THE EFFECT OF MICROBIAL AND PLANT EXTRACT PRESERVATIVES ON THE CHEMICAL, MICROBIAL AND SENSORY QUALITY OF A TRADITIONAL FRESH SOUTH AFRICAN SAUSAGE

By

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DECLARATION

I declare that the Doctoral Degree research thesis, that I herewith submit, for the Doctoral Degree qualification in Ph.D. Food Science, in the Faculty of Natural and Agricultural Science at the University of the Free State, is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

A. Freitag

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TABLE OF CONTENTS

The language, formatting and reference style of this thesis are in accordance with the requirements for *Food Research International*.

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LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF ABBREVIATIONS

*a**	Redness
AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
APT	All-purpose Tween 80
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
aw	Water activity
AWCD	Average well colour development
*b**	Yellowness
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BP	Baird-Parker
BPW	Buffered peptone water
°C	Degrees Celsius
*C**	Chroma
CD	Colour development
cfu	Colony forming units
CLPP	Community-level physiological profiling
cm	Centimeter
CO_2	Carbon dioxide
°	Degrees
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DoH	Department of Health (South Africa)
E	Shannon evenness
e.g.	Exempli gratia; for example

EPS	Expanded polystyrene
et al.	*Et alia*
etc.	Etcetera
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FSA	Food Standards Agency
g	Gram
GRAS	Generally recognized as safe
H	Shannon diversity
h	Hours
*H**	Hue angle
HACCP	Hazard analysis and critical control points
HNO_3	Nitric acid
HSO_3^-	Bisulphite
H_2O	Water
H_2SO_3	Sulphurous acid
i.e.	Id est; that is
kg	Kilogram
KD1	Kalsec Duralox plant extract 1 (0.35% NR20)
KD2	Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30)
L*	Lightness
LAB	Lactic acid bacteria
M	Molar
MAP	Modified atmosphere packaging
MDA	Malondialdehyde
mEq	Milliequivalents

Mg	Magnesium
mg	Milligram
mg/100 g	Milligrams per 100 grams
min	Minute
ml	Millilitre
mm	Millimetre
MPN	Most probable number
MSG	Monosodium glutamate
MUG	4-methylumbelliferyl-β-D-glucuronide
N	Normality
n	Population size
NA	Not applicable
Na	Sodium
NAD	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
Na_2SO_3	Sodium sulphite
$Na_2S_2O_5$	Sodium metabisulphite
NC	Negative control with no added preservative
NCSS	Number Cruncher Statistical System
ND	Not detected
NH_4Cl	Ammonium chloride
nm	Nanometres
No.	Number
Nr.	Number
NS	Not significant
NSA	Not statistically analysed
O2	Oxygen
OD	Optical density
p	Significance level
PC	Positive control with inclusion level SO_2 for Boerewors

PCA	Principle Component Analysis
ppm	Parts per million
PrC1	Protective culture 1 (0.025% BLC 20)
PrC2	Protective culture 2 (0.0125% BLC 48)
PVC	Polyvinyl chloride
%	Percentage
R	South African Rand
RBC	Rose-Bengal Chloramphenicol
rH	Relative humidity
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Shannon richness
s	Seconds
SO_2	Sulphur dioxide
SO_3^{2-}	Sulphite
SAPP	Sodium acid pyrophosphate
STEC	Shiga toxin-producing *E. coli*
STPP	Sodium tripolyphosphate
SANS	South African National Standard
SHMP	Sodium hexametaphosphate
SPCA	Standard plate count agar
spp.	Species
ssp.	Subspecies
TBC	Total bacterial count
tBHQ	Tert-butylhydroquinone
TSPP	Tetrasodium pyrophosphate
TBARS	Thiobarbituric acid reactive substances
TVC	Total viable counts
UK	United Kingdom
USA	United States of America

USDA	United States Department of Agriculture
VRBGA	Violet red bile glucose agar
WHC	Water holding capacity
WHO	World Health Organization
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

Meat from slaughtered animals and packaged meat that has not undergone treatment other than refrigeration fall under the term “fresh meat” (Zhou, Xu, & Liu, 2010). According to the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA-FSIS, 2014), fresh sausages are a coarse or finely comminuted (reduced to minute particles) meat product. They must be refrigerated and thoroughly cooked before eating. Boerewors is a traditional South African fresh sausage that is available throughout the year and is usually manufactured in butcheries, meat processing plants and even at home (Mathenjwa, Hugo, Bothma, & Hugo, 2012). The concept of meat quality is ambiguous; high-quality meat may be defined as meat that satisfies the needs and demands of consumers, in particular, organoleptic quality, nutritional quality, hygienic quality, and processing suitability. Fresh meat has a diverse nutrient composition which makes it susceptible to spoilage caused by microorganisms and oxidation. Therefore, it is essential to provide adequate preservation to maintain the quality and safety of the product (Aymerich, Picouet, & Monfort, 2008).

Sulphur dioxide (SO_2) has been used as the main preservative in meat products for many decades and is also the preservative in Boerewors at an inclusion level of 450 mg/kg of meat. It has been successful in preserving food by inhibiting microorganisms growth and preventing reactions, such as oxidation, that could cause discolouration or off-flavours (D’Amore Di Taranto, Berardi, Vita, Marchesani, Chiaravalle, & Iammarino, 2020). Sulphites are chemical preservatives that are generally regarded as safe (GRAS); however, they can trigger allergic reactions, such as asthmatic attacks and urticaria, in hypersensitive people (Vally, Misso, & Madan, 2009). In recent years, technologies in the food industry have improved and consumers have changed their expectations, leading to a more diverse and natural approach to the use of safer additives (Ribeiro, Santos, Silva, Pereira, Santos, da Silva Lannes, & da Silva, 2019).

Many natural alternatives have been investigated as potential SO_2 replacements, and the most researched would be plant derived preservatives (Teshome, Forsido, Rupasinghe, & Olika Keyata, 2022). The bioactive compounds in plant extracts have antimicrobial, antioxidant and health-promoting properties, which makes it an ideal option for natural food preservation (Awad, Kumar, Ismail-Fitry, Jusoh, Ab Aziz, & Sazili, 2022). The use of protective cultures and bacteriocins, known as biopreservation, is fairly new in the food industry. Protective cultures can exert an inhibitory effect against spoilage and pathogenic microorganisms as a

consequence of their competition for nutrients. Another way these cultures inhibit microorganisms is by producing bacteriocins or other compounds, such as lactic acid (Melero, Vinuesa, Diez, Jaime, & Rovira, 2013).

Even with healthy alternatives on the market or natural preservatives in the product, consumers are not keen on changing their habits or compromising on sensory aspects (Verbeke, 2006). The use of natural preservatives should not alter the safety and qualities of the product negatively, and for it to be a suitable replacement, it should maintain these qualities just as well as SO_2, without any adverse health effects (Marchante, Loarce, Izquierdo-Cañas, Alañón, García-Romero, Pérez-Coello, & Díaz-Maroto, 2019).

The first aim of this study was to determine whether natural preservatives could maintain the quality and stability of Boerewors.

The following hypothesis was formulated:

In addition to food safety, maintaining the particular taste of Boerewors is probably the most important factor for consumers. Consumers have generally developed a disapproving attitude towards food that compromises the sensory experience, in exchange for potential health benefits (Verbeke, 2006). When looking at potential replacers for SO_2, a few purposes are considered: preventing microbial growth (Lisanti, Blaiotta, Nioi, & Moio, 2019), maintaining sensory quality, such as colour and taste (Chen, Shih, Tsai, Jiang, Hou, & Huang, 2021), and preventing adverse changes, such as oxidation, in the chemical composition of the product (Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2009). Finding natural preservatives that can adequately fulfil these purposes is the key to consumer satisfaction and health (Mesías, Martín, & Hernández, 2021).

The null hypothesis would be that replacing SO_2 with natural preservatives resulted in Boerewors with unacceptable changes in physico-chemical, microbial and/or sensory quality. This hypothesis will be tested in Chapter 3 of the thesis.

