Fig. 4.8). However, all these counts were lower than the limits suggested (SA DAFF, 2018). The S450, GR2, H050 + S100 and R025 + S100 had significantly ($p < 0.001$) lower *E. coli* counts than the NC. Kaempherol (flavonone) found in honeybush, possesses antibacterial activity (McKay

& Blumberg, 2007). According to Makarewicz et al. (2021), a study has shown that flavonoids enter the hydrophobic core of membranes (lipid bilayer). Kaempherol was one of the flavonoids that decreased membrane fluidity of *E. coli* the best.

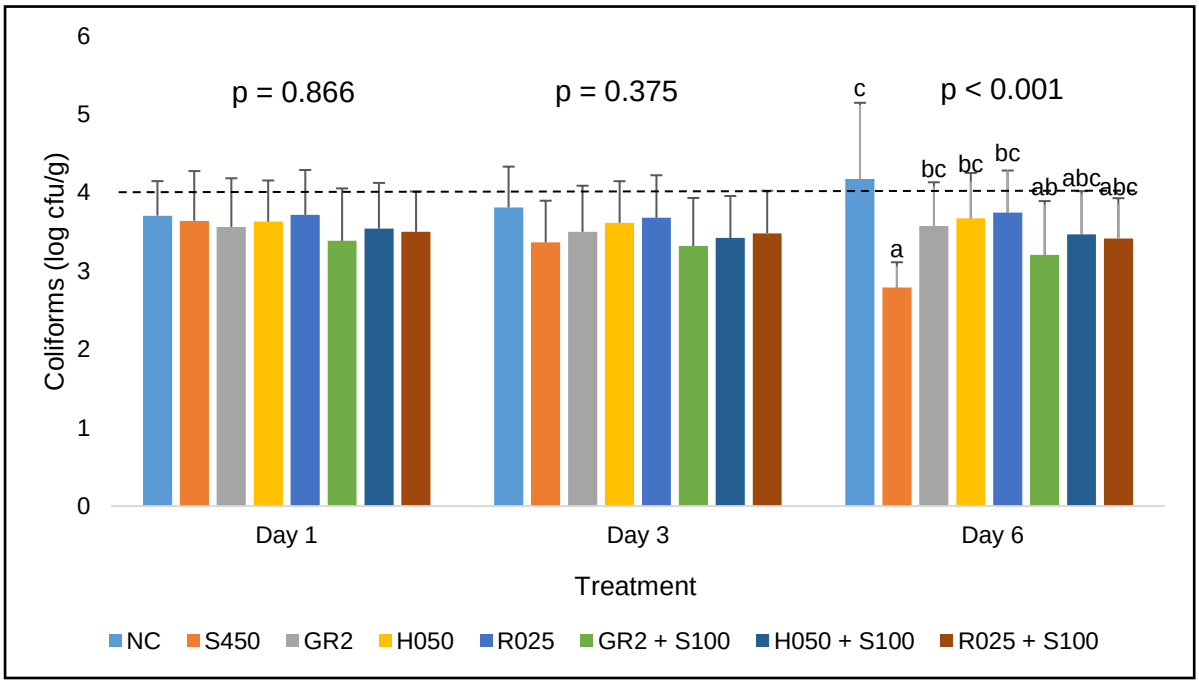

Fig. 4.7. Coliform count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 °C. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly ($p < 0.05$). The dotted line represents the microbial limit of < 4 log cfu/g for coliforms in raw sausages (Shapton & Shapton, 1991).

According to D'Amore et al. (2020), by means of passive transport, sulphiting agents enter the cell membrane of micro-organisms, which leads to inactivation or even cell death. This is carried out by the reaction of disulfide bonds with the thiol groups of the enzymes/proteins of micro-organisms. The use of sulphite with polyphenols, may provide a synergistic effect by preventing the loss of SO_2, which may increase the antimicrobial activity against *E. coli*. As the co-addition of chelating agents, scavenger compounds and antioxidants prevent the formation of sulphate ions in meat that causes the loss of SO_2 (D'Amore et al., 2020). According to a study by Roller et al. (2002), Gram-negative bacteria were inhibited by using the combination of low sulphite and chitosan in pork sausages. There were no significant

differences in the *E. coli* counts between all the treatments on days 3 and 6 (p = 0.474 and p = 0.419, respectively).

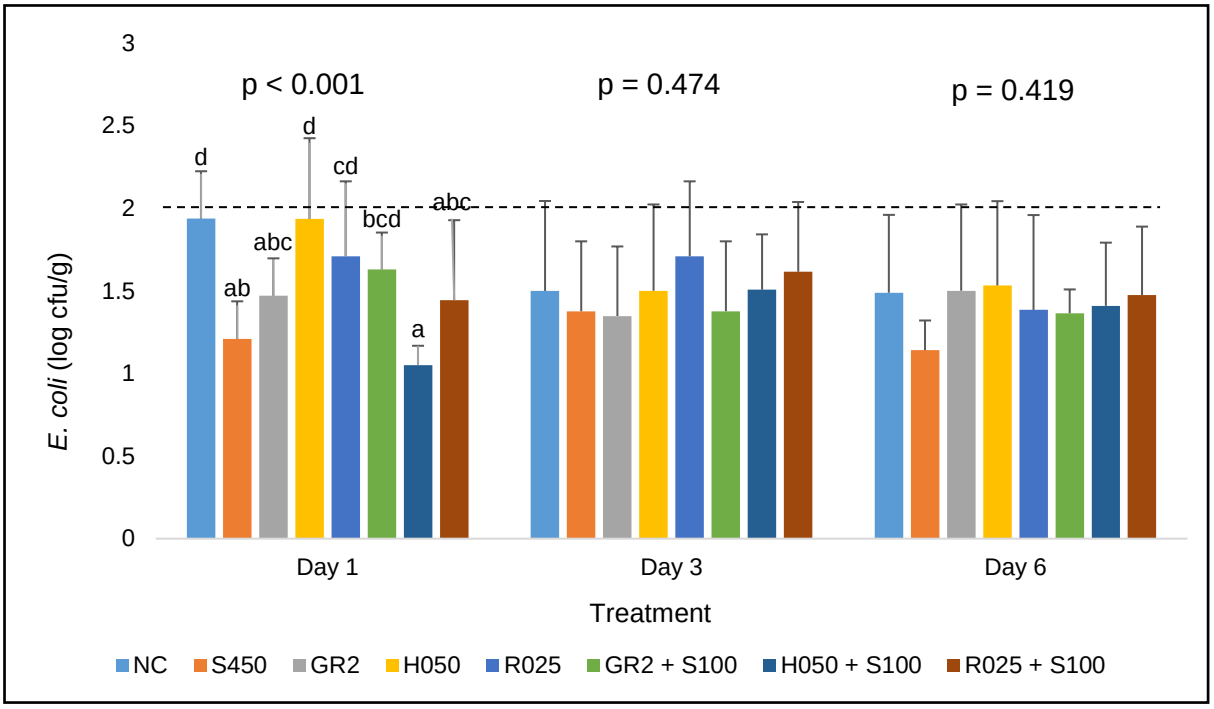

Fig. 4.8. *Escherichia coli* count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly (p < 0.05). The dotted line represents the microbial limit of < 2 log cfu/g for *E. coli* in fresh sausage (SA DAFF, 2018).

4.3.2.6. Staphylococcus aureus

Significant differences for *S. aureus* counts between the treatments on days 1 and 6 (p < 0.001 and p = 0.005, respectively), were observed (Fig. 4.9). The GR2 and GR2 + S100 had significantly (p < 0.001) lower *S. aureus* counts than the NC and the S450 treatments. It was also the only two treatments with *S. aureus* counts conforming to the microbial limit of < 2 log cfu/g for fresh sausage (SA DAFF, 2018). According to Makarewicz et al. (2021), tea catechins could sensitize methicillin-resistant *S. aureus* strains to antimicrobials. This might explain the greater inhibition of GR2 and GR2 + S100 against *S. aureus*, as catechins are found in green Rooibos (McKay & Blumberg, 2007).

Treatment NC with the absence of a preservative, had the highest count on day 1 and differed significantly from S450, GR2, H050 and GR2 + S100. Treatment GR2 and GR2 + S100 had a significant lower count compared to NC and S450 but did not differ from the other treatments. This might be explained as the combination of plant extracts (containing polyphenols) with SO_2 might provide a synergistic effect, which increases the antimicrobial activity thereof.

On day 3, there were no significant differences between any of the treatments (p = 0.141) and no treatment conformed to the microbial limit of < 2 log cfu/g. The higher *S. aureus* counts on day 3 are an indication that the *S. aureus* was able to multiply from day 1 to day 3 resulting in all the treatments not conforming to the microbial limit of < 2 log cfu/g anymore.

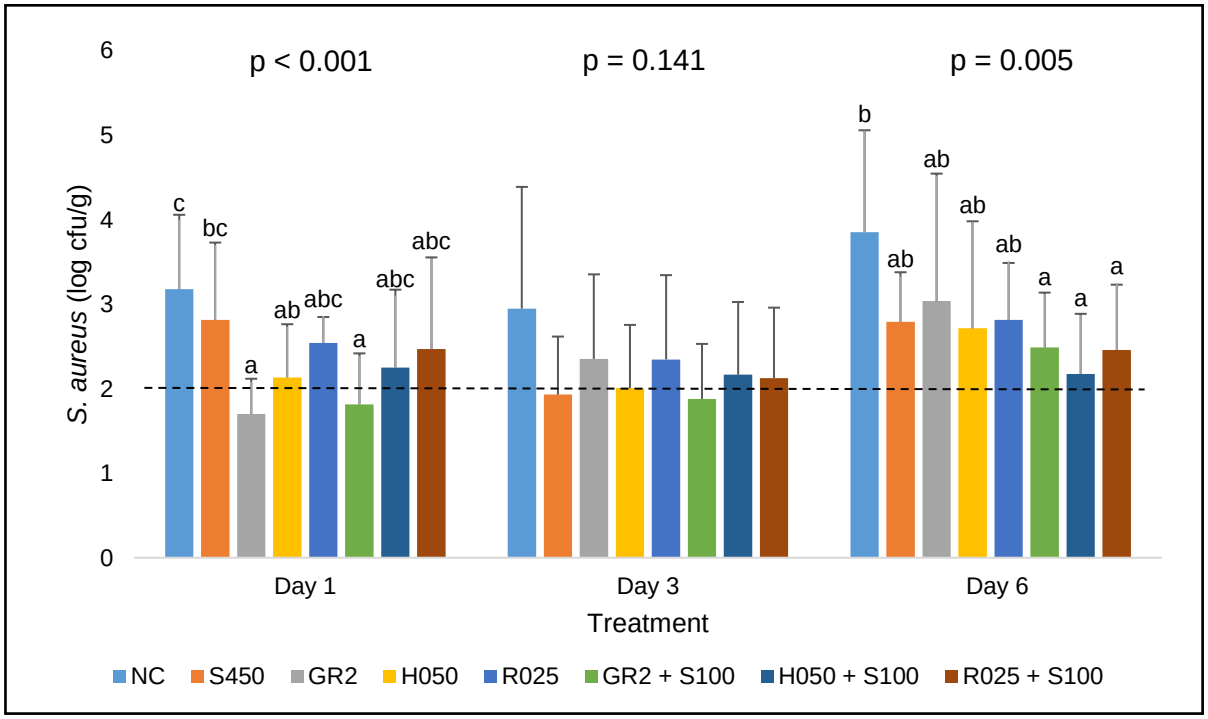

Fig. 4.9. *Staphylococcus aureus* count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly (p < 0.05). The dotted line represents the microbial limit of < 2 log cfu/g for *S. aureus* in fresh sausage (DAFF, 2018).

By day 6, none of the eight treatments had *S. aureus* counts that adhered to the microbial limit of < 2 log cfu/g (SA DAFF, 2018) (Fig. 4.9), indicating the negative effect of storage time. The high *S. aureus* count present in the Boerewors may be due to the initial microbial load of the

meat before manufacturing of the Boerewors. On day 6, the three plant extracts combined with S100, were the only treatments that had significantly ($p = 0.005$) lower *S. aureus* counts than treatment NC. Hence, the combination of plant extracts with low SO_2 have reduced the counts of *S. aureus* more effectively. According to Makarewicz et al. (2021), a study has shown that tea catechins could cause the strains of *S. aureus* to become more susceptible towards antimicrobials. Some studies have shown that antibiotics can have a synergistic effect in combination with polyphenols of green tea (Efenberger-Szmechtyk et al., 2020). Researchers have also shown that the combination of Rosemary and SO_2, have reduced microbial growth more effectively rather than when used separately (Mathenjwa et al., 2012).

The inhibition action of these natural preservatives cannot guarantee the best effect against micro-organisms, as their antimicrobial and antioxidant activity depend largely on the species and the strains of micro-organisms as well as the molecular structure of the polyphenols responsible for the antioxidant activity (Efenberger-Smechtyk et al., 2020; Markarewicz et al., 2021).

4.3.2.7 Listeria monocytogenes

Listeria monocytogenes is recognised as a pathogen that may cause listeriosis in humans. There have been many historical outbreaks caused by this pathogen (WHO, 2018). In South Africa, 216 deaths were recorded during 2017 to 2018 due to the outbreak of listeriosis in processed meat products (Tchatchouang et al., 2020). Therefore, it was necessary to test for the presence of this pathogen beforehand in meat. *Listeria monocytogenes* was absent in all the three replicates during the production of the Boerewors treatments.

4.3.2.8 Yeasts

There was a significant difference in the yeast counts between all the treatments on days 1, 3 and 6 ($p < 0.001$) (Fig. 4.10). Over the 6-day storage period, the yeast count increased for all the treatments and none of the yeasts counts of the treatments conformed to the microbial limit of < 2 log cfu/g for yeast and mould counts in raw sausage (Shapton & Shapton, 1991).

On day 1, all the treatments were significantly ($p < 0.001$) lower than the NC (Fig. 4.10). Treatment S450 was, however, the most effective in inhibiting the yeasts with the rest of the treatments having significantly ($p < 0.001$) higher yeast counts. This same trend was also seen on days 3 and 6. Yeasts are known to be destroyed by chemical preservatives, such as SO_2 (Walker, 1985). Yeasts are inhibited at an a_w of below 0.80 according to literature (Mathenjwa et al., 2012). The water activity of all the treatments in the current study were in the range of 0.93 to 0.95. This may explain the increased growth of yeasts observed by all the treatments.

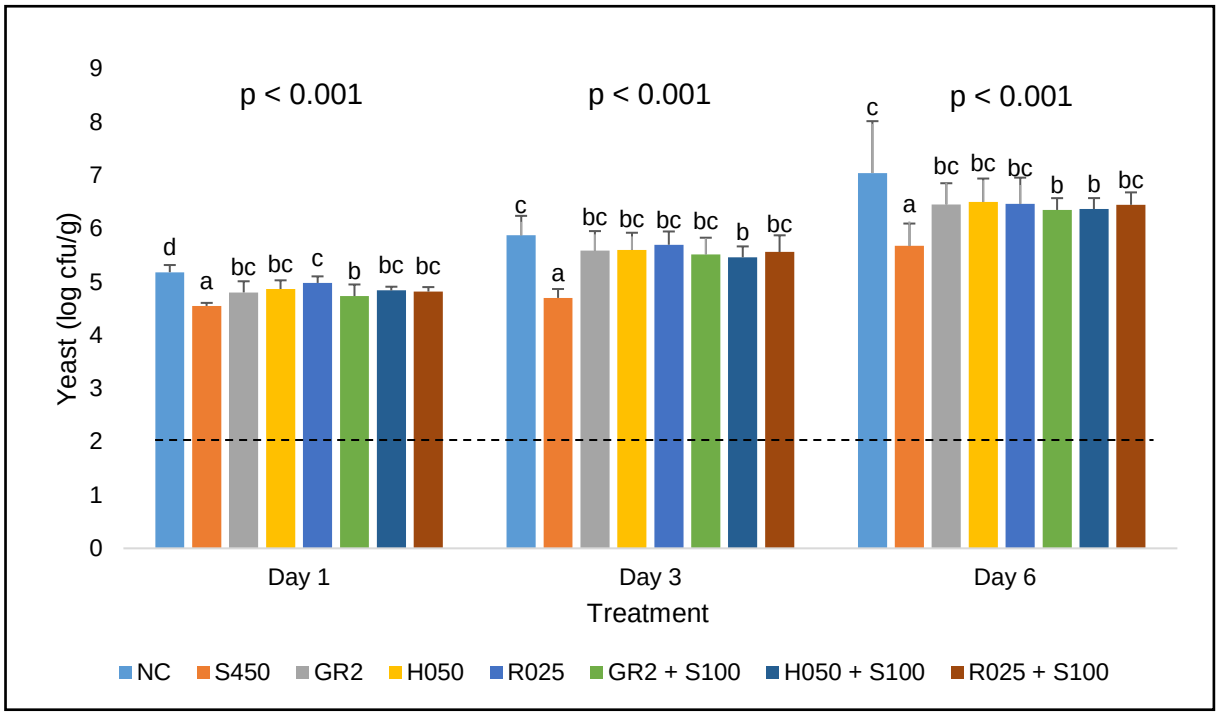

Fig. 4.10. Yeast count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly ($p < 0.05$). The dotted line represents the microbial limit of < 2 log cfu/ g for yeast and mould counts on raw sausage (Shapton & Shapton, 1991).

4.3.2.9. Moulds

The mould counts differed significantly between all the treatments on days 1 ($p = 0.002$), 3 ($p < 0.001$), as well as on day 6 ($p = 0.006$), with mould counts ranging between 1 and 1.6 log cfu/g (Fig. 4.11). Treatment GR2 + S100 had the significantly ($p = 0.002$) lowest count on day 1 and differed significantly from NC, GR2, H050 and H050 + S100, but did not differ significantly from treatment S450, R025 and R025 + S100. Treatments S450 and GR2 + S100 were the only treatments able to control the mould counts to below the microbial limit of < 2 log cfu/g for yeast and mould counts on raw sausage (Shapton & Shapton, 1991).

On day 3, the treatment without any preservatives (NC), as well as all the treatments with SO_2 (S450, GR2 + S100, H050 + S100 and R025 + S100) had significantly ($p < 0.001$) lower counts

compared to the treatments containing only plant extracts. Moulds may be more resistant towards plant extracts, as a study has shown that fungi were in general more resistant than bacteria to extracts containing polyphenols (Efenberger-Szmechtyk et al., 2020).

The mould counts decreased with increasing time, as day 6 had much lower counts compared to days 1 and 3. The reason for this might be attributed to the moulds not being able to survive at the low temperature of 4 ºC. While six of the treatments had significantly ($p < 0.006$) lower mould counts than the NC, GR2 did not seem to have any inhibitory effect on the mould counts on day 6. According to Shapton & Shapton (1991), the maximum mould count allowed in raw sausages is 2 log cfu/g. No treatment exceeded this limit at the end of shelf-life.

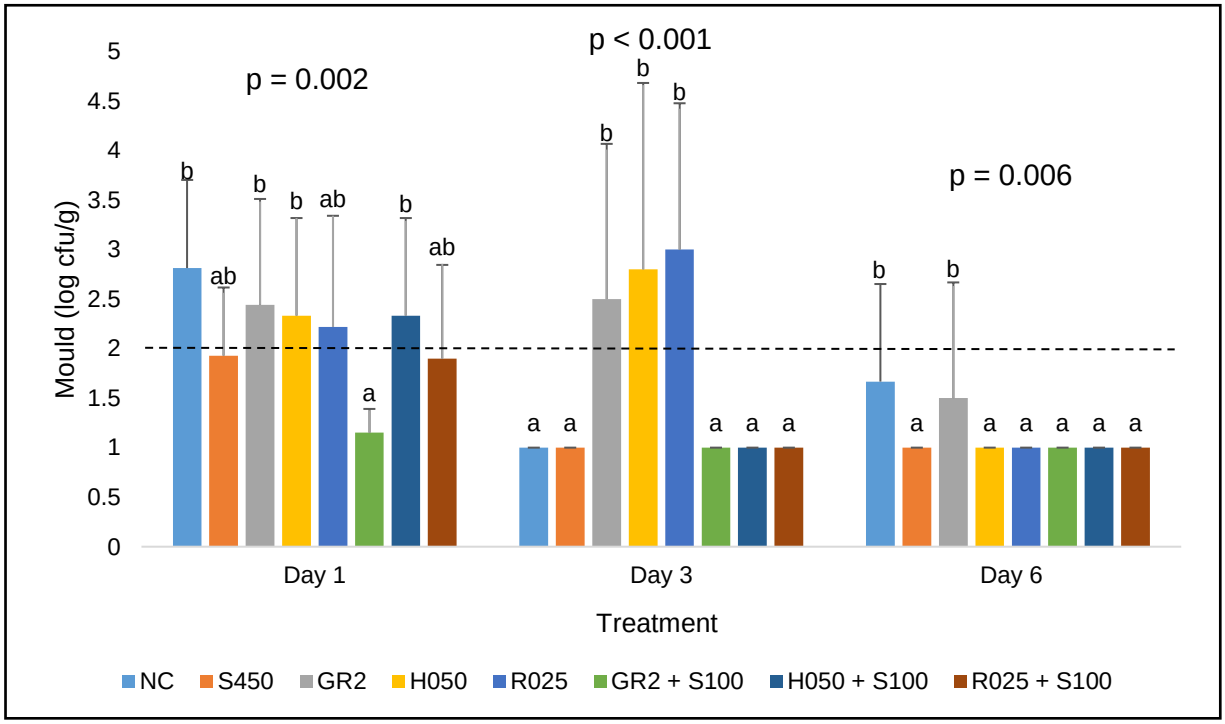

Fig. 4.11. Mould count (mean log cfu/g, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 ºC. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same day differed significantly ($p < 0.05$). The dotted line represents the < 2 log cfu/g microbial limit for yeasts and mould counts on raw sausage (Shapton & Shapton, 1991).

4.3.3. Sensory analysis

4.3.3.1. Colour analysis

The difference in colour of the different Boerewors treatments can be seen in Fig. 4.12. GR2 and GR + S100, indicated as “C” and “F”, had a darker colour compared to the other treatments. It can also be seen that S450, indicated as “B”, was lighter in colour than the rest of the treatments.

Fig. 4.12. A picture of the eight Boerewors treatments after production. A = NC (negative control with no preservatives); B = PC (Positive control with 450 mg/kg SO_2); C = 2% Green Rooibos extract; D = 0.50% Honeybush extract; E = 0.25% Rooibos extract; F = 2% Green rooibos extract with 100 mg/kg SO_2; G = 0.50% Honeybush extract with 100 mg/kg SO_2 and H = 0.25% Rooibos extract with 100 mg/kg SO_2.

The results obtained from the colour analysis in terms of *L** values of the treatments, are presented in Table 4.5. The *L** values represented the lightness of the treatment. Treatments GR2, H050 and GR2 + S100 had significantly ($p < 0.001$) lower *L** values than the rest of the treatments on day 1, while S450 had the highest value. This may be due to the dark yellow/orange colour that the green Rooibos extract possesses. The treatments with low added SO_2, had greater *L** value than those consisting only of the plant extract. SO_2 may be responsible for this, as it is known to give a bright pinkish red colour, as it is used as an anti-browning agent in most food products (Garcia-Fuentes et al., 2015). Both treatment NC and S450 had the highest value compared to the rest of the treatments, although it did not differ from treatment R025 + S100. The negative control had no preservative; therefore, it may be expected to have the highest *L** value compared to the other treatments.

The same trend was seen on day 3 and day 6 between the different treatments. Higher concentrations of singular plant extracts tended to decrease the *L**, while those with SO_2, had increased *L** with lower concentrations of plant extracts (R025 + S100 > H050 + S100 > GR2 + S100).

Table 4.5

Colour *L** values (mean ± Standard deviation, n = 12) of eight Boerewors treatments on days 1, 3 and 6 stored at 4 °C and after 30 min bloom. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

	Mean colour *L value ± Standard deviation (n = 12)**		
Treatment	**Day 1**	**Day 3**	**Day 6**
NC	$55.008^{d} \pm 3.937$	$55.367^{d} \pm 4.641$	$53.578^{e} \pm 3.949$
S450	$55.316^{d} \pm 4.969$	$55.317^{d} \pm 4.906$	$52.458^{de} \pm 4.361$
GR2	$46.663^{a} \pm 3.144$	$44.672^{a} \pm 3.016$	$44.691^{a} \pm 2.772$
H050	$48.986^{ab} \pm 3.841$	$47.975^{b} \pm 3.271$	$47.800^{bc} \pm 2.797$
R025	$52.209^{c} \pm 3.658$	$52.422^{c} \pm 4.450$	$50.112^{cd} \pm 2.824$
GR2 + S100	$46.787^{a} \pm 3.144$	$44.672^{a} \pm 3.016$	$44.691^{a} \pm 2.722$
H050 + S100	$50.307^{bc} \pm 3.588$	$48.434^{b} \pm 3.455$	$46.429^{ab} \pm 3.423$
R025 + S100	$52.679^{cd} \pm 3.317$	$52.868^{cd} \pm 4.145$	$50.271^{d} \pm 3.336$
p-value	<0.001	<0.001	<0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

There was a significant difference in the redness (*a** value) between all the Boerewors treatments on days 1, 3 and 6 ($p < 0.001$) as can be seen in Table 4.6. The S450 treatment had the significantly highest *a** value on day 1 as it differed from all the other treatments. According to D'Amore et al. (2020), SO_2 is known for preventing browning in food products by inhibiting the oxidation of myoglobin to metmyoglobin (Walker, 1985). Thus, this explains the increased *a** value of the S450 treatment. The increased concentration of the plant extracts (specifically R025, H050 and GR2), showed an increase in *a** value, but when combined with low SO_2, the redness increased even more. According to Bañón et al. (2007), previous studies showed that green tea and grape seed extract used in beef patties did not delay the colour deterioration thereof, therefore, the addition of sulphur dioxide was needed. The powder of Rooibos and honeybush itself possess a yellow orange colour, which may change the colour of the meat, which therefore, may decrease the a* value of the treatments compared to those with SO_2.