The second aim of this study was to investigate the effect of natural preservatives in Boerewors on the bacterial community profiles, by using Biolog™ EcoPlate™.

The following hypothesis was formulated:

Analysing microbial communities in food products can possibly predict the effectiveness of treatments, such as SO_2 replacement, to control microbial quality and product safety (Weber & Legge, 2010; Furtak, Grządziel, Gałązka, & Niedźwiecki,

2019). The advantages of using the community-level physiological profiling approach include the low manpower requirements, and the reliance on metabolic traits, leading to functional characterization of temporal and spatial changes in microbial communities. A concerning disadvantage is potential bias due to the growth phase that requires 2–7 days for colour development in the wells (Garland, 1997). The growth patterns, and differences in substrates utilized, could give insight into what the microbial communities prefer and what possibly hinders the growth, as each microbial community utilizes different amounts of chemical compounds and also at different rates (Yeh, Line, Hinton, Gao, & Zhuang, 2019).

The null hypothesis would be that replacing SO_2 with natural preservatives would result in adverse changes in the microbial communities in Boerewors. This hypothesis will be evaluated in Chapter 4 of the thesis.

The third and final aim of this study was to determine the effect of plant derived, natural preservatives in Boerewors, on the growth and survival of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) as potential pathogens.

The following hypothesis was formulated:

An additional concern to food spoilage is the growth of potential pathogens, like *E. coli* and *S. aureus*, that may have detrimental effects on the food safety of the product (Godfree, & Farrell, 2005). Most preservatives are added to fresh food products for this exact reason, and a change or disruption in the preservation process may allow for pathogens to emerge or re-emerge (Miller, Smith, & Buchanan, 1998). Fresh meat products are complex systems, and natural additives may have unexpected effects on the growth, and even viability, of microorganisms. In this study, it was suspected that the natural preservatives in Boerewors formulations would have synergistic antimicrobial effects, in combination with low storage temperature (4 °C), to restrict the growth and survival of *E. coli* or *S. aureus* strains.

The null hypothesis would be that replacing SO_2 with plant derived natural preservatives would allow for the survival and growth of *E. coli* and/or *S. aureus*. This hypothesis will be tested in Chapter 5 of the thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Fresh meat products, such as Boerewors, are highly perishable due to their biological composition; it is an ideal environment for the growth of food spoilage microorganisms and common pathogens. It is, therefore, imperative that adequate preservation techniques are applied to ensure food safety and quality (Aymerich, Picouet, & Monfort, 2008). Most of the fresh meat preservation technologies are focused on the inhibition of microorganisms, although, it is also important to minimise other deteriorations, like colour and oxidative changes.

Chemical preservatives, such as sulphur dioxide (SO_2), are often used in fresh meat products, to improve the shelf-life and maintain the colour stability (Wedzicha & Mountfort, 1991). However, consumers are more aware about preservatives and the negative health effects associated with chemical preservatives and they are now seeking naturally preserved food products (Bañón, Diaz, Rodriguez, Garrido, & Prince, 2007). The demand for natural preservatives has led to renewed searches for preservatives derived from plant, animal, and microbial sources (Dillon & Board, 1994). Some of these natural preservatives, like rosemary extract, nisin, citrox and chitosan, have been analysed and have shown antimicrobial and antioxidant properties in food products (Bañón et al., 2008).

Protective cultures and bacteriocins are considered promising alternatives to chemical preservatives. It is mostly used to inhibit the growth of spoilage bacteria and foodborne pathogens; improving the shelf-life and quality of the food product (Hammami, Fliss, & Corsetti, 2019). Bacteriocins are mainly applied in the form of protective cultures, because purified bacteriocins are expensive and only three, nisin, pediocin PA-1, and micocin, have been allowed in food due to strict food regulations (Naskar & Kim, 2021).

The aims of this review were to expand the knowledge on 1) Boerewors, including the background and formulations, 2) the role and functions of chemical preservatives, especially SO_2, in fresh meat products, and the reasons for reduction and/or replacement, and 3) the use of natural preservatives in meat products, focusing on the benefits of plant extracts and biopreservation.

2.2 Boerewors

2.2.1 Background

Boerewors is a fresh traditional South African sausage, with a coarse texture, made from minced beef, pork, or beef fat. Spices and vinegar are added for flavour and preservation (Charimba, Hugo, & Hugo, 2012). Boerewors consists of two words; 'boer', meaning farmer and 'wors', which means sausage (Allen, 2015). The word 'sausage' comes from the Latin word "salsus", which means salted or preserved (Lonergan, Topel, & Marple, 2019). Boerewors originated from farms in South Africa and after scandals of substandard raw materials, e.g., offal, bone meal and udder tissue used in Boerewors erupted in the 1960's, consumers demanded that it should be a high-quality sausage, with high quality ingredients and a standardised recipe (Biltongmakers, 2018).

Regulations on the traditional South African sausage were, therefore, implemented to uphold the good quality of Boerewors. According to these regulations, Boerewors should consist of 90% total meat content (beef lean meat and fat); maximum 30% fat content; maximum 1.5% salt content; no soya protein source; vinegar; spices; herbs; and only permitted additives (which will be discussed later) and flavourings. The only non-meat proteins that are allowed in Boerewors, are cereal products derived from the seed of cultivated grasses of the *Poaceae* family, with a maximum of 15.0% protein content. Originally, the meat was stuffed into cattle, hog, or sheep casings, using cattle horn; nowadays, only clean, natural casings should be used, with a minimum diameter of 12.55 mm and a maximum diameter of 28.0 mm, when filled (Steyn, 1989; South African Department of Health (DoH), Regulation No. R.2718, 1990). There are many varieties of Boerewors, which will be discussed in this literature review, but it can only be labelled Boerewors if it conforms to the above-mentioned regulations.

2.2.2 Classification of sausages

There are a few different types of sausages which can be classified as fresh, dry, or cooked (Table 2.1). Boerewors forms part of the fresh sausages, which is made from raw beef and pork, and therefore, should be refrigerated or frozen during storage and cooked prior to consumption (Romans, Costello, Jones, Carlson, & Ziegler, 2001).

There are various types of Boerewors in terms of flavour (Table 2.2), but the basic formulation in each remains the same, due to the regulations mentioned in section 2.2.1.

Table 2.1. Classification of sausages (Romans et al., 2001).

Classification	Characteristics	Examples
Fresh sausages	Fresh meat; uncured; comminuted; seasoned; stuffed in casings; must be cooked before serving.	Fresh pork sausage Bratwurst Boerewors
Dry/semi-dry sausages	Cured meat; fermented; air dried, may be smoked before drying; served cold.	Gonoa salami Pepperoni Len Bologna Droë wors
Cooked sausages	Cured or uncured meat; comminuted; seasoned; stuffed into casings; cooked, sometimes smoked; served cold.	Liver sausage Braun-Schweiger Liver cheese
Cooked, smoked sausages	Cured meat; comminuted; seasoned; stuffed into casings; smoked and fully cooked; do not require further cooking; sometimes heated before serving.	Frankfurters Bologna Cotto salami
Uncooked, smoked sausages	Fresh meat, cured or uncured; stuffed into casings; smoked; not cooked; cooked before serving.	Smoked pork country-style sausages Mettwurst Kielbasa
Cooked meat specialties	Specially prepared meat; cured or uncured; cooked but rarely smoked; often made in loaves; generally sold in sliced, packaged form; usually served cold.	Loaves Head cheese (brawn) Scrapple

Table 2.2. Different flavoured Boerewors found in supermarkets in South Africa (Woolworths, 2021).

Type of Boerewors	Main flavour additives
Classic	Nutmeg
	Clove
Traditional	Roasted coriander
	Clove
Grabouw	Roasted coriander
Drakensberg	Coriander
	Clove

2.3 Microbial quality of fresh meat products

2.3.1 Spoilage microorganisms

Beef lean meat and fat are used in the production of Boerewors. Generally, carcass meat is sterile immediately after slaughter. However, the environment and equipment used for slaughter, can be sources of contamination. The microbial quality of carcass meats mostly depends on the conditions under which the animals are reared, slaughtered, and processed (Aiyegoro, 2014). Some of the spoilage microorganisms will originate from the animals' intestines and others from the environment. Moreover, psychrotolerant bacteria, also known as psychrotrophs, are often found on the animals' hides and work surfaces within abattoirs, thus, contaminating the meat at all stages of processing. If the pre-slaughter environment is excessively stressful, the meat's ultimate pH increases above normal ranges of 5.5 to 6.2, which increases the growth of various microorganisms, resulting in unwanted odours and flavours, even at relatively low bacterial numbers. The extent of the contamination usually reflects the standard hygiene of the meat (Nychas & Drosinos, 2014).

The shelf-life of red meat is relatively short, because the composition (near neutral pH, nutrient content and high water activity), provides a favourable growth medium for microorganisms (Mathenjwa, Hugo, & Hugo, 2012). Spoilage becomes noticeable when amino acids are broken down by bacteria, and this spoilage can cause off-odours, off-flavours, discolouration, as well as sliminess (Iulietto, Sechi, Borgogni, & Cenci-goga, 2015; Erkman & Bozoglu, 2016).

Fresh meat products are usually stored under refrigerated conditions; hence, psychrotolerant bacteria would be present in the surrounding environment (Gill & Newton, 1978).

Psychrotolerant bacteria usually linked to the spoilage of Boerewors, are *Lactobacillus* spp., *Enterobacteriaceae*, *Pseudomonas* spp., *Leuconostoc* spp., *Shewanella putrefaciens* (Borch et al., 1996), *Aeromonas* and *Alteromonas putrefaciens* (Kotula & Kotula, 2000). Although bacteria are the main source of spoilage in fresh meat, yeasts also contribute and cause spoilage by producing off-flavours. Some yeast strains can metabolize preservatives, such as sodium nitrite and organic acids, which can shorten the shelf-life of the products even more (Dalton, Board, & Davenport, 1984; Fleet, 1992).

2.3.2 Pathogens

Nowadays, consumers are more aware of food safety due to the increasing outbreaks of foodborne illnesses. Foodborne pathogenic bacteria are the leading cause of death and illness in less developed countries (Mead, Slutsker, Dietz, McGraig, Bresee, Shapiro, Griffin, & Tauxe, 1999). The pathogens most frequently associated with fresh meat products, are *Escherichia (E.) coli*, *Staphylococcus (S.) aureus*, *Listeria (L.) monocytogenes*, *Campylobacter jejuni* and *Salmonella* spp. (Huffman, 2002). Although pathogens mostly cause gastroenteritis, they can also cause more invasive illnesses and diseases (Mor-Mur & Yuste, 2010). In the United States of America, almost 1.4 million cases are caused by non-typhoidal *Salmonella* and about 270 000 cases are caused by *E. coli* O157:H7, annually (Hoelzer, Moreno Switt, & Wiedmann, 2011).

Escherichia coli is Gram-negative, rod shaped, non-spore forming, motile with peritrichous flagella or nonmotile. It naturally occurs in the intestines of humans and animals. Meat can become contaminated with *E. coli* during the slaughtering process, when the *E. coli* in the animal's intestines gets onto the meat cuts or from contaminated water sources used during processing or preparation. It is also possible to ingest *E. coli* from contaminated hands, where the *E. coli* is transferred from hands to food or mouth. Hands become contaminated from changing diapers, not washing after bowel movements, or petting farm animals (Martin & Beutin, 2011). The presence of *E. coli* in fresh meat products may indicate poor hygiene practices during slaughter. When fresh meat products are contaminated with *E. coli*, it should be thoroughly cooked to destroy the bacteria, because *E. coli* food poisoning is reported to be associated with the consumption of raw meat (Mor-Mur, & Yuste, 2010). Moreover, pathogenic *E. coli* strains are known to cause some serious illnesses, such as diarrhoea, pneumonia, meningitis, and septicaemia (Ekici, & Dümen, 2019). Like most pathogens, *E. coli* has an optimum growth temperature of 37 °C and a near neutral optimum pH (Adams & Moss, 2008).

Staphylococcus aureus is Gram-positive cocci in clusters, nonmotile and non-spore-forming. It can be carried on hands, throat, and the nasal passage of humans, which is why food plant

personnel are often the source of contamination. *Staphylococcus aureus* is commonly found in ground meat and other processed meat products (Baird-Parker, 2000). According to Genigeorgis (1989), food poisoning is caused by the enterotoxin produced by *S. aureus*, which is also one of the leading causes of food borne illnesses. Some of the symptoms, caused by the enterotoxin, include diarrhoea, vomiting and stomach cramps. It is rarely fatal, and the symptoms usually disappear after 24 – 48 h (De Boer, Zwartkruis-Nahuis, Wit, Huysdens, De Neeling, Bosch, van Oosterom, Vila, & Heuvelink, 2009). To avoid staphylococcal food poisoning, the food needs to undergo a heat treatment before the enterotoxin, which can survive the heat treatment, is produced (Le Loir, Baron, & Gautier, 2003). The optimum growth conditions for *S. aureus* are a temperature of 36 °C and a pH of 6.0 – 7.0 (Adams & Moss, 2008).

Listeria monocytogenes is a facultatively anaerobic, Gram-positive non-spore former. It has coccoid to rod shaped cells exhibiting a tumbling motility. It is considered the only significant human pathogen among the recognised species within the genus *Listeria*. This microorganism causes concern due to its psychrotolerant nature and the illness, listeriosis, has a high mortality rate. There have been outbreaks of human listeriosis, but the cases remain low when compared to other foodborne infections (Adams & Moss, 2008). However, in 2017 – 2018, South Africa had the world's largest listeriosis outbreak which resulted from contaminated processed meat. There were 1,060 confirmed cases of listeriosis during the outbreak, and about 216 deaths (Kaptchouang Tchatchouang, Fri, De Santi, Brandi, Schiavano, Amagliani, & Ateba, 2020). *Listeria monocytogenes* is capable of growing over a wide range of temperature from 0 – 42 °C, with an optimum range between 30 and 35 °C. It also has received some attention for the ability to survive some thermal treatments. After the outbreak of listeriosis in the United States, associated with pasteurized milk, it was suggested that the pathogen could survive commercial pasteurization. There are conflicting data surrounding the thermal survival characteristics, although, it appears that the heat resistance is similar to that of other Gram-positive non-spore formers. All *Listeria* strains are inhibited at pH values below 5.5, however; the minimum growth pH is dependent on strain and acidulant and has been reported as 4.4 – 5.6. *Listeria monocytogenes* is salt tolerant, being able to grow at 10% NaCl and survive in 16% NaCl at pH 6 for approximately one year (Adams & Moss, 2008).

Other pathogens found in fresh meat products are *Salmonella*, *Campylobacter jejuni*, *Clostridium perfringens* and *Yersinia enterocolitica* (Bhandare, Sherikar, Paturkar, Waskar, & Zende, 2007; Bantawa, Rai, Limbu, & Khanal, 2018). *Campylobacter jejuni* is also one of the leading causes of food associated bacterial illnesses. Some serotypes, like *Campylobacter jejuni* O:19, are etiological agents of Guillian-Barre syndrome, a neuropathy due to an autoimmune response. A few *Salmonella* serotypes have been found to be multi-drug resistant

(Mor-Mur & Yuste, 2010). Most of these pathogens can be destroyed using antimicrobial methods, like high temperature treatments, lowering the water activity, or lowering the pH (Zhang, Zhang, Zhao, Liu, Chen, Yang, Xia, & Cao, 2019).