On day 6, the NC, H050 and R025 had significantly ($p < 0.001$) lower redness than the rest of the treatments, with NC having the lowest redness. Because NC contained no preservatives to prevent oxidation processes in meat, it may be expected to change colour near the end of

shelf-life, by turning brown/pale which is the cause of enzymatic browning that occur in meat, as well as protein and lipid oxidation (Jumayi et al., 2022).

Table 4.6

Colour *a** values (mean ± Standard deviation, n = 12) of the eight treatments on days 1, 3 and 6 stored at 4 °C and after 30 min bloom. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

	Mean colour *a value ± Standard deviation (n = 12)**		
Treatment	**Day 1**	**Day 3**	**Day 6**
NC	$17.025^{b} \pm 2.666$	$14.134^{ab} \pm 2.460$	$10.548^{a} \pm 1.423$
S450	$18.867^{c} \pm 2.909$	$16.948^{d} \pm 2.304$	$15.703^{c} \pm 2.917$
GR2	$16.825^{ab} \pm 1.394$	$14.952^{bc} \pm 1.097$	$13.897^{b} \pm 0.914$
H050	$16.243^{ab} \pm 2.830$	$14.138^{ab} \pm 1.871$	$11.422^{a} \pm 1.379$
R025	$15.176^{a} \pm 2.378$	$13.294^{a} \pm 1.875$	$10.730^{a} \pm 1.875$
GR2 + S100	$15.665^{ab} \pm 1.336$	$14.354^{ab} \pm 1.188$	$13.279^{b} \pm 1.742$
H050 + S100	$16.726^{ab} \pm 2.653$	$15.791^{cd} \pm 1.822$	$13.345^{b} \pm 1.774$
R025 + S100	$17.073^{b} \pm 2.240$	$15.806^{cd} \pm 2.449$	$13.735^{b} \pm 1.816$
p-value	<0.001	<0.001	<0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

The yellowness (*b**) of all the treatments differed significantly ($p < 0.001$) from each other on days 1, 3 and 6. On day 1, yellowness of the GR2 treatment had a significantly higher *b** value (18.398) than treatments NC, S450 and H050 + S100 (Table 4.7). This may be due to the yellow/orange colour of the green Rooibos extract. Similar results were reported by Cullere et al. (2019), who found that the use of increased concentrations of Rooibos in meat patties, showed increased *b** values. The NC treatment and the S450 treatment did not differ from each other on day 1. Both treatments had significantly lower *b** values than the rest of the treatments. This may be expected from S450, as SO_2 is known to retain the red colour of the Boerewors (D'Amore et al., 2020; Garcia-Fuentes et al., 2015). Sulphur dioxide inhibits enzymatic browning in meat, by removing the heme of hemoglobin, which is responsible for the browning of meat (D'Amore et al., 2020). Therefore, a decreased yellowness is to be expected.

On day 3, NC and S450 had significantly ($p < 0.001$) lower *b** values compared to the other treatments. It appeared that the *b** value of the plant extract treatments may be associated

with the yellow/orange colour of the plant extracts. The polyphenols of plant extracts are known to cause denaturing of proteins in meat at high concentrations, while at low concentrations, it only affects the activity of enzymes (Tiwari et al., 2009). This may explain the higher *b** values obtained in the treatments containing higher concentrations of plant extracts.

The yellowness of all the treatments did, however, decrease with increasing time, as can be seen on day 3 and day 6. Polyphenols are known to retard the deterioration of lipids in meat (Jumayi et al., 2022). Myoglobin and hemoglobin (both heme-proteins) are known to be pro-oxidants of lipid oxidation in meat (Wu et al., 2022). According to Wu et al. (2022), polyphenols may prevent the brown discolouration in fresh meat by reducing hemin into heme from myoglobin and hemoglobin due to the ability of chelating metal ions and their strong potential of donating hydrogen to oxygen radicals. This may explain the reduced *b** value with time. The fermented and unfermented Rooibos extracts had a higher *b** value than the treatments with honeybush extract. This may be associated with the different polyphenol compounds in the two different plant extracts (McKay & Blumberg, 2007).

Table 4.7

Colour *b** values (mean ± Standard deviation, n = 12) of the eight treatments on days 1, 3 and 6 stored at 4 °C and after 30 min bloom. NC = negative control (0% preservatives); S450 = positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050 = 0.50% honeybush extract; R025 = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos extract with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2.

	Mean colour *b value ± Standard deviation (n = 12)**		
Treatment	**Day 1**	**Day 3**	**Day 6**
NC	$14.504^{a} \pm 1.732$	$13.786^{a} \pm 1.483$	$13.797^{a} \pm 1.248$
S450	$14.980^{a} \pm 1.505$	$14.428^{a} \pm 1.498$	$13.804^{a} \pm 1.629$
GR2	$18.398^{c} \pm 1.189$	$17.623^{d} \pm 1.319$	$17.118^{d} \pm 1.391$
H050	$17.368^{bc} \pm 2.272$	$15.750^{b} \pm 2.105$	$15.834^{bc} \pm 2.184$
R025	$17.773^{bc} \pm 2.168$	$17.506^{d} \pm 1.895$	$16.887^{cd} \pm 2.009$
GR2 + S100	$17.489^{bc} \pm 1.163$	$16.833^{bcd} \pm 1.512$	$16.043^{bcd} \pm 2.003$
H050 + S100	$16.936^{b} \pm 1.997$	$16.261^{bc} \pm 1.699$	$14.994^{ab} \pm 1.740$
R025 + S100	$17.719^{bc} \pm 1.446$	$17.129^{cd} \pm 1.721$	$16.470^{cd} \pm 1.528$
p-value	<0.001	<0.001	<0.001

Means with different superscripts in the same column differed significantly ($p < 0.05$).

4.3.3.2. Sensory analysis

For the sensory analysis, the four attributes that were tested, were colour, taste, texture and overall acceptability. The attribute aroma was not tested for, as aroma and taste were seen as the same attribute. Consumers cannot differentiate between aroma and taste, therefore, only taste was used to represent both. The consumer panel consisted mostly of female participants and most consumers were between the age of 18 to 24 years (Table 4.8). The minority of the consumers were aged between 55 to 65 years. (Fig. 4.13.).

Table 4.8

Demographic profile of the consumer panel in this study.

Gender	Total	Percentage	Age group	Total	Percentage
Female	42	58	18-24	44	60
Male	31	42	25-34	11	15
			35-44	9	13
			45-54	6	8
			55-65	3	4

Most participantsin the sensory evaluation were students and lecturers on campus and only a few people by word of mouth from off-campus. Most participants (41%) spoke Afrikaans or Zulu (19%). The rest of the participants spoke either English, Ndebele, Northern Sotho, Southern Sotho, Tswana, Venda or other languages as can be seen in Fig. 4.14.

4.3.3.3. Evaluation of the sensory attributes

A total of 73 observations were made per sample on the following attributes: colour, taste, texture and overall acceptability (Fig. 4.15). The results obtained from sensory evaluation showed that in terms of all the attributes evaluated, GR2 differed significantly ($p < 0.001$) from all the other treatments, except from GR2 + S100 treatment. The taste and texture of both GR2 and GR2 + S100, were slightly disliked by the panel, compared to the other treatments.

In terms of colour, the panel did neither like nor dislike the colour of GR2 and GR2 + S100 treatments as it had a score near 5. Therefore, it may be concluded that consumers did not like the brown reddish colour of green Rooibos, as it was different from the bright red/pink colour of Boerewors normally expected by consumers. R025 and H050 had a significantly ($p < 0.001$) higher mean score for colour than the S450 treatment. This suggested that the panel preferred the colour of R025 and H050 above that of S450, as they only slightly liked the colour of S450 (score = 6.2). There were no significant differences observed in the colour of NC, S450, H050 + S100 and R025 + S100 by the sensory panel.

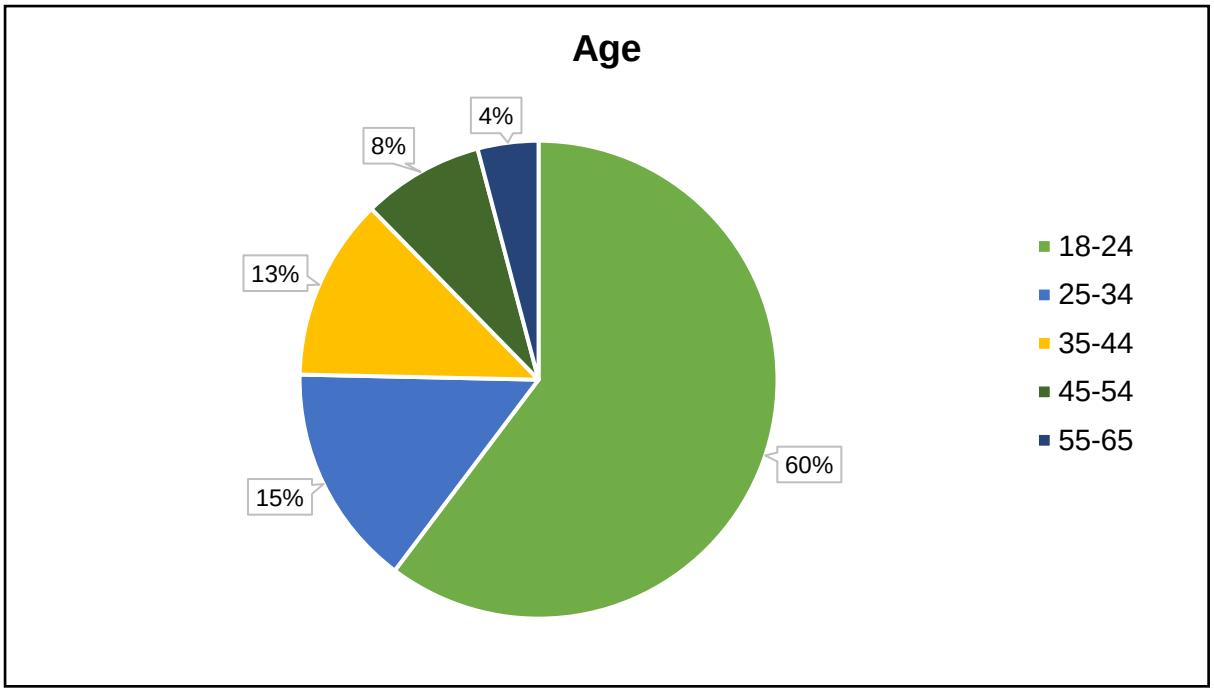

Fig. 4.13. The percentage (%) of different age groups that participated in the sensory evaluation.

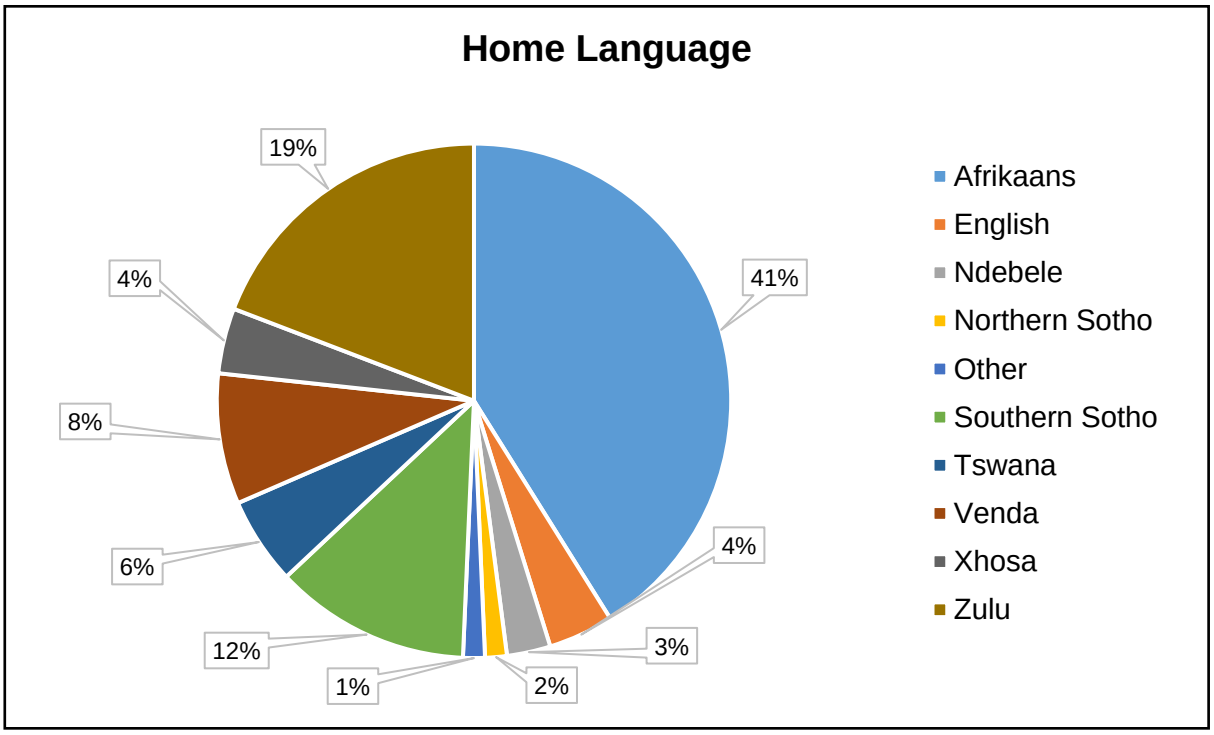

Fig. 4.14. The percentage (%) of participants of different language participating in this study.