2.3.3 Methods of microbial quality determination

Determining the number of microorganisms, in any food product, is important, because it indicates the microbial quality of the product. Microbial cells are not determined individually, instead, the colonies formed are determined, by culturing the microorganisms on specific media. The results are noted as colony forming units per millilitre or gram (cfu/ml or cfu/g) (Nemati, Hamidi, Dizay, Javaherzadeh, & Lotfipour, 2016). Conventional and rapid methods are used to determine the microbial quality. The microbiological standards for fresh meat and minced meat are shown in Tables 2.3 and 2.4, respectively.

The total viable count (TVC), also known as total bacterial count (TBC), indicates the shelf-life and microbial load of the meat products. These counts should not exceed the limits of the given microbiological standards (Table 2.3). Standard plate count agar, which is a non-selective medium, is usually used for this procedure (R-Biopharm, 2016). Incubation is at 15 – 32 ^{o}C for 48 – 72 h, depending on the product, since the total viable microorganisms are regarded mesophiles (Harrigan, 1998).

The coliform count, as well as the *E. coli* count, should be included when the microbial quality is determined, as it serves as a hygiene indicator. *Escherichia coli* is a human pathogen frequently found in fresh products. High coliform counts can point out possible contamination during slaughter, dirty equipment and/or environment. The two traditional methods used to determine the coliform count, are 1) agar plate count methods, using violet red bile agar (VRBA) with methylumbelliferyl-β-glucuronidase (MUG); and, the Most Probable Number (MPN) method. The MPN method is used when higher sensitivity is required (BioLumix, 2011).

When measuring the hygiene of food products, it is important to include the *Enterobacteriaceae* count, and not just the coliform count, because coliforms are a group of bacteria in the *Enterobacteriaceae* family, that forms gas and acid, by fermenting lactose, whereas *Enterobacteriaceae* is a large family of Gram-negative bacteria, that produces acid from glucose (Harrigan, 1998). The *Enterobacteriaceae* count is determined by using VRBA with glucose, incubated at 30 °C, and coliforms are incubated at 35 – 37 °C (Harrigan, 1998). *Enterobacteriaceae* includes several human pathogens, like *Shigella dysenteriae*, *Yersinia pestis* and *Salmonella enterica* (Kurtzman, James, & Blackburn, 2006; Smoot & Cordier, 2006).

Table 2.3. Microbiological standards for fresh meat (Shapton & Shapton, 1991).

Organism	**Limits (cfu/g)**
Total viable count	3×10^4
Coliforms or *Enterobacteriaceae*	1×10^3
Escherichia coli or Faecal coliforms	20
Salmonella spp.	0
Staphylococcus aureus	10
Clostridium (*perfringens*)	10
Faecal streptococci	1×10^3
Yeasts and moulds	1×10^3

Table 2.4. Microbiological standards for comminuted meat products (Shapton & Shapton, 1991).

Organism	**Limits (cfu/g)**
Total viable count	1×10^7
Coliforms	2×10^4
Escherichia coli	5×10^1
Enterobacteriaceae	2×10^4
Staphylococcus aureus	1×10^3
Salmonella spp.	0
Clostridium perfringens	1×10^2
Listeria monocytogenes	1×10^2

The *S. aureus* count should be part of meat quality and safety determination because it is pathogenic, can easily contaminate meat from meat handlers, and the fact that meat is a favourable medium for *S. aureus* to grow in (Das & Mazunder, 2016). Baird-Parker agar is used to detect and enumerate *S. aureus* colonies with incubation at 37 °C for 24 – 48 h; the colonies are characteristically black, with a surrounding clear zone (Oxoid, 2020).

Yeasts and moulds can possibly cause spoilage in meat products and should, therefore, be included in meat quality analysis (Doyle & Glass, 2010). Yeasts are more frequently found in fresh meat products than moulds, but both may be cultured on rose-bengal chloramphenicol agar with incubation at 25 °C for 4 – 5 days (Oxoid, 2020).

2.4 Sulphur dioxide (SO_2) as preservative

Sulphur dioxide is the main preservative in Boerewors, maintaining sensory and microbial quality of fresh meat. It will be discussed in terms of forms and solubility, role and function in food products, and the problems associated with SO_2.

2.4.1 Different forms and solubility

Three types of chemical substances form when SO_2, or compounds containing SO_2, such as bisulphite and metabisulphite, are dissolved in water: sulphurous acid (H_2SO_3), bisulphite (HSO_3^-) and sulphite (SO_3^{2-}). The amount of substances formed, depends on the pH of the solution, which is regulated by the SO_2 concentration, or by the addition of acid or alkali (Payne, Beavers, & Cain, 1969). Sulphur dioxide is available in various forms: gas, aqueous solutions, and salts. Sulphur dioxide in the form of a salt is much easier to store and handle than gaseous or liquid SO_2. Two of the most commonly used sulphites in food are sodium sulphite (Na_2SO_3) and sodium metabisulphite ($Na_2S_2O_5$). Sodium metabisulphite is more easily controlled, but it acts less rapidly and can result in flavour changes (Nicolas, Billaud, Rouer-Mayer, & Philippon, 2003). Sulphur dioxide and the various forms of SO_2 used in the food industry and the SO_2 availability are shown in Table 2.5.

Table 2.5. Approximate theoretical available sulphur dioxide (SO_2) content of various sources (Joslyn & Braverman, 1954).

Compound	Formula	Availability (%)
Liquid sulphur dioxide	SO_2	100.00
Sulphurous acid (6%)	H_2SO_3	6.00
Potassium sulphite	K_2SO_3	33.00
Sodium sulphite	Na_2SO_3	50.80
Potassium bisulphite	$KHSO_3$	53.30
Sodium bisulphite	$NaHSO_3$	61.60
Potassium metabisulphite	$K_2S_2O_5$	67.40
Sodium metabisulphite	$Na_2S_2O_5$	57.70

2.4.2 Role and function in food products

2.4.2.1 Antimicrobial

The antimicrobial action of SO_2 is based on enzyme-catalysed reaction inhibition. Sulphur dioxide has a substantial inhibitory effect on enzymes with thiol groups. The antimicrobial

effects of sulphurous acids are most pronounced when it is in an un-ionized form (Gehman, & Osman, 1954; Davidson, Taylor, & David, 2020). According to Ough (1993), the action of SO_2 is more intense against bacterial growth than the growth of yeasts and moulds. Yeasts react differently to SO_2, depending on the strain (Rehm & Whitman, 1962). In the case of yeasts, SO_2 blocks the reaction stage of glyceraldehyde 3-phosphate to 1,3-di-P-glycerate, while with *E. coli*, it is mainly the inhibition of NAD-dependant formation of oxaloacetate from malate. Another way SO_2 inhibits enzymatic reactions, is by capturing the end products or intermediate products. Thus, in the decomposition of carbohydrates, acetaldehyde is immediately bound after formation (Wallnöfer & Rehm, 1965).

2.4.2.2 Antioxidant effect and colour preservation

Some chemical reactions can cause oxidative rancidity in meat products; molecules are broken down, which changes the organoleptic properties of meat. These changes have a big impact on the food industry in terms of economic cost and consumer acceptability (Papuc, Goran, Predescu, & Nicorescu, 2017).

There are mainly two types of browning: enzymatic browning and non-enzymatic browning. Sulphites are added to products because it can reduce or inhibit the effects of both these processes (Wedzicha, Bellion, & Goddard, 1991; Ough & Were, 2005).

Enzymatic browning and the degradation of lipids and proteins, and oxidative processes, are directly linked. Sulphites prevent proteolytic breakdown and lipid peroxidation by inactivation of the major enzymes, like lipase and peroxidase. It also interferes in the three stages of lipid oxidation: 1) initiation, 2) propagation, and 3) termination (Roberts & McWeeny, 1972; Kuijpers, Gruppen, Sforza, Berkel, & Vincken, 2013). The formation of sulphate ions in meat is essential in controlling the initiation stage of oxidation. Furthermore, sulphites can inhibit meat discolouration, which is the oxidation of oxymyoglobin and oxyhemoglobin, to ferric pigments, which are pro-oxidants and compounds that start meat oxidation. Sulphite agents do not associate with myoglobin and haemoglobin like nitrites. Sulphites rather stabilize oxygen binding forms. Thus, sulphites retain meat colour and flavour, by delaying the development of oxidative changes (Taylor, Higley, & Bush, 1986; Wedzicha et al., 1991).

Non-enzymatic browning reactions cause brown pigmentation in meat. This process is known as the Maillard reaction. Amino acids and reducing sugars rearrange, then arranging themselves in collections of rings, called melanoidins, that reflect light in a way that makes the meat brown. Sulphites inhibit this reaction by binding with the carbonyl groups (Wedzicha et

al., 1992; Modernist Cuisine, 2013). According to van Boekel (2006), SO_3^{2-} ions act as terminators of polymer formation, by attacking intermediates, like glycosylamine.

2.4.3 Problems associated with SO_2

Sulphur dioxide is used in food products and drinks for its preserving properties. When used in recommended concentrations, it is harmless to healthy people, but it can cause asthma in sensitive people (Freedman, 1980). Sulphites are regulated in many countries and are Generally Regarded As Safe (GRAS) in the USA, with some exceptions when it comes to fresh fruit and vegetables (Garcia-Fuentes, Writz, Vos, & Verhagen, 2015). When an excess SO_2 is added to a product, it can cause organoleptic changes to the final product; by neutralizing the aroma or even produce aromatic defects and it can negatively affect the taste (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006).

Some people are allergic to SO_2, and various reactions have been recorded: abdominal pain, diarrhoea, urticaria, dermatitis, bronchoconstriction and even anaphylaxis (Qin & Meng, 2009; Vally, Misso, & Madan, 2009; Fredericks, du Toit, & Krügel, 2011). According to Vanier, Paraginski, Berrios, Oliviera, & Elias (2015), treating food products with sulphites can notably reduce the thiamine content of the product. It has also been suggested that consuming beverages that contain SO_2, may strongly reduce the overall levels of thiamine in the diet (Hötzel, & Bitsch, 1976). Thiamine plays a crucial role in the glucose metabolism; it is needed to form adenosine triphosphate (ATP), which the body uses for energy. It is also essential for nerve, heart and muscle function (Abdou & Hazell, 2015).

According to the regulations of the South African Department of Health (DoH, 2020), SO_2 and other compounds classified as ‘additives’, must be mentioned on the label if the food product contains more than 10 mg/kg as packed. There are different permitted quantities specified for each foodstuff; sausages and sausage meat have a maximum permitted SO_2 level of 450 mg/kg (DoH, 2023).

2.5 Natural preservatives

With chemical preservatives raising health concerns, and consumers demanding ‘cleaner’, more natural products, natural preservatives have become a major focus in the food industry. Although several types of natural preservatives are known, e.g., plant derivatives, animal derivatives, microbial (bio) derivatives, only plant extracts and biopreservation, will be discussed in the following sections.

2.5.1 Plant derived products

For many years, spices and herbs have been used in food, not just for flavouring, but also for preservation purposes. Phytochemicals, like flavonoids, phenylpropanoids, terpenes and anthocyanins, have antioxidant and antimicrobial properties. The antimicrobial properties are attributed to the hydroxyl group of the phenolic compounds; it disrupts the cell membrane function, shifting proton exchanges and proton motive force. It also inhibits the synthesis of ATP, causing cell death (Tajkarimi, Ibrahim & Cliver, 2010). In Table 2.6, some plant extracts that have been used in the food industry, are given.

Many plants possess antioxidant properties, which is why they are often added to food products, especially meat. Lipid peroxidation is one of the main reasons for meat and meat products being rejected. The antioxidant prevents or lowers the rate of lipid peroxidation (Burri, Ekholm, Bleive, Jensen, Hellstr, Mńkinen, Korpinen, Mattila, Radenkovs, Segliņa, & Rumpunen, 2020).

Lipid oxidation and oxidation of proteins and pigments, lower the quality and nutritional value of meat products. Adding antioxidants to these products can slow the reaction, and therefore, preserve the quality and extend the shelf-life (Ribeiro, Santos, Silva, Pereira, Santos, Lannes, & da Silva, 2019). Due to the demand from consumers, most antioxidants added to meat products are natural plant extracts. Fresh sausages contain fat and are highly susceptible to oxidation, especially when stored at refrigeration temperatures, and in semi-permeable packaging with oxygen (Hugo & Hugo, 2015; Lu & Pham-Mondala, 2018).

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate, are still used in some cases. The food industry generally prefers to use a combination of BHA and BHT, due to their temperature stability, while still preserving the colour, odour, and flavour of the product (Dave & Ghaly, 2011).

Table 2.6. Plant extracts commonly used as preservatives in food products, the bioactive compounds in each of these extracts, and the bacteria it inhibits. *S., Staphylococcus; E., Escherichia; P., Pseudomonas; B., Bacillus; C., Clostridium; L., Listeria*

Plant extract	Bioactive compounds	Effective against	Reference
Rosemary	Carnosic acid, rosmarinic acid, 1,8-cineole, and camphor	*S. aureus, E. coli, P. aeruginosa, Salmonella enteritidis* and *B. subtilis*	Wang, Luo, Zu, and Efferth (2012) Antolak & Kregiel (2017)
Cinnamon	Eugenol and cinnamaldehyde	*C. perfringens, S. aureus, E. coli* and streptococci	Antolak, Czyzowska, & Kregiel (2017)
Cranberry	Coumaroyl iridoid glycosides and ellagic acid	*E. coli, L. monocytogenes* and staphylococci	LaPlante, Sarkisian, Woodmansee, Rowley, & Seeran (2012) Harich, Maherani, Salmieri, & Lacroix (2017)
Basil	Eugenol, p-coumaric acid and rosmarinic acid	*S. aureus, E. coli* and *P. aeruginosa*	Moghaddam Shayegh, & Mikaili (2011)
Thyme	Thymol and p-cymene	*B. subtilis, S. aureus, E. coli, P. aeruginosa* and *L. monocytogenes*	Soković, Glamočlija, Marin, Brkić, & van Griensven (2010) Abdollahzadeh, Rezaei. & Hosseini (2014)
Cumin	Gallic acid, rutin and catechin	*E. coli, P. aeruginosa, S. aureus* and *B. pumilus*	Dua, Gaurav, Balkar, & Mahajan (2013) Allaithy (2017)
Rooibos	Aspalathin and catechin	*E. coli, S. aureus, B. cereus* and *L. monocytogenes*	Simpson et al. (2013)
Ginger	6-gingerol	*S. aureus, Streptococcus pyogenes, E. coli* and *P. aeruginosa*	Stoilova, Krastanov, Stoyanova, Denev, & Gargova (2007) Kim & Park (2013) Dhiman & Aggarwal (2019)

Plant-derived compounds are mostly secondary metabolites, including phenols and derivatives, which have antimicrobial properties. Natural pigments, like carotenoids, flavonoids, and anthocyanidins, which are also secondary metabolites, have excellent antioxidant properties and could possibly add colour to products (Petcu, Mihai, Tăpăloagă, Gheorghe-Irimia, Pogurschi, Militaru, Borda, & Ghimpețeanu, 2023). The different types of antioxidants and how they work when added to meat products, are shown in Figure 2.1.