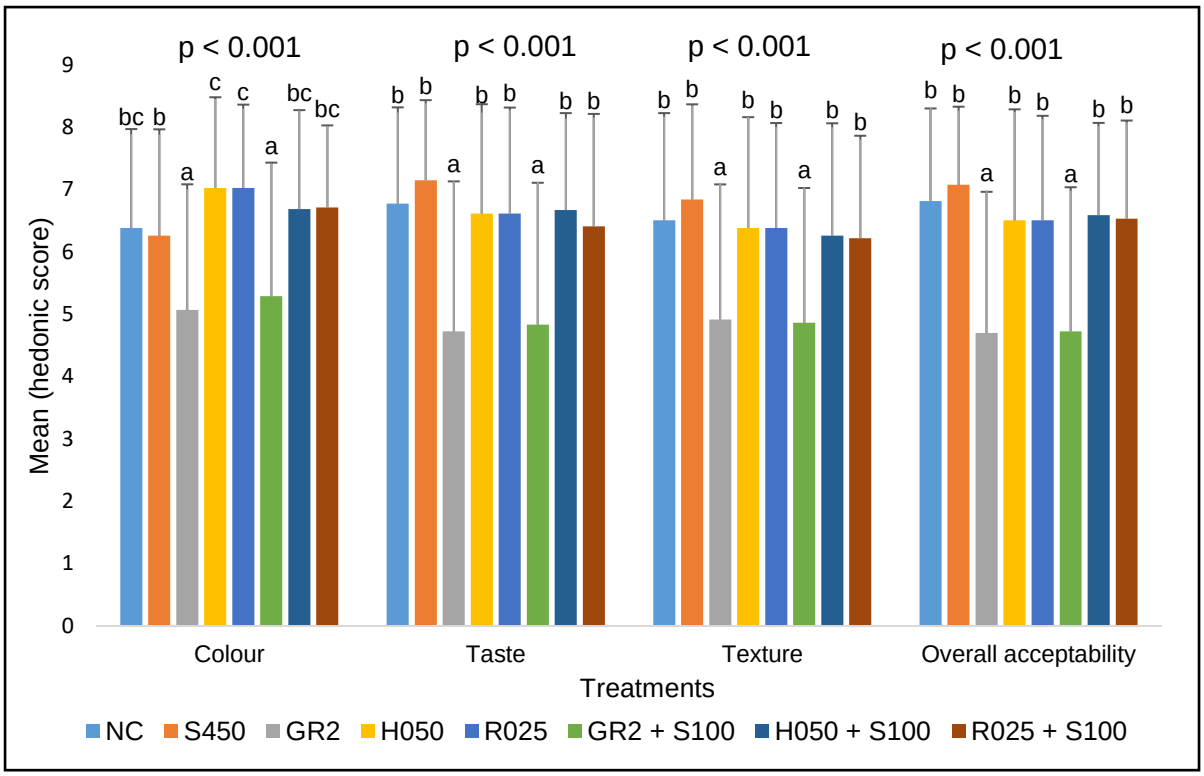

Fig. 4.15. The mean (n = 73) hedonic scale scores of the eight cooked Boerewors treatments in terms of the colour, taste, texture, and overall acceptability by a consumer panel. NC = negative control (0% preservatives); S450 = Positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050% = 0.50% honeybush extract; R025% = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts on the same attribute differed significantly ($p < 0.05$).

The GR2 treatment had the significantly ($p < 0.001$) lowest mean score (4.7) in terms of taste. This suggested that the sensory panel slightly disliked the taste of green Rooibos. Because of the high concentration used, it may have caused an overwhelming flavour. There was no significant difference in the taste between the rest of the treatments, although S450 had the highest mean score (7.1). This might be expected, as SO_2 enhances the flavour/taste of Boerewors (D'Amore et al., 2020).

The texture of the GR2 was also slightly disliked, this can be due to the high concentration of green Rooibos extract used, as the texture was mealier/pastier and softer compared to the others. The S450 (positive control) treatment, was moderately liked in terms of texture and overall acceptability, but it also had the significantly ($p < 0.001$) highest mean score (7.1) compared to the other treatments. This can be expected, as SO_2 is known as a flavour enhancer, gives stability to texture and retains the red colour of Boerewors (D'Amore et al.,

2020; Hugo & Hugo, 2015). This suggested that all the plant extract treatments, excluding 2% green Rooibos, may be accepted as a preservative in Boerewors by consumers.

4.3.4. Thaw-, cooking- and total losses

4.3.4.1. Thaw losses

Both the treatments of GR2 and GR2 + S100 showed significant ($p < 0.001$) losses during thawing of the sausages when compared to the rest of the treatments (Fig. 4.16). The high concentration of polyphenols in the GR2, may have caused an increase in the moisture release by meat proteins. The exact mechanism or compound that might be responsible for this, still needs further investigation.

Treatment S450 had the least amount of loss during thawing (0.615%), but did not differ significantly from NC, H050, R025, H050 + S100 and R025 + S100. The GR2 (2.257%) did not differ significantly from R025 (1.142%), H050 + S100 (0.987%), R025 + S100 (0.986%) and GR2 + S100 (3.763%).

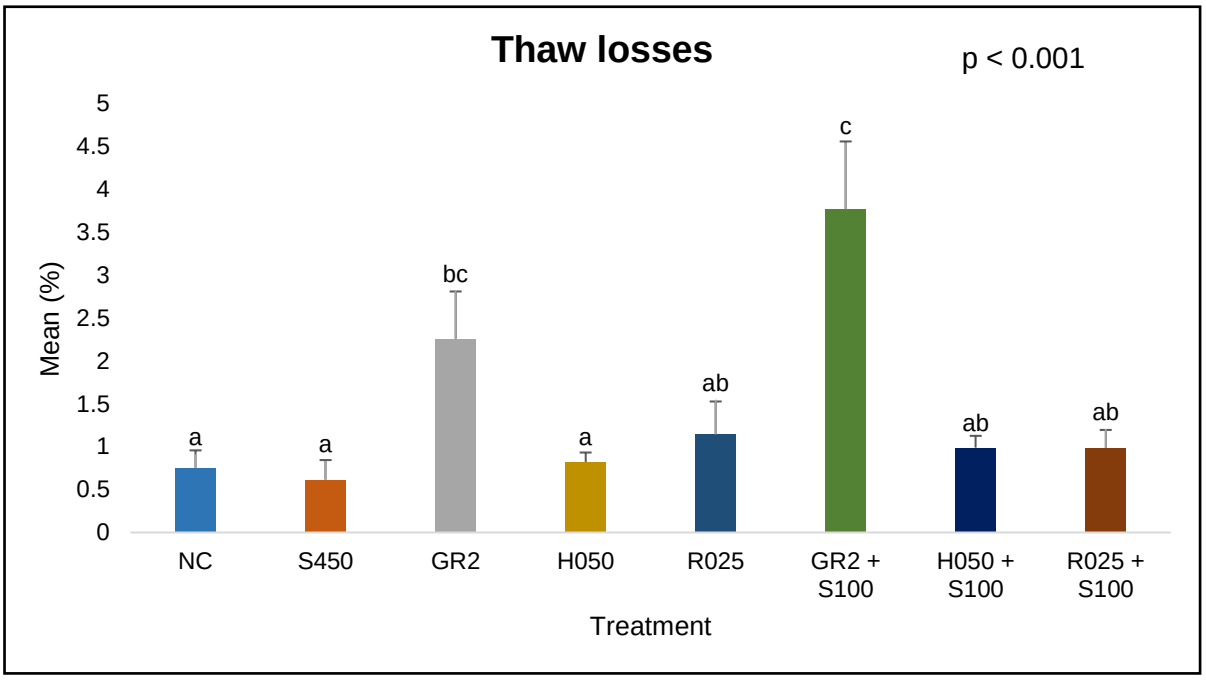

Fig. 4.16. The mean percentage (n = 12) thaw losses of the eight treatments. NC = negative control (0% preservatives); S450 = Positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050% = 0.50% honeybush extract; R025% = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts differed significantly ($p < 0.05$).

4.3.4.2. Cooking losses

The greatest cooking loss was found within GR2 and GR2 + S100, which was significantly ($p < 0.001$) higher than the rest of the treatments in this study (Fig. 4.17). There was no significant difference between GR2 + S100 and GR2. The rest of the treatments also did not differ significantly from each other. The great loss of water during cooking can be related to the low pH levels of the treatments. GR2 had a pH near the iso-electric point of meat (pH = 5.2), which causes the water holding capacity of meat to reduce and therefore, release more water/fluid (Warner, 2017). According to Aaslyng et al. (2003), high cooking losses were to be expected when the pH and water holding capacity was low. As the treatments containing green Rooibos extract had the highest thawing loss (Fig. 4.16.) compared to the other treatments, it can be expected that the cooking loss of these two treatments, will be high as well.

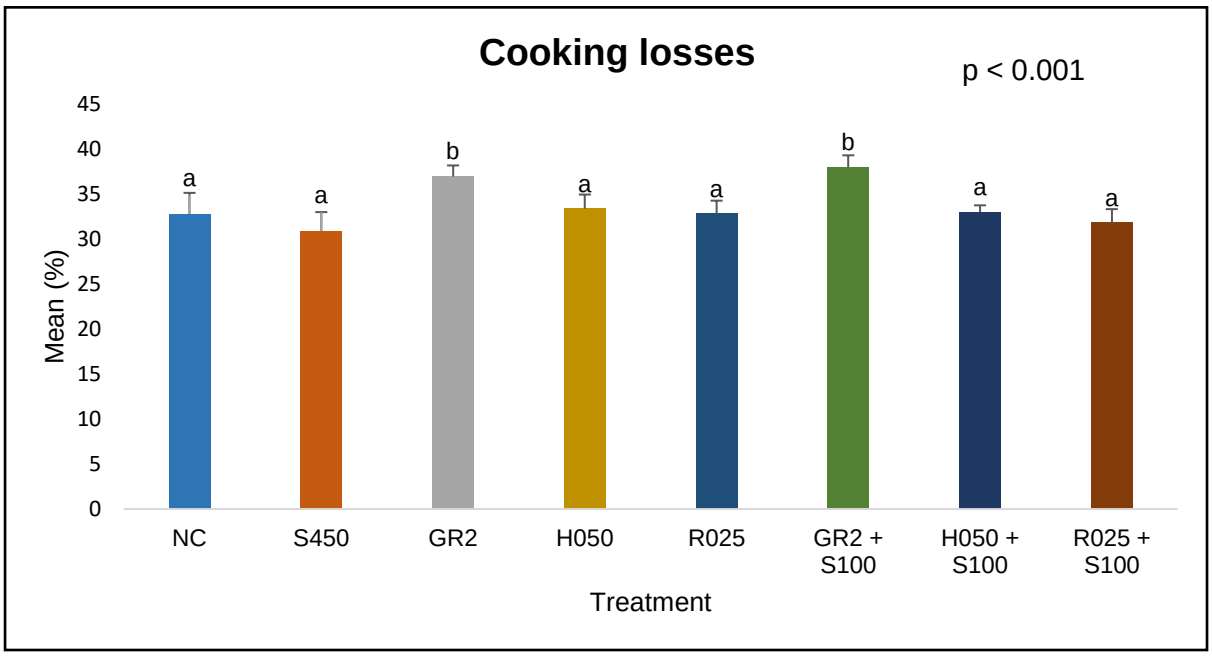

Fig. 4.17. The mean percentage cooking losses (n = 12) of the eight treatments. NC = negative control (0% preservatives); S450 = Positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050% = 0.50% honeybush extract; R025% = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts differed significantly ($p < 0.05$).

4.3.4.3. Total losses

S450 had a total loss of 31.48% moisture and was the treatment that had the lowest moisture loss, which differed significantly ($p < 0.001$) from GR2, GR2 + S100 and H050 + S100 (Fig. 4.18). However, it did not differ from NC, R025 and R025 + S100. GR2 + S100 had the highest

% total moisture loss (41.70%) and differed significantly ($p < 0.001$) from all the treatments. Both green Rooibos extract treatments (GR2 = 41.70% and GR2 + S100 = 39.17%) had a significantly higher % total moisture loss compared to the other treatments, which may be expected as it was also the treatments with the highest thaw- and cooking losses. These two treatments also differed significantly ($p < 0.001$) from each other. The higher the concentration of the plant extract used and with increased temperature, the more moisture it releases due to the increased denaturation of meat proteins (Ishiwatari et al., 2013).

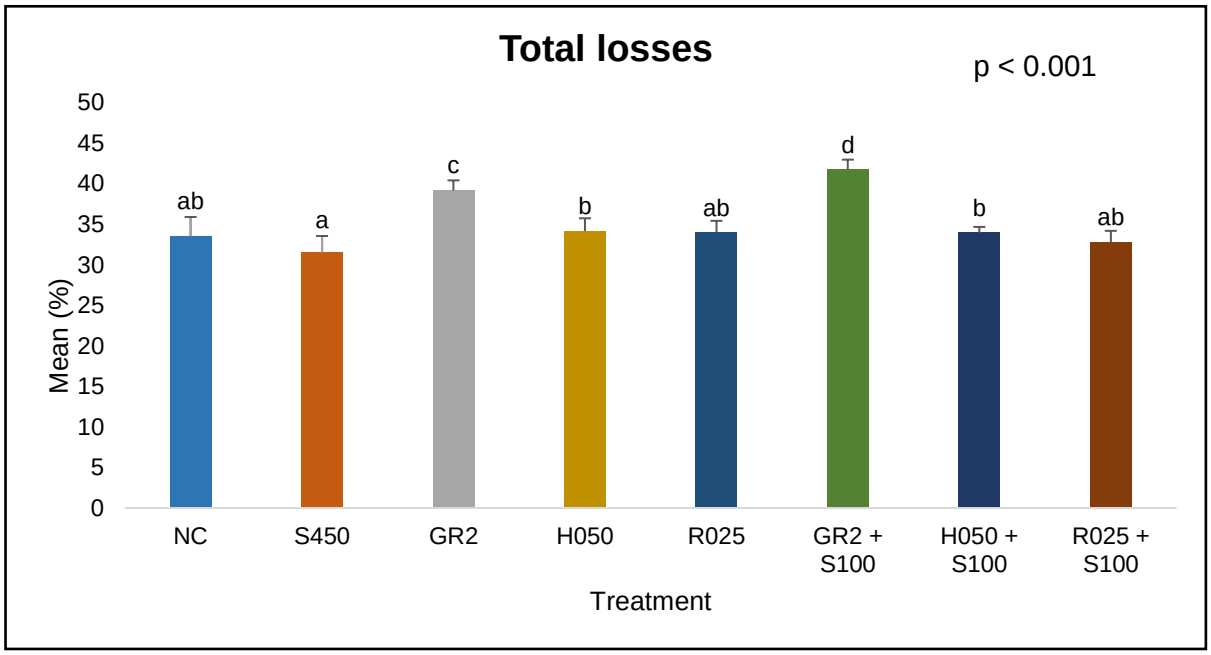

Fig. 4.18. The mean percentage total losses (n = 12) of eight treatments. NC = negative control (0% preservatives); S450 = Positive control (450 mg/kg SO_2); GR2 = 2% green Rooibos extract; H050% = 0.50% honeybush extract; R025% = 0.25% Rooibos extract; GR2 + S100 = 2% green Rooibos with 100 mg/kg SO_2; H050 + S100 = 0.50% honeybush extract with 100 mg/kg SO_2 and R025 + S100 = 0.25% Rooibos extract with 100 mg/kg SO_2. Means with different superscripts differed significantly ($p < 0.05$).

4.4. Conclusions

The physico-chemical, microbial and sensory effect of Rooibos, green Rooibos and honeybush as possible preservative replacers of SO_2 in Boerewors were explored in this Chapter.

The plant extracts and their combinations with S100 were like S450 in terms of pH, water activity and lipid stability (TBARS) values, with no significant differences on days 1, 3 and 6 at 4 ^oC and on days 1 and 90 at -18 ^oC for TBARS.

The microbial analysis indicated some positive and negative effects of both the natural preservatives and the S450. The polyphenols of the plant extracts were able to control total bacteria counts, *Enterobacteriaceae* counts, coliform counts and *E. coli* equally as well as the S450 treatment, especially up to 3 days of storage at 4 ^{o}C. When the three plant extracts were combined with S100, these counts were equally well controlled as the S450 up to 6 days of storage.

The three natural plant extracts were, however, not able to control the LAB counts and the *S. aureus* counts over 6 days storage at 4 ^{o}C as well as the S450 but were able to control these counts if the plant extracts were combined with S100. This could be due to a synergistic effect that takes place between polyphenols and S100 as both contain high antioxidant activity, which may have increased the antimicrobial action against Gram-positive bacteria.

The three plant extracts were not able to control the yeast and mould counts. Not S450, nor the plant extracts, were able to control the yeast counts, although S450 did control the yeast counts significantly better than the three plant extracts. In the case of mould counts, the controlling effect of the three plant extracts and the S450 increased up to 6 days of storage at 4 ^{o}C, except for GR2.

With colour analysis, the lightness (*L**)-value indicated that the GR2, H050 and GR2 were significantly ($p < 0.001$) darker than the S450, which could deter consumers. The red colour, indicated by the a*-value, was better preserved by the S450 treatment than with the three plant extracts, although the addition of S100 to the three plant extracts, increased the a*-value. The yellowness (b*)-values over the 6-days storage at 4 ^{o}C, were negatively influenced by the plant extracts when compared to the S450 treatment.

With the sensory analysis, consumers preferred the colour, taste, texture and overall acceptability of the H050 and R025 with their combinations with S100, equally well to the S450, but did not prefer the GR2 and GR2 + S100 treatments.

With thaw-, cooking and total losses, GR2 and GR2 + S100 gave very high losses, with H050, R025, H050 + S100 and R025 + S100 having similar values to the S450 treatment.

This study indicated that S450 was still the best preservative in terms of microbial, colour and sensory analysis. Honeybush and Rooibos and its combination with S100, are very good candidates as replacers of SO_2 in Boerewors, while the colour that green Rooibos imparted on the Boerewors, could possibly negatively influence consumers.

Chapter 5
General discussion and conclusions

Boerewors is a fresh meat product that can be easily spoiled by many factors. The presence of micro-organisms, enzymes, oxygen, temperature and the oxidation of lipids, are among these factors that contribute to the reduced shelf-life of sausages and many other meat products (Mathenjwa et al., 2012). Boerewors normally contains 450 mg/kg SO_2 as a chemical preservative. The use of SO_2 in Boerewors is known to retain the red colour of the meat and act as antimicrobial. However, SO_2 has been associated with negative health effects, especially in asthmatic persons (D'Amore et al., 2020).

There has been a growing need for natural preservatives, as they may replace these chemical preservatives in sausages, which might even increase the quality/shelf-life of meat products. Studies at the University of the Free State on Boerewors as a model, have already investigated rosemary extract and chitosan (Mathenjwa et al., 2012), Citrox® (van Schalkwyk et al., 2013) and commercial plant extracts and protective cultures (Freitag, 2023). These studies indicated promising results for the partial replacement of SO_2 in Boerewors.

According to the literature review, plant extracts may impart beneficial characteristics to Boerewors and may reduce health risks caused by SO_2. The use of Rooibos and honeybush extracts have many health benefits due to the presence of polyphenols. Flavonoids and phenolic acids are the major polyphenols found within these plant extracts. These phenolic compounds are known to have antioxidant as well as antimicrobial properties (McKay & Blumberg, 2007).

The general aim of this study was, therefore, to investigate the effect of Rooibos, green Rooibos and honeybush plant extracts as natural preservatives on the physico-chemical, microbial and sensory quality of Boerewors.

In Chapter 3, the antimicrobial activity of three types of plant extracts (Rooibos, honeybush and green Rooibos) were investigated in vitro by means of a disc diffusion assay. As the use of plant extracts have not been studied in Boerewors before, it was needed to evaluate which concentrations would be sufficient to be used in Boerewors models. The objectives of this chapter were, therefore, to:

- Evaluate the inhibitory effect/antimicrobial activity of three concentrations of all three types of extracts against five different strains each of *E. coli* and *S. aureus*.

- Evaluate the antimicrobial activity effect of two concentrations of SO_2 as a control in the form of sodium metabisulphite against five different strains each of *E. coli* and *S. aureus*.
- Determine which concentration, as well as which type of extract, will perform the best for acting as a preservative in Boerewors.

From the results obtained, GR2, H050 and R025 had the largest zones of inhibition against the strains of *E. coli* and *S. aureus*. From these three plant extracts, GR2 had the best inhibitory activity against strains of both *E. coli* and *S. aureus*. This may be attributed to the flavonoids present in green Rooibos, especially aspalathin and quercetin, as they could cause alteration to the cell membranes of organisms, which may lead to cell death (Makarewicz et al., 2021). The antimicrobial activity of S100 and S450 was also assessed against these two micro-organisms. S100 had no inhibitory effect on the strains of both *S. aureus* and *E. coli*. The antimicrobial activity depends on the concentration of sodium metabisulphite used, as well as on the bacterial species and strain. However, S450 had a small inhibitory effect against *E. coli*, but no effect on *S. aureus*. This may be due to the higher concentration used and that SO_2 has a greater inhibitory effect on Gram-negative bacteria than on Gram-positive bacteria (Walker, 1985).

In chapter 4, the aim was to determine the preservative effect of GR2, H050 and R025, on their own, or in combination with lowered levels (100 mg/kg) of SO_2 in a Boerewors model in terms of their physico-chemical, microbial and sensory characteristics.

The plant extracts, as well as their combinations with low SO_2 (S100) levels, were similar to S450 (positive control) in terms of pH, water activity and TBARS values, as there were no significant differences on days 1, 3 and 6 at 4 ^oC and on days 1 and 90 at -18 ^oC for TBARS. The use of SO_2 and plant extracts were both effective in Boerewors, as both have a high antioxidant potential and could reduce the oxidation of lipids by their ability to scavenge free radicals and oxygen in sausages (Mulaudzi et al., 2022).

The microbial analysis indicated some positive and negative effects of both the natural preservatives and the S450. The polyphenols of the plant extracts were able to control total bacteria counts, *Enterobacteriaceae* counts, coliform counts and *E. coli* equally as well as the S450 treatment, especially up to 3 days of storage at 4 ^oC. When the three plant extracts were combined with S100, these counts were equally well controlled to the S450 up to 6 days of storage. Both polyphenols and SO_2 have antioxidant as well as antimicrobial properties against Gram-negative bacteria. The combination of both may increase the antimicrobial action against these bacteria. Other studies have also reported that the presence of more than one antioxidant may improve the quality of meat (Bañón et al., 2007; Roller et al., 2002).

The three natural plant extracts were, however, not able to control the LAB counts and the *S. aureus* counts over 6 days storage at 4 ^{o}C as well as the S450, but they were able to control these counts if the plant extracts were combined with S100. This could be due to a synergistic effect that takes place between polyphenols and S100 as both contain high antimicrobial and antioxidant activity, which may have increased the antimicrobial action against Gram-positive bacteria. The low pH (pH5.4) in the sausages may have contributed to the increased LAB counts on day 6.

The three plant extracts were not able to control the yeast and mould counts. Not S450, nor the plant extracts, were able to control the yeast counts, although S450 did control the yeast counts significantly ($p < 0.001$) better than the three plant extracts. In the case of mould counts, the controlling effect of the three plant extracts and the S450 increased up to 6 days of storage at 4 ^{o}C, except for GR2.

With colour analysis, the lightness (*L**) -value indicated that the GR2, H050 and GR2 were significantly ($p < 0.001$) darker than the S450, which could deter consumers. The red colour, indicated by the a*-value, was better preserved by the S450 treatment than with the three plant extracts, although the addition of S100 to the three plant extracts, increased the a*-value. The yellowness (b*)-values over the 6-days storage at 4 ^{o}C, were negatively influenced by the plant extracts when compared to the S450 treatment. This may be due to the colour of the Rooibos or honeybush extracts, which depends on specific polyphenols found within the extract. Sulphur dioxide is well known to retain the colour of meat and does not possess polyphenols that may produce a colour by itself (Garcia-Fuentes et al., 2015).

With the sensory analysis, consumers preferred the colour, taste, texture and overall acceptability of the H050 and R025 with their combinations with S100, equally well to the S450, but did not prefer the GR2 and GR2 + S100 treatments. The high concentrations of polyphenols in green Rooibos contributed to the increased brown colour of the green Rooibos treatment. This suggested that the use of lower concentrations of plant extracts may be preferred above the use of higher concentrations for the use as preservatives in Boerewors in the future.