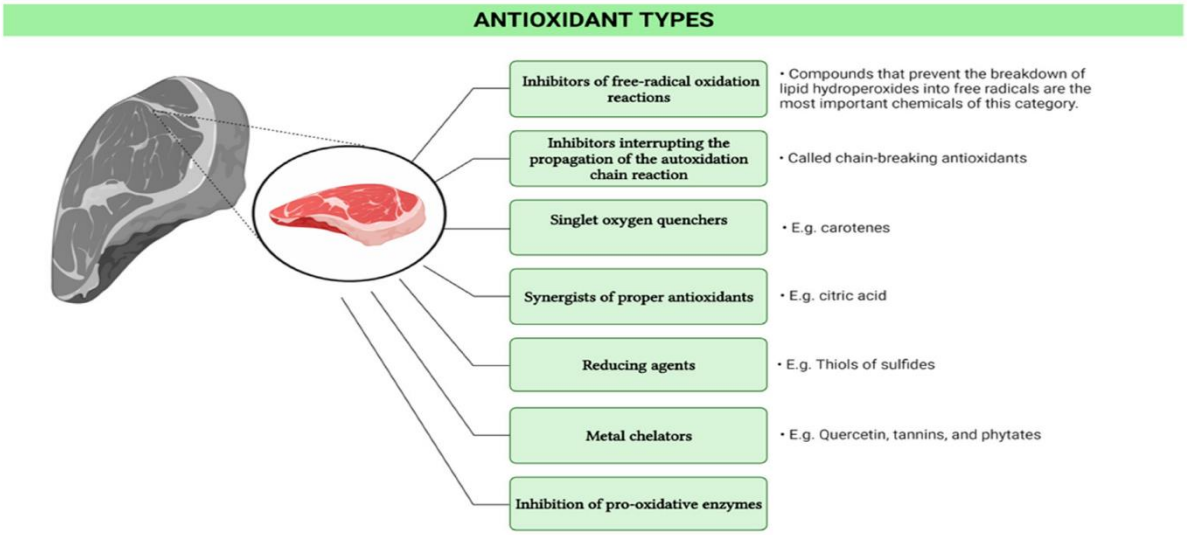

Figure 2.1. Types of antioxidants and their mechanisms when added to meat products (Petcu et al., 2023).

The conventional method of antioxidant application in meat and meat products, is incorporating it in the product during processing, which has been proven to be more effective than spraying it on the surfaces. Newer approaches, like edible coatings or casings, or active packaging containing antioxidants, make the most of the beneficial effects of the antioxidants. With these new technologies, the quantities that are required, are reduced, thus, improving the cost-benefit ratio (Ahmed, Lin, Zou, Brody, Li, Qazi, & Pavase, 2017; Ganiari, Choulitoudi, & Orepoulou, 2017; Cunha, Monteiro, Lorenzo, Munekata, Muchenje, de Carvalho, & Conte-Junior, 2018).

Anti-browning agents are compounds that preserve colour by acting on either the enzymes or the intermediates of pigment formation. The most used anti-browning agents in the food industry, include ascorbic acid, citric acid, and sulphites. However, the use of these agents is constrained due to issues such as cost, toxicity, or the altering effects on other sensory properties (Iyengar & McEvily, 1992).

Many companies are nowadays involved in the production of natural preservatives from plants, however, this study used plant-based preservatives produced by Kalsec™. Kalsec™ is a company based in Michigan (United States of America), specializing in natural food and beverage ingredients since 1958, founded by Paul H. Todd Jr. Kalsec™ products are derived from herbs, spices, hops, and vegetables. These derivatives are translated to powders or liquids that can easily be incorporated in most food formulations. Duralox® is an antioxidant/antimicrobial blend produced by Kalsec™, which protects the food against microbial and oxidative spoilage. These blends are made up of rosemary extract, green tea, acerola, and tocopherols, with quenchers and/or chelators (Kalsec.com, 2023).

Rosemary is a commonly used additive in meat products because of its flavour and preserving effects. Research showed that rosemary is one of the most effective preservatives, especially when the rosemary extracts were high in carnosic acid; it has antimicrobial properties even at low concentrations (Gachkar, et al., 2007). Rosemary is also a low-cost preservative and easily available; it is an evergreen shrub that can grow spontaneously in Mediterranean regions. The oil is extracted by a steam distillation procedure, which is cost-effective and easy to handle, giving a high yield of good quality product based on sensory and functional properties (Berreta, Artali, Faccino, & Gelmini, 2011). However, variants of the same species can differ in their essential oil composition (Mangena & Muyima, 1999). According to a study on essential oils (Gachkar, et al., 2007), *Rosmarinus officinalis* oil was one of the best performing extracts in terms of the ability to prevent oxidation by neutralizing free radicals, and antimicrobial activity. Another study by Stoick, Gray, Booren, & Buckley (1991), reported that rosemary extract was highly effective in delaying lipid oxidation in different food products. In the case of meat, it was effective at 200 – 1000 mg oil/kg meat.

Green tea (*Camellia sinensis*) leaves are rich in epicatechin, epicatechin gallate, epigallocatechin, teaflavin gallate, teaflavin monogallate A and B, and teaflavin digallate, which are responsible for its antioxidant characteristics. Other studies have suggested that green tea extracts may have an antibacterial effect *in vitro* (Bañón et al., 2007).

Acerola cherry, *Malpighia emarginata* D.C., is an edible tropical fruit with a characteristic flavour, and high ascorbic acid (± 1677 mg/100 g fruit) content and carotenoid phenolics, anthocyanins, and flavonoids. These characteristics result in the colour stability and high antioxidation potential of acerola extract (Prakash & Baskaran, 2018).

Natural mixed tocopherols (forms of vitamin E), consisting of alpha, beta, gamma, and delta tocopherol, can help maintain the freshness and shelf life of products. They are a natural alternative to synthetic antioxidants, such as BHT, BHA and tBHQ (Prepared foods, 2023).

2.5.2 Biopreservatives

2.5.2.1 Definition

Biopreservation can be defined as the controlled usage of natural macrobiotics or antimicrobials to preserve food and extend product shelf-life (Settanni & Corsetti, 2008; Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2014). Microorganisms used in biopreservation, are referred to as protective cultures and the antimicrobial metabolites produced by bacteria, are known as bacteriocins. Hansen (2002) defined protective cultures as preparations consisting of live microorganisms of pure cultures or concentrated cultures, that are added to food products with the purpose of reducing risks by pathogenic or toxigenic bacteria. The term 'bacteriocin' was first used in 1953, to define the antimicrobial metabolite, colicin, which is produced by *E. coli* (Settanni & Corsetti, 2008). Bacteriocins have been consumed by humans for many years, as products of lactic acid bacteria, and therefore, may be considered as natural food ingredients (Cotter, Hill, & Ross, 2005). Some companies manufacture protective cultures that can be used in various food products (Table 2.7).

Antimicrobial phenolic compounds and peptides, produced by lactic acid bacteria, have been successfully applied in food product preservation against yeasts and moulds. Apart from decreasing mycotoxins in food, some of the bacteriocins from lactic acid bacteria have antioxidant activity and potential anti-cancer properties. In some cases, protective cultures and bacteriocins can enhance the nutritional value of food products (Varsha & Nampoothiri, 2016).

Bacteriocins may have a broad spectrum of antimicrobial action, which means it inhibits a wide variety of microorganisms, or it can have a narrow spectrum, by inhibiting microorganisms that are taxonomically close. Bacteriocins are selected for specific purposes based on these abilities and effectiveness of eliminating pathogens (Cotter et al., 2005; Mills, Stanton, Hill, & Ross, 2011). Bacteriocins were initially classified into four groups, however; one of the groups were aborted and renamed bacteriolysins, which consists of complexes with lipid or carbohydrate parts. Examples of bacteriolysins are leuconocin S and lactocin 27 (Güllüce, Kardayi, & Baris, 2013). Therefore, bacteriocins are divided into three major classes (Table 2.8).

Bacteriocins have different modes of actions; those that primarily target Gram-positive bacteria attacks the cell envelope (Figure 2.2). Specifically, class Ia bacteriocins, like nisin, eliminate the synthesis of peptidoglycan by inhibiting lipid-II on the cell membrane. Others kill or inhibit target bacteria by forming pores in the cell membrane (Cotter et al., 2005). Some of the bacteriocins that inhibit Gram-negative bacteria also forms pores in the cell membrane,

but most of the bacteriocins that target Gram-negative bacteria controls it by interfering with RNA or DNA and the protein metabolism (Figure 2.3; Cotter et al., 2013).

Table 2.7. Commercially available biopreservatives (Gensler, 2019).

Manufacturer	**Product Name**	**Organism**	**Intended Purpose**
Chr. Hansen	BLC 20	*Pediococcus acidilactici*	*Listeria monocytogenes* control in meat products
	BLC 48	*Lactobacillus curvatus*	*Listeria monocytogenes* control
	BS 10	*Lactococcus lactis* ssp. *lactis*	Control of Gram-positive bacteria: *Clostridium* sp., *Bacillus* sp.
SACCO	LPAL	*Lactobacillus plantarum*	*Listeria monocytogenes* control on cheese surfaces
	CNBAL	*Carnobacterium divergens* sp.	*Listeria monocytogenes* control in soft cheeses
	LRB	*Lactobacillus rhamnosus*	Inhibits *Listeria monocytogenes*, yeasts and moulds
Danisco	Holdbac	*Lactobacillus plantarum*	*Listeria monocytogenes* control in dairy products
Cerbios Pharma	SF 68	*Enterococcus faecium*	Pharmaceutical probiotic
Lallemand Lalcult	B16	*Hafnia alvei*	Shiga Toxin-producing *Escherichia coli* control in milk
	XF01	*Staphylococcus xylosus*	*Listeria monocytogenes* control in dairy products

Table 2.8. Classification of bacteriocins.

Class	Description	Subclass	Examples	Reference
Class I	Typically comprises of 19 – 50 amino acids, extensively modified	Class Ia (Lantibiotics) Class Ib (Labyrinthopeptins) Class Ic (Sanctibiotics)	Nisin Labyrinthopeptin A1 Thuricin CD	Parada, Caron, Medeiros, & Soccol (2007); Le Lay, Dridi, Bergeron, & Ouellette (2016)
Class II	Small, heat stable, non-modified peptides	Class IIa (Pediocin-like) Class IIb (Two-peptides) Class IIc (Circular) Class IId (Linear)	Pediocin PA-1 Lactococcins G Gassericin A Bactofencin A	Drider, Fimland, Héchard, McMullen, & Prévost (2006); O'Shea, O'Connor, O'Sullivan, Cotter, Ross, & Hill (2013)
Class III	Large molecules, sensitive to heat		Colicin	Heng & Tagg (2006); Kumariya, Garsa, Rajput, Sood, Akhtar, & Patel (2019)

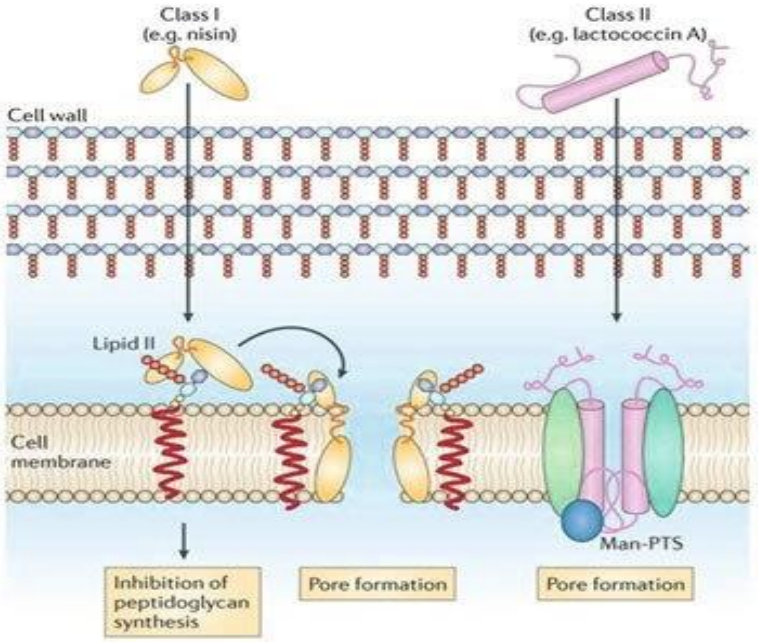

Figure 2.2. Bacteriocin mechanism against Gram-positive bacteria (Cotter et al., 2013).

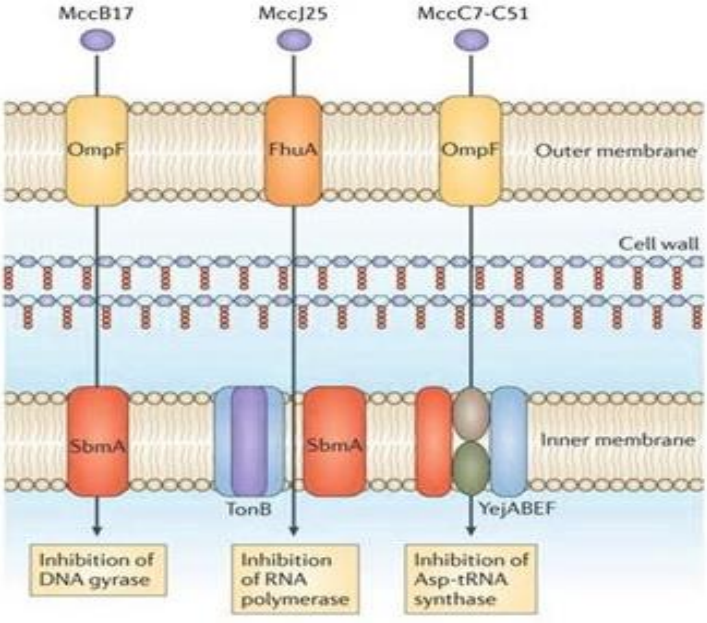

Figure 2.3. Bacteriocin mechanism against Gram-negative bacteria (Cotter et al., 2013).

2.5.2.3 Applications in meat and fresh sausage production

Bacteriocins and their producing strains, have shown efficacy in inhibiting pathogens in different food matrices, including vegetables, meat, and cheese. Bacteriocins and protective cultures have shown potential in ensuring quality and safety of minimally processed foods,

ready-to-eat products and extended-shelf-life foods without chemical preservatives (Hammani, Fliss, & Corsetti, 2019). Protective cultures also have minimal effects on the foods' nutritional and phytochemical composition (Gill & Holley, 2003). The use of lactic acid bacteria (LAB) as biopreservatives in meat and meat products, have increased over the last 10 years. Lactic acid bacteria can be directly incorporated into meat products as a functional ingredient, or as a starter culture in the case of fermentation processes (Figure 2.4) (Barcenilla et al., 2022).

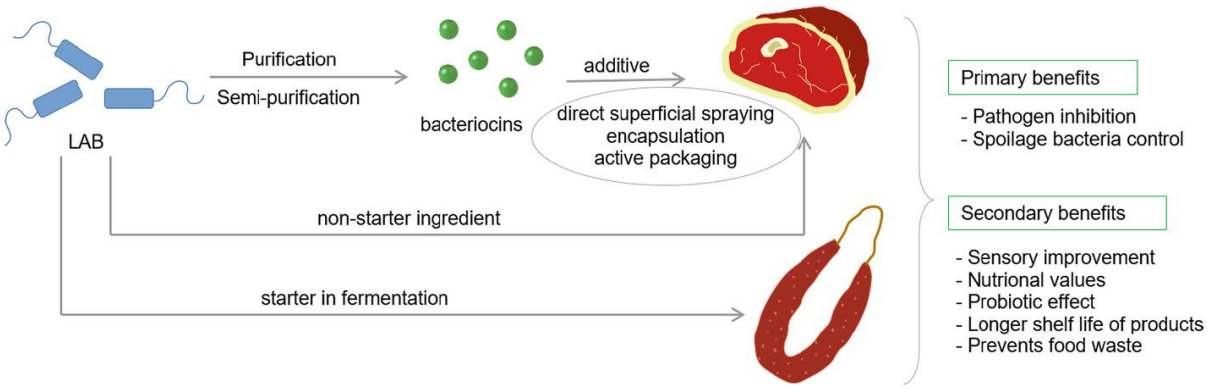

Figure 2.4. Biopreservatives and their application in meat products with potential benefits (Barcenilla et al., 2022).