With thaw-, cooking- and total losses, GR2 and GR2 + S100 gave very high losses, with H050, R025, H050 + S100 and R025 + S100 having similar values to the S450 treatment. The high concentration of GR2, with the increased temperature during cooking, may have caused an increased denaturation of meat proteins, thus the release of more fluid will be expected (Ishiwatari et al., 2013).

The efficiency of the antimicrobial action of plant extracts (Rooibos, honeybush and green Rooibos extracts) against micro-organisms lies within the concentration and type of

polyphenols. It also depends on the strains and species of the pathogens. The use of SO_2 as a preservative in Boerewors, was still the best in terms of microbial, colour and sensory analysis. However, this study indicated that honeybush and Rooibos and their combinations with low SO_2, may be used as natural replacers for SO_2 in Boerewors. Green Rooibos may not be a good replacement, as the reddish-brown colour of the green Rooibos, as well as the high cooking losses, could be rejected by consumers.

For future research, plant extracts combined with low SO_2 which had an inhibitory effect in reducing the growth of micro-organisms, as well as reducing the oxidation of lipids, could be further explored in terms of the polyphenol content of Rooibos or honeybush, as well as different incorporation methods. The use of Rooibos or honeybush extracts can be combined with other natural extracts that may be more effective in Boerewors, or even make it possible to replace SO_2 completely. The use of Rooibos combined with honeybush may be further explored in Boerewors as preservatives, as both had the same good sensory properties than the SO_2.

Chapter 6
References

Aaslyng, M. D., Bejerholm, C., Ertbjerg, P., Bertram, H. C., & Andersen, H. J. (2003). Cooking loss and juiciness of pork in relation to raw meat quality and cooking procedure. *Food Quality and Preference*, *14*(4), 277–288. https://doi.org/10.1016/S0950-3293(02)00086-1

Abdul, N. S., & Marnewick, J. L. (2023). What has been the focus of rooibos health research? A bibliometric overview. *Journal of Herbal Medicine*, *37*, 100615. https://doi.org/10.1016/j.hermed.2022.100615

Agresti, A. (2017). *Statistical Methods for the Social Sciences*. (5th Edn.), Pearson.

Ahn, J., Grün, I. U., & Mustapha, A. (2004). Antimicrobial and antioxidant activities of natural extracts in vitro and in ground beef. *Journal of Food Protection*, *67*(1), 148–155. https://doi.org/10.4315/0362-028X-67.1.148

Ajourloo, M., Khanjari, A., Misaghi, A., Akhondzadeh Basti, A., Kamkar, A., Yadegar, F., Gholami, F., Khansavar, F., & Fallah, F. (2021). Combined effects of *Ziziphora clinopodioides* essential oil and lysozyme to extend shelf-life and control *Listeria monocytogenes* in Balkan-style fresh sausage. *Food Science and Nutrition*, *9*(3), 1665–1675. https://doi.org/10.1002/fsn3.2141

Ajuwon, O. R., Ayeleso, A. O., & Adefolaju, G. A. (2018). The potential of South African herbal tisanes, Rooibos and honeybush in the management of type 2 diabetes mellitus. *Molecules*, *23*, 3207. https://doi.org/10.3390/molecules23123207

Ajuwon, O., Marnewick, J., & Davids, L. (2015). Rooibos (*Aspalathus linearis*) and its major flavonoids — Potential against oxidative stress-induced conditions. *IntechOpen*. https://www.intechopen.com/books/basic-principles-and-clinical-significance-of-oxidative-stress/rooibos-aspalathus-linearis-and-its-major-flavonoids-potential-against-oxidative-stress-induced-cond. Retrieved on 17 February 2021.

Alara, O., Abdurahman, N., Afolabi, H., & Olalere, O. (2018). Efficient extraction of antioxidants from *Vernonia cinerea* leaves: Comparing response surface methodology and artificial neural network. *Beni-Suef University Journal of Basic and Applied Sciences*, *7*(3), 276–285. https://doi.org/10.1016/j.bjbas.2018.03.007

Al-Hijazeen, M., & Al-Rawashdeh, M. (2019). Preservative effects of rosemary extract (*Rosmarinus officinalis* L.) on quality and storage stability of chicken meat patties. *Food Science and Technology*, *39*(1), 27–34. https://doi.org/10.1590/1678-457x.24817

Almasaudi, S. (2021). The antibacterial activities of honey. *Saudi Journal of Biological Sciences*, *28*(4), 2188–2196. https://doi.org/10.1016/j.sjbs.2020.10.017

Aminzare, M., Hashemi, M., Ansarian, E., Bimkar, M., Azar, H., & Mehrasbi, M., et al. (2019). Using natural antioxidants in meat and meat products as preservatives: A review. *Advances in Animal and Veterinary Sciences*, *7*(5), 417–419. https://doi.org/10.17582/journal.aavs/2019/7.5.417.426

Bañón, S., Díaz, P., Rodríguez, M., Garrido, M. D., & Price, A. (2007). Ascorbate, green tea and grape seed extracts increase the shelf-life of low sulphite beef patties. *Meat Science*, *77*, 626–633. https://doi.org/10.1016/j.meatsci.2007.05.015

Boles, J. A., & Parrish, F. C. (1990). Sensory and chemical characteristics of precooked mircrowave-reheatable pork roasts. *Journal of Food Science*, *55*(3), 618–620. https://doi.org/10.1111/j.1365-2621.1990.tb05190.x

Chibane, L. B., Degraeve, P., Ferhout, H., Bouajila, J., & Oulahal, N. (2018). Plant antimicrobial polyphenols as potential natural food preservatives. *Journal of the Science of Food and Agriculture*, *99*(4), 1457–1474. https://doi.org/10.1002/jsfa.9357

Carballo, D. E., Mateo, J., Andrés, S., Giráldez, F. J., Quinto, E. J., Khanjari, A., Operta, S., & Caro, I. (2019). Microbial growth and biogenic amine production in a Balkan-style fresh sausage during refrigerated storage under a CO_2-containing anaerobic atmosphere: Effect of the addition of *Zataria multiflora* essential oil and hops extract. *Antibiotics*, *8*(4), 227. https://doi.org/10.3390/antibiotics8040227

Carballo, J. (2021). Sausages: nutrition, safety, processing and quality improvement. *Foods*, *10*(4), 890. https://doi.org/10.3390/foods10040890

Chan, E. W. C., Soh, E. Y., Tie, P. P., & Law, Y. P. (2011). Antioxidant and antibacterial properties of green, black, and herbal teas of *Camellia sinensis*. *Pharmacognosy Research, 3*, 266–272. https://doi.org/10.4103/0974-8490.89748

Charimba, G., Hugo, C. J., & Hugo, A. (2010). The growth, survival and thermal inactivation of *Escherichia coli* O157:H7 in a traditional South African sausage, *Meat Science*, *85*(1), 89–95. https://doi.org/10.1016/j.meatsci.2009.12.010

Charimba, G., Hugo, C. J., & Hugo, A. (2012). The incidence of diarrhoeagenic *Escherichia coli* in minced beef and boerewors. *Food Research International*, *47*, 353–358. https://doi.org/10.1016/j.foodres.2011.08.002

Chizzolini, R., Novelli, E., & Zanardi, E. (1998). Oxidation in traditional Mediterranean meat products. *Meat Science*, *49*, S87–S99. https://doi.org/10.1016/s0309-1740(98)90040-7

Clinical and Laboratory Standards Institute. (2007). Performance Standards for Antimicrobial Susceptibility Testing, 17th Informational Supplement M100-S17. *Journal of Clinical Microbiology*, *27*, 1–182. https://webstore.ansi.org/standards/clsi/m100s17

Coetzee, G., Marx, I. J., Pengilly, M., Bushula, V. S., Joubert, E., & Bloom, M. (2016). Effect of rooibos and honeybush tea extracts against *Botrytis cinerea*. *South African Journal of Enology and Viticulture*, *29*(1). https://doi.org/10.21548/29-1-1449

Compusense Inc. (2023). Consumer and sensory testing made simple. Compusense Inc. https://compusense.com/

Cullere, M., Hoffman, L., & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products. *Meat Science*, *95*(1), 72–77. https://doi.org/10.1016/j.meatsci.2013.04.018

Cullere, M., Tasoniero, G., Secci, G., Parisi, G., Smit, P., Hoffman, L. C., & Dalle Zotte, A. (2019). Effect of the incorporation of a fermented rooibos (*Aspalathus linearis*) extract in the

manufacturing of rabbit meat patties on their physical, chemical, and sensory quality during refrigerated storage. *LWT – Food Science and Technology*, *108*, 31–38. https://doi.org/10.1016/j.lwt.2019.03.051

Damodaran, S., Parkin, Kirk, L., & Fennema, O. R. (2008). *Fennema's Food Chemistry* (4th ed.). CRC Press.

D'Amore, T., Di Taranto, A., Berardi, G., Vita, V., Marchesani, G., Chiaravalle, A., & Iammarino, M. (2020). Sulfites in meat: Occurrence, activity, toxicity, regulation, and detection. A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *19*(5), 2701–2720. https://doi.org/10.1111/1541-4337.12607

de Beer, D., Schulze, A., Joubert, E., de Villiers, A., Malherbe, C., & Stander, M. (2012). Food ingredient extracts of *Cyclopia subternata* (honeybush): Variation in phenolic composition and antioxidant capacity. *Molecules*, *17*(12), 14602–14624. https://doi.org/10.3390/molecules171214602

Domínguez, R., Pateiro, M., Gagaoua, M., Barba, F., Zhang, W., & Lorenzo, J. (2019). A comprehensive review on lipid oxidation in meat and meat products. *Antioxidants*, *8*(10), 429. https://doi.org/10.3390/antiox8100429

du Toit, J., Joubert, E., & Britz, T. (1998). Honeybush tea – A rediscovered indigenous South African herbal tea. *Journal of Sustainable Agriculture*, *12*(2–3), 67–84. https://doi.org/10.1300/j064v12n02_06

Dube, P. (2015). *The antimicrobial and associated antioxidant activity of Rooibos (Aspalathus linearis) and Honeybush (Cyclopia intermedia) herbal teas*. M.Sc. thesis, Cape Peninsula University of Technology, Belville, South Africa.

Dube, P., Meyer, S., & Marnewick, J. (2017). Antimicrobial and antioxidant activities of different solvent extracts from fermented and green honeybush (*Cyclopia intermedia*) plant material. *South African Journal of Botany*, *110*, 184–193. https://doi.org/10.1016/j.sajb.2016.10.010

Efenberger-Szmechtyk, M., Nowak, A., & Czyzowska, A. (2020). Plant extracts rich in polyphenols: antibacterial agents and natural preservatives for meat and meat

products. *Critical Reviews in Food Science and Nutrition* 61(1), 149–178. https://doi.org/10.1080/10408398.2020.1722060

Erickson, L. (2003). Rooibos tea: Research into antioxidant and antimutagenic Properties.–. *Herbalgram*, *59*, 34–45. https://www.herbalgram.org/resources/herbalgram/authors/erickson-laurie/ Retrieved on 10 April 2021.

European Commission. (2021). Agriculture: the Commission approves a new protected geographical indication from South Africa. *European Commission – Daily News* 31 May 2021. Brussels, Belgium. — https://ec.europa.eu/commission/presscorner/detail/en/mex_21_2785 Retrieved on 25 June 2021.

Falowo, A. B., Fayemi, P. O., & Muchenje, V. (2014). Natural antioxidants against lipid–protein oxidative deterioration in meat and meat products: A Review. *Food Research International*, *64*, 171–181. https://doi.org/10.1016/j.foodres.2014.06.022

Falowo, A. B., Muchenje, V., Hugo, C., & Charimba, G. (2016). In vitro antimicrobial activities of *Bidens 88ilosa* and *Moringa oleifera* leaf extracts and their effects on ground beef quality during cold storage. *CyTA – Journal of Food*, *14*(4), 541–546. https://doi.org/10.1080/19476337.2016.1162847

Falowo, A. B., Mukumbo, F., & Muchenje, V. (2019). Phytochemical constituents and antioxidant activity of *Artemisia afra* and *Bidens 88ilosa* essential oil in ground pork. *Journal of Essential Oil-Bearing Plants*, *22*(1), 176–186. https://doi.org/10.1080/0972060x.2019.1574212

Fontana, A. (2000). Understanding the importance of water activity in food. *Cereal Foods World*, *45*(1), X–10. https://www.researchgate.net/publication/286871018_Understanding_the_importance_of_water_activity_in_food. Retrieved on 26 June 2021.

Freitag, A. (2023). The effect of microbial and plant extract preservatives on the chemical, microbial and sensory quality of a traditional fresh South African sausage. Ph.D. Thesis. University of the Free State, Bloemfontein, South Africa.

Garba, L., Muhammad, M., Adamu, M., Isa, S., Abdullahi, M., & Yarma, A. A. (2021). Potential inhibitory effects of *Moringa oleifera* leaf extracts on extended-spectrum β-lactamase-producing bacteria. *Journal of Fundamental and Applied Sciences*, *13(1)*, 137–150. https://doi.org/10.4314/jfas.v13i1.8

Garcia-Fuentes, A., Wirtz, S., Vos, E., & Verhagen, H. (2015). Short review of sulphites as food additives. *European Journal of Nutrition and Food Safety*, *5*(2), 113–120. https://doi.org/10.9734/ejnfs/2015/11557

Gaupp, R., Ledala, N., & Somerville, G. A. (2012). Staphylococcal response to oxidative stress. *Frontiers in Cellular and Infection Microbiology*, *33*, 1–19. https://doi.org/10.3389/fcimb.2012.00033

Gould, G. W., Russell, N. J. (2003). Sulfite. In N. J. Russell, & G. W. Gould (Eds.), *Food preservatives* (pp. 85–101). Boston, MA, Springer. https://doi.org/10.1007/978-0-387-30042-9_5

Gouws, P., Hartel, T., & van Wyk, R. (2014). The influence of processing on the microbial risk associated with Rooibos (*Aspalathus linearis*) tea. *Journal of the Science of Food and Agriculture*, *94*(15), 3069–3078. https://doi.org/10.1002/jsfa.6719

Hadidi, M., Orellana-Palacios, J. C., Agahababaei, F., Gonzalez-Serrano, D. J., Moreno, A., & Lorenzo, J. M. (2022). Plant by-product antioxidants: Control of protein-lipid oxidation in meat and meat products. *LWT – Food Science and Technology*, *169*, 114003. https://doi.org/10.1016/j.lwt.2022.114003

Harrigan, W. F. (1998). *Laboratory Methods in Food Microbiology*. San Diego, Academic Press.

Hertz, R., & Benington, M. (2020). *Biology: The Dynamic Science* (5th Edn., p. 576). Cengage. https://www.cengage.uk/

Hoffman, L. C., Jones, M., Muller, M., Joubert, E., & Sadie, A. (2014). Lipid and protein stability and sensory evaluation of ostrich (*Struthio camelus*) droëwors with the addition of rooibos tea

extract (*Aspalathus linearis*) as a natural antioxidant. *Meat Science*, *96*, 1289–1296. https://doi.org/10.1016/j.meatsci.2013.10.036

Hübsch, Z., Van Vuuren, S., & Van Zyl, R. (2014). Can rooibos (*Aspalathus linearis*) tea have an effect on conventional antimicrobial therapies? *South African Journal of Botany*, *93*, 148–156. https://oi.org/10.1016/j.sajb.2014.04.004

Hugo, A., Roberts, J. J., Erasmus, A. H., & Uys, R. C. B. (1992). Proximate and non-meat protein analysis of boerewors – a preliminary survey. In I. Zondagh, & P. Heinze (Eds.), *Meat science and technology in service of the meat industry* (pp. 147, 161–167). Irene Animal Production Institute (Private Bag X2, Irene 1675), Pretoria, South Africa.

Hugo, C., & Hugo, A. (2015). Current trends in natural preservatives for fresh sausage products. *Trends in Food Science and Technology* *45*(1), 12–23. https://doi.org/10.1016/j.tifs.2015.05.003

Human, C., de Beer, D., Aucamp, M., Marx, I. J., Malherbe, C. J., Viljoen-Bloom, M., van der Rijst, M., & Joubert, E. (2020). Preparation of rooibos extract-chitosan microparticles: Physicochemical characterisation and stability of aspalathin during accelerated storage. *LWT – Food Science and Technology*, *117*, 108653. https://doi.org/10.1016/j.lwt.2019.108653

Ishiwatari, N., Fukuoka, M., & Sakai, N. (2013). Effect of protein denaturation degree on texture and water state of cooked meat. *Journal of Food Engineering*, *117*(3), 361–369. https://doi.org/10.1016/j.jfoodeng.2013.03.013

Jones, M. (2013). *The addition of rooibos tea extract (Aspalathus linearis) as a natural antioxidant to South African droëwors*. M.Sc. Thesis, University of Stellenbosch, Cape Town, South Africa.

Jones, M., Hoffman, L. C., & Muller, M. (2015). Oxidative stability of blesbok, springbok and fallow deer droëwors with added rooibos extract. *South African Journal of Science*, *111*, 1–8. https://doi./10.17159/sajs.2015/20140347

Joubert, E., & De Beer, D. (2012). Phenolic content and antioxidant activity of rooibos food ingredient extracts. *Journal of Food Composition and Analysis, 27*, 45–51. https://doi.org/10.1016/j.jfca.2012.03.01

Joubert, E., Joubert, M. E., Bester, C., de Beer, D., & De Lange, J. H. (2011). Honeybush (*Cyclopia* spp.): From local cottage industry to global markets — The catalytic and supporting role of research. *South African Journal of Botany*, *77*, 887–907. https://doi.org/10.1016/j.sajb.2011.05.014

Joubert, E., Richards, E., van der Merwe, J., De Beer, D., Manley, M., & Gelderblom, W. (2008). Effect of species variation and processing on phenolic composition and in vitro antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry*, *56*(3), 954–963. https://doi.org/10.1021/jf072904a

Joubert, E., Winterton, P., Britz, T. J., & Gelderblom, W. C. A. (2005). Antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos (*Aspalathus linearis*). *Journal of Agricultural and Food Chemistry*, *53*, 10260–10267. https://doi.org/10.1021/jf051355a

Kalogianni, A., Lazou, T., Bossis, I., & Gelasakis, A. (2020). Natural phenolic compounds for the control of oxidation, bacterial spoilage, and foodborne pathogens in meat. *Foods*, *9*(6), 794. https://doi.org/10.3390/foods9060794.

Kamara, B. I., Brandt, E. V., Ferreira, D., & Joubert, E. (2003). Polyphenols from honeybush tea (*Cyclopia intermedia*). *Journal of Agricultural and Food Chemistry, 51*(13), 3874–3879. https://doi.org/10.1021/jf0210730

Kamara, B., Brand, D., Brandt, E., & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, *52*(17), 5391–5395. https://doi.org/10.1021/jf040097z

Kanatt, S., Chander, R., & Sharma, A. (2008). Chitosan and mint mixture: A new preservative for meat and meat products. *Food Chemistry*, *107*(2), 845–852. https://doi.org/10.1016/j.foodchem.2007.08.088

Keshebo, D., Washe, A., & Alemu, F. (2016). Determination of antimicrobial and antioxidant activities of extracts from selected medicinal plants. *American Scientific Research Journal for Engineering, Technology, and Sciences, 16*, 212–222. http://asrjetsjournal.org/index.php/American_Scientific_Journal/article/download/948/685 . Retrieved on 23 May 2021.

Kim, M. K. (2006). Impact of temperature and pH on the survival of *Listeria monocytogenes* in souse meat. http://www.lib.ncsu.edu/theses/available/etd-11012006-153559/unrestricted/etd.pdf. Retrieved on 3 July 2021.

Koch, I., Muller, M., Joubert, E., van der Rijst, M., & Næs, T. (2012). Sensory characterization of rooibos tea and the development of a rooibos sensory wheel and lexicon. *Food Research International, 46*(1), 217–228. https://doi.org/10.1016/j.foodres.2011.11.028

Krafczyk, N., & Glomb, M. (2008). Characterization of phenolic compounds in rooibos tea. *Journal of Agricultural and Food Chemistry*, *56*(9), 3368–3376. https://doi.org/10.1021/jf703701n

Kumar, Y., Yadav, D. N., Ahmad, T., & Narsaiah, K. (2015). Recent trends in the use of natural antioxidants for meat and meat products. *Comprehensive Reviews in Food Science and Food Safety*, *14*(6), 796–812. https://doi.org/10.1111/1541-4337.12156

Lawless, H. T., & Heymann, H. (1998). *Sensory evaluation of food: Principles and practices*. Aspen.

Lumivero. (2023). XLSTAT. The leading Data Analysis and Statistical Solution for Microsoft Excel. https://www.xlstat.com/en

Mahdavikia, F., Saharkhiz, M., & Karami, A. (2017). Defensive response of radish seedlings to the oxidative stress arising from phenolic compounds in the extract of peppermint (*Mentha × piperita* L.). *Scientia Horticulturae*, *214*, 133–140. https://doi.org/10.1016/j.scienta.2016.11.029

Makarewicz, M., Drożdż, I., Tarko, T., & Duda-Chodak, A. (2021). The interactions between polyphenols and micro-organisms, especially gut microbiota. *Antioxidants*, *10*(2), 188. https://doi.org/10.3390/antiox10020188

Manessis, G., Kalogianni, A., Lazou, T., Moschovas, M., Bossis, I., & Gelasakis, A. (2020). Plant-derived natural antioxidants in meat and meat products. *Antioxidants*, *9*(12), 1215. https://doi.org/10.3390/antiox9121215

Mantzourani, I., Daoutidou, M., Dasenaki, M., Nikolaou, A., Alexopoulos, A., Terpou, A., Thomaidis, N., & Plessas, S. (2022). Plant extract and essential oil application against food-borne pathogens in raw pork meat. *Foods*, *11*(6), 861. https://doi.org/10.3390/foods11060861

Marnewick, J. L., Joubert, E., Joseph, S., Swanevelder, S., Swart, P., & Gelderblom, W. C. A. (2005). Inhibition of tumour promotion in mouse skin by extracts of rooibos *(Aspalathus linearis)* and honeybush *(Cyclopia intermedia)*. *South African Cancer Letters, 224*, 193–202. https://doi.org/10.1016/j.canlet.2004.11.014

Marnewick, J. L., Rautenbauch, F., Venter, I., Neethling, H., Blackhurst, D. M., Wolmarans, P., & Macharia, M. (2011). Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. *Journal of Ethnopharmacology*, *133*, 46–52. https://doi.org/10.1016/j.jep.2010.08.061

Márquez-Rodríguez, A., Nevárez-Baca, S., Lerma-Hernández, J., Hernández-Ochoa, L., Nevárez-Moorillon, G., & Gutiérrez-Méndez, N., et al. (2020). In vitro antibacterial activity of *Hibiscus sabdariffa* L. phenolic extract and its' in situ application on shelf-life of beef meat. *Foods*, *9*(8), 1080. https://doi.org/10.3390/foods9081080

Mathenjwa, S. A. (2010). *Evaluation of natural preservatives for use in a traditional South African sausage*. M.Sc. dissertation, University of the Free State, Bloemfontein, South Africa.

Mathenjwa, S. A., Hugo, C. J., Bothma, C., & Hugo, A. (2012). Effect of alternative preservatives on the microbial quality, lipid stability and sensory evaluation of boerewors. *Meat Science*, *91*(2), 165–172. https://doi./10.1016/j.meatsci.2012.01.014

McKay, D., & Blumberg, J. (2007). A review of the bioactivity of South African herbal teas: rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*). *Phytotherapy Research*, *21*(1), 1–16. https://doi.org/10.1002/ptr.1992

Mena, P., Cirlini, M., Tassotti, M., Herrlinger, K., Dall'Asta, C., & Del Rio, D. (2016). Phytochemical profiling of flavonoids, phenolic acids, terpenoids, and volatile fraction of a

rosemary (*Rosmarinus officinalis* L.) extract. *Molecules*, *21*(11), 1576. https://doi.org/10.3390/molecules21111576

Miklasińska-Majdanik, M., Kępa, M., Wojtyczka, R., Idzik, D., & Wąsik, T. (2018). Phenolic compounds diminish antibiotic resistance of *Staphylococcus aureus* clinical strains. *International Journal of Environmental Research and Public Health*, *15*(10), 2321. https://doi.org/10.3390/ijerph15102321

Mladenović, K. G., Grujović, M. Ž., Kiš, M., Furmeg, S., Tkalec, V. J., Stefanović, O. D., & Kocić-Tanackov, S. D. (2021). Enterobacteriaceae in food safety with an emphasis on raw milk and meat. *Applied Microbiology and Biotechnology*, *105*(23), 8615–8627. https://doi.org/10.1007/s00253-021-11655-7

Mudenda, S., Hikaambo, C. N., Chabalenge, B., Mfune, R. L., Mufwabi, W., Ngazimbi, M., Matafwali, S., & Daka, V. (2023). Antibacterial activities of honey against *Escherichia coli* and *Staphylococcus aureus*: A potential treatment for bacterial infections and alternative to antibiotics. *Journal of Pharmacognosy and Phytochemistry*, *12*, 6–13. https://doi.org/10.22271/phyto.2023.v12.i3a.14655

Mulat, M., Khan, F., Muluneh, G., & Pandita, A. (2020). Phytochemical profile and antimicrobial effects of different medicinal plant: Current knowledge and future perspectives. *Current Traditional Medicine*, *6*(1), 24–42. https://doi.org/10.2174/2215083805666190730151118

Mulaudzi, N., Combrink, S., Vermaak, I., Joubert, E., & Viljoen, A. (2022). High performance thin layer chromatography fingerprinting of rooibos (*Aspalathus Linearis*) and honeybush (*Cyclopia genistoides*, *Cyclopia intermedia* and *Cyclopia subternata*) teas. *Journal of Applied Research on Medicinal and Aromatic Plants*, *30*, 100378. https://doi.org/10.1016/j.jarmap.2022.100378

Murali, N. (2011). *Antibacterial activity of plant extracts against foodborne pathogenic bacteria and food spoilage bacteria*. M.Sc. Thesis, University of Arkansas, Fayettteville, United States of America.