2.5.2.4 Effect of extrinsic/intrinsic factors on biopreservatives in meat

When environmental conditions are not optimal, and deviate to extreme levels, it can inflict stress on microorganisms. The extent of the physiological deviation usually determines whether the microorganism stops growing, dies or experiences an extended lag period or slow growth rate (Russell, Evans, Tersteeg, Hellemans, Verheul, & Abee, 1995). Most bacteria, including protective cultures, can tolerate minor environmental changes and even adapt in time (Hill, O'Driscoll, & Booth, 1995).

Meat products have an ultimate pH of about 5.5 or above, which makes it prone to microbial spoilage. The meat of animals that are fatigued prior to slaughter, have a higher pH level, closer to neutral, and therefore, prone to faster spoilage. The microorganism's ability to proliferate depends on how well the microorganism can change the environmental pH to a more optimum value. There are other environmental factors that also interact with the pH level of the meat; when the temperature rises, the substrates become more acidic, and salt above a certain level, narrows the pH growth range (Jay, Loessner, & Golden, 2005).

Another way of preserving food, is to lower the moisture content, which is a direct consequence of binding or removing the water, which the microorganisms cannot live without. Water activity (a_w) is regarded as an indicator of microbial stability and anything, for example, changing the salt concentration, can tip the balance from safe to unsafe (Taormina, 2010). Lowering the moisture content also affects the protective cultures in meat products; most bacteria have a minimum a_w level of 0.91.

Microorganisms need more than water to survive; they also need sources of energy, nitrogen, vitamins, and minerals. Gram-positive bacteria and yeasts have the highest nutritional requirement, and moulds have the lowest requirements (Jay et al., 2005). Protective cultures utilize carbon sources at a faster rate than other microorganisms and they are also classified as mildly proteolytic, which makes them useful and efficient in meat preservation (Sperber, 1983).

Temperature is a key factor when it comes to meat biopreservation. Since meat products are generally stored under refrigerated conditions, the protective cultures used in these products should be able to grow at a low temperature, and still elicit their inhibitory effect on the pathogens. Specific strains, that can adapt to these low temperatures, must be selected, although, under eventual higher temperatures, the protective cultures would grow rapidly and still inhibit pathogens by releasing antimicrobial compounds more readily (Galvez, Pulido, Abriouel, Omar, & Burgos, 2012).

Three types of packaging can be used when packaging meat and meat products: vacuum, modified atmosphere packaging (MAP) and air. Fresh sausages, like Boerewors, are generally packaged aerobically, or in some cases with MAP. The shelf-life of meat decreases in the following order: 100% CO_2, no O_2 MAP, vacuum, high O_2 MAP and air (Borch et al., 1996). Protective cultures can be affected by the packaging method. Using CO_2 MAP has been shown to result in an immediate selection of the microbial composition, whereas vacuum packaging showed gradual selection of LAB growing at a slower rate (Greer, & Dilts, 1995).

2.5.2.5 Safety and regulations

The human microbiota is a complex system and plays a key role in health (Mosca, Gianni, & Rescigno, 2019). With an increasing population and consumers having more knowledge about products, there have been increased demands for food products with high quality and safety. More research has provided evidence for the beneficial effects of some bacteria, especially in the intestines. Protective cultures used in the food industry, like *Lactobacillus* spp. and *Bifidobacterium* spp., have Generally Recognized as Safe (GRAS) status, which means it is

safe for human consumption (von Wright, 2005). However, protective cultures are live bacteria, and therefore, have not been regarded as risk-free for groups like the immunocompromised, elderly people and infants; it can cause negative effects, such as gastrointestinal symptoms and infections (Sotoudegan, Daniali, Hassani, Nikfar, & Abdollahi, 2019). Bacteriocins have fewer clinical evidence of risks associated with it, but technically, bacteriocins are not live bacteria, and therefore, pose no risk of infection (Pearce, Coia, Karl, Pantoja-Feliciano, Zachos, & Racicot, 2018; Wegh, Geerlings, Knol, Roeselers, & Belzer, 2019).

2.6 Conclusions

Boerewors is a popular product in South Africa, which makes it important to improve the product in terms of its effect on the health of consumers. There are different varieties of Boerewors available, but the basic recipes should adhere to the required standards and regulations. Fresh meat and meat products are highly susceptible to spoilage, thus, adding preservatives to prolong the shelf-life, is common practice. Nowadays, consumers demand that products contain natural ingredients, which forces the meat industry to consider replacing the chemical preservatives with natural preservatives.

The most prominent chemical preservative in Boerewors, SO_2, has shown to cause adverse health problems in some people. However, it is highly effective in preserving colour, oxidation, and microbial quality, making it difficult to replace. The natural preservatives that show the most potential as SO_2 replacements, include plant derived preservatives, e.g., rosemary extract, and biopreservatives, such as protective cultures. Rosemary is especially known for its antimicrobial properties. Biopreservation is a newer approach as more research is highlighting the benefits of using bacteria to control the growth of pathogens and spoilage bacteria in foods.

Biopreservation can be the use of protective cultures, which is live bacteria, or bacteriocins, which are peptidic compounds produced by bacteria. Lactic acid bacteria and their metabolites meet the consumers' demands and ensures the safety of the product. The main challenges of using biopreservation agents could be regulatory, as the approval of novel bacteriocins and their application in meat products, could be constrained. The quantities of these agents need to be accurate and specific so that preservation is achieved, without changing the end product.

Nevertheless, food safety and quality will always be a concern when product formulations need to be changed, and it seems possible to positively manage it through replacing chemical preservatives, such as SO_2, with plant extracts and biopreservatives.

CHAPTER 3

CHEMICAL, MICROBIAL AND SENSORY EFFECTS OF NATURAL PRESERVATIVES AS SULPHUR DIOXIDE REPLACERS IN BOEREWORS

ABSTRACT

The purpose of this study was to determine if natural preservatives in Boerewors had an effect on the a_w, pH, and moisture content; lipid oxidative, microbial and colour stability; and sensory quality. Sulphur dioxide inclusion at 0.035% (positive control), formulation with no preservatives (negative control), protective cultures; Pediococcus acidilactici (PrC1) and Lactobacillus curvatus (PrC2), and plant extract blends; rosemary extract and acerola oil (KD1) and rosemary extract, buffered vinegar, and acerola powder (KD2), were evaluated. The goal was to best match the quality characteristics of the positive control, while complying to the consumer demands for natural ingredients. The use of KD2 increased the pH on days 0 and 6, while the a_w of were only affected on day 0, with PrC1 having the highest a_w and KD1 the lowest. Both plant extract preservatives increased the lipid oxidative stability more than any other treatment, including the positive control. The microbial stability results were inconclusive due to the protective cultures which increased the total bacteria count. The pathogens, coliform and Enterobacteriaceae counts were analysed, but there were no significant differences between the treatments. KD1 maintained the highest lightness colour score and lowest TBC at the same level as the positive control but increased the yellowness score. The treatments had no effect on the sensory evaluation. The use of plant preservatives as the best candidates for SO_2 replacement were confirmed for use in Boerewors.

Keywords: sulphur dioxide replacement, Boerewors, plant preservatives, protective cultures, shelf-life

3.1 Introduction

Sulphur dioxide (SO_2) is a common food additive in meat products, used as a preservative and antioxidant. In addition to these functions, SO_2 also delays discolouration in fresh meat, caused by oxidation (Freedman, 1980). Sulphur dioxide is of low toxicity and, upon normal consumption, pose no adverse health effects on humans. However, there are hypersensitive

individuals that may experience uncomfortable symptoms, like shortness of breath and nausea (D'Amore, Di Taranto, Berardi