Mwanza, E. P., Hugo, A., Charimba, G., & Hugo, C. J. (2022). Pathogenic potential and control of *Chryseobacterium* species from clinical, fish, food and environmental sources. *Microorganisms*, *10*, 895. https://doi.org/10.3390/microorganisms10050895

Nikmaram, N., Budaraju, S., Barba, F., Lorenzo, J., Cox, R., Mallikarjunan, K., & Roohinejad, S. (2018). Application of plant extracts to improve the shelf-life, nutritional and health-related properties of ready-to-eat meat products. *Meat Science*, *145*, 245–255. https://doi.org/10.1016/j.meatsci.2018.06.031

Orlando, P., Chellan, N., Louw, J., Tiano, L., Cirilli, I., Dludla, P., Joubert, E., & Muller, C. J. F. (2019). Aspalathin-rich green rooibos extract lowers LDL-cholesterol and oxidative status in high-fat diet-induced diabetic vervet monkeys. *Molecules*, *24*, 1713. https://doi.org/10.3390/molecules24091713

Pateiro, M., Gómez-Salazar, J., Jaime-Patlán, M., Sosa-Morales, M., & Lorenzo, J. (2021). Plant extracts obtained with green solvents as natural antioxidants in fresh meat products. *Antioxidants*, *10*(2), 181. https://doi.org/10.3390/antiox10020181

Pfukwa, T., Fawole, O., Manley, M., Gouws, P., Opara, U., & Mapiye, C. (2019). Food preservative capabilities of grape (*Vitis vinifera*) and clementine mandarin (*Citrus reticulata*) by-products extracts in South Africa. *Sustainability*, *11*(6), 1746. https://doi.org/10.3390/su11061746

Prinsloo, J. (2021). Behind boerewors. *Whetstone*. https://www.whetstonemagazine.com/journal/behind-boerewors

Rahardiyan, D. (2018). Antibacterial potential of catechin of tea (*Camellia sinensis*) and its applications. *Food Research*, *3*(1), 1–6. https://doi.org/10.26656/fr.2017.3(1).097

Ripoll, R., Joy, M., & Munoz, F. (2001). Use of dietary vitamin E and selenium (Se) to increase the shelf-life of modified atmosphere packaged light lamb meat. *Meat Science*, *89*, 1–5. https://doi: 10.1016/j.meatsci.2010.09.008

Rock, C. M., Brassill, N., Dery, J. L., Carr, D., McLain, J. E., Bright, K. R., & Gerba, C. P. (2019). Review of water quality criteria for water reuse and risk-based implications for irrigated produce under the FDA (Food Safety Modernization Act), produce safety rule. *Environmental Research*, *172*, 616–629. https://doi.org/10.1016/j.envres.2018.12.050

Rodford, R. (1997). Safety evaluation of preservatives. *International Journal of Cosmetic Science*, *19*(6), 281–290. https://doi.org/10.1111/j.1467-2494.1997.tb00192.x

Roller, S., Sagoo, S., Board, R., O'Mahony, T., Caplice, E., Fitzgerald, G., Fogden, M., Owen, M., & Fletcher, H. (2002). Novel combinations of chitosan, carnocin and sulphite for the preservation of chilled pork sausages. *Meat Science*, *62*(2), 165–177. https://doi.org/10.1016/s0309-1740(01)00243-1

Rooibos Limited. (2023). *Our products*. Available at: https://www.rooibosltd.co.za/our-products

Schilling, M., Pham, A., Williams, J., Xiong, Y., Dhowlaghar, N., Tolentino, A., & Kin, S. (2018). Changes in the physiochemical, microbial, and sensory characteristics of fresh pork sausage containing rosemary and green tea extracts during retail display. *Meat Science*, *143*, 199–209. https://doi.org/10.1016/j.meatsci.2018.05.009

Senanayake, S. (2018). Rosemary extract as a natural source of bioactive compounds. *Journal of Food Bioactives*, *2*(2), 51–57. https://doi.org/10.31665/jfb.2018.2140

Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, *98*, 21–23. https://doi.org/10.1016/j.meatsci.2014.03.020

Shapton, D. A., & Shapton, N. F. (1991). Criteria for ingredients and finished products. *Principles and practices for the safe processing of foods* (pp. 377–444). Oxford, Butterworth-Heinemann Ltd. https://doi.org/10.1016/b978-0-7506-1121-3.50020-7

Simpson, M., Hjelmqvist, D., López-Alarcón, C., Karamehmedovic, N., Minehan, T., Yepremyan, A., Salehani, B., Lissi, E., Joubert, E., Udekwu, K., & Alarcon, E. (2013). Anti-peroxyl radical quality and antibacterial properties of rooibos infusions and their pure glycosylated polyphenolic constituents. *Molecules*, 18(9), 11264–11280. https://doi.org/10.3390/molecules180911264

Smit, P. (2020). *The effect of honeybush (Cyclopia subternata) extract in dry-cured and fermented warthog salami with reduced added nitrate*. M.Sc. Thesis, University of Stellenbosch, Cape Town, South Africa.

Smit, P., Cullere, M., Dalle Zotte, A. D., Balzan, S., Hoffman, L., & Novelli, E. (2020). Effects of honeybush (*Cyclopia subternata*) extract on physico-chemical, oxidative and sensory traits

of typical Italian salami. *Food Science and Nutrition*, *8*(5), 2299–2306. https://doi.org/10.1002/fsn3.1509

Snijman, P. W., Joubert, E., Ferreira, D., Li, X.-C., Ding, Y., Green, I. R., & Gelderblom, W. C. (2009). Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and Trolox. *Journal of Agricultural and Food Chemistry*, *57*(15), 6678–6684. https://doi.org/10.1021/jf901417k

Snijman, P. W., Swanevelder, S., Joubert, E., Green, I. R., & Gelderblom, W. C. A. (2007). The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*): Some dose-response effects on mutagen activation-flavonoid interactions. *Mutation Research – Genetic Toxicology and Environmental Mutagenesis*, *632*, 111–123. https://doi.org/10.1016/j.mrgentox.2007.03.009

South African Department of Agriculture, Forestry and Fisheries (SA DAFF). (2018). Guidelines on the verification of compliance with process hygiene control and microbiological food safety performance objectives under the Meat Safety Act. Notice No. VPN/52/2018. http://www.rmaa.co.za/wp-content/uploads/2018/12/VPN-52-Microbiological-Reference-Criteria-for-Meat-19-October 2018-Draft.pdf

South African Department of Health. (1990). Regulation governing the composition and labelling of raw Boerewors, raw species sausage and raw mixed-species sausage. Published under Government Notice No. R2718 of 23 November 1990. www.doh.gov.zadocsregulations1990reg2718. Retrieved on 25 April 2021.

South African Department of Health. (2001). Meat annexure: Fresh and Processed meat. http://www.doh.gov.za/docs/factsheets/guidelines/foodservice/meat.pdf. Retrieved on 25 April 2021.

South African Department of Health. Directorate: Food Control. (2005). Guidelines for Environmental Health Officers on the Interpretation of Microbiological Analysis Data of Food. https://www.thefoodsafetynetwork.co.za/images/stories/docs/consumers/Directorate_Food_Control_information.pdf. Retrieved on 26 April 2021.

SPSS. (2022). IBM SPSS Statistics for Windows, Version 29.0. Armonk, NY, IBM Corp.

Stander, M., Van Wyk, B., Taylor, M., & Long, H. (2017). Analysis of phenolic compounds in rooibos tea (*Aspalathus linearis*) with a comparison of flavonoid-based compounds in natural populations of plants from different regions. *Journal of Agricultural and Food Chemistry*, *65*(47), 10270–10281. https://doi.org/10.1021/acs.jafc.7b03942

Stone, H., & Sidel, J. L. (2004). *Sensory evaluation practices*. Amsterdam: Elsevier Academic Press.

Sun, Y., Li, Y., Song, H., & Zhu, Y. (2011). Microbial fermentation for food preservation. In M. Rai, & M. Chikindas (Eds.), *Natural antimicrobials in food safety and quality* (pp. 77–94). Oxfordshire, UK, CAB International. https://doi.org/10.1079/9781845937690.0077

Surendran Nair, M., Nair, D., Kollanoor Johny, A., & Venkitanarayanan, K. (2020). Use of food preservatives and additives in meat and their detection techniques. *Meat Quality Analysis, 12*, 187–213. https://doi.org/10.1016/b978-0-12-819233-7.00012-4

Tchatchouang, C. K., Fri, J., De Santi, M., Brandi, G., Schiavano, G. F., Amagliani, G., & Ateba, C. N. (2020). Listeriosis outbreak in South Africa: A comparative analysis with previously reported cases worldwide. *Microorganisms*, *8*(1), 135. https://doi.org/10.3390/microorganisms8010135

Tiwari, B. K., Valdramidis, V. P., O' Donnel, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. https://doi.org/10.1021/jf900668n

Uličná, O., Vančová, O., Božek, P., Čársky, J., Šebeková, K., Boor, P., Nakano, M., & Greksák, M. (2006). Rooibos tea (*Aspalathus linearis*) partially prevents oxidative stress in streptozotocin-induced diabetic rats. *Physiological Research*, *55*(2), 157–164. https://doi.org/10.33549/physiolres.930778

Vally, H., Misso, N., & Madan, V. (2009). Clinical effects of sulphite additives. *Clinical & Experimental Allergy*, *39*(11), 1643–1651. https://doi.org/10.1111/j.1365-2222.2009.03362.x

van Schalkwyk, C. P. B. (2010). *Evaluation of a natural preservative in a boerewors model system*. M.Sc. Thesis, University of the Free State, Bloemfontein, South Africa.

van Schalkwyk, C. P. B., Hugo, A., Hugo, C. J., & Bothma, C. (2013). Evaluation of a natural preservative in a boerewors model system. *Journal of Food Processing and Preservation*, *37*(5), 824–834. https://doi.org/10.1111/j.1745-4549.2012.00706.x

Vattem, D. A., Lin, Y. T., Ghaedian, R., & Shetty, K. (2005). Cranberry synergies for dietary management of *Helicobacter pylori* infections. *Process Biochemistry*, *40*(5), 1583–1592. https://doi.org/10.1016/j.procbio.2004.06.024

Walker, R. (1985). Sulphiting agents in foods: Some risk/benefit considerations. *Food Additives and Contaminants*, *2*(1), 5–24. https://doi.org/10.1080/02652038509373522

Warner, R. D. (2017). The eating quality of meat—IV water-holding capacity and juiciness. In *Lawrie's meat science* (8th Edn., pp. 419–459). Woodhead Publishing. https://doi.org/10.1016/B978-0-08-100694-8.00014-5.

WHO Codex Alimentarius. (2019). *General standard for food additives (GSFA)—Codex Standard 192–1995*. Rome, Italy: Secretariat of the Joint FAO/WHO Food Standards Programme, FAO.

Windvogel, S. (2020). Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.): From bush teas to potential therapy for cardiovascular disease. In M. C. Hueda (Ed.), *Nutraceuticals - past, present and future*. IntechOpen. https://doi.org/10.5772/intechopen.86410

World Health Organization. (2018). *Listeriosis*. Available at: https://www.who.int/news-room/fact-sheets/detail/listeriosis

Wu, H., Bak, K. H., Goran, G. V., & Tatiyaborworntham, N. (2022). Inhibitory mechanisms of polyphenols on heme protein-mediated lipid oxidation in muscle food: New insights and advances. *Critical Reviews in Food Science and Nutrition*, *30*, 1–19. https://doi.org/10.1080/10408398.2022.2146654

Xie, Y., Chen, J., Xiao, A., & Liu, L. (2017). Antibacterial activity of polyphenols: Structure-activity relationship and influence of hyperglycemic condition. *Molecules*, *22*(11), 1913. https://doi.org/10.3390/molecules22111913

Yildirim, A., Mavi, A., Oktay, M., Kara, A. A., Algur, Ö. F., & Bilaloglu, V. (2000). Comparison of antioxidant and antimicrobial activities of Tilia (*Tilia argentea Desf ex DC*), sage (*Salvia triloba L.*), and black tea (*Camellia sinensis*) extracts. *Journal of Agricultural and Food Chemistry*, *48*(10), 5030–5034. https://doi.org/10.1021/jf000590k

Yu, H. H., Chin, Y., & Paik, H. (2021). Application of natural preservatives for meat and meat products against food-borne pathogens and spoilage bacteria: A review. *Foods*, *10*(10), 2418. https://doi.org/10.3390/foods10102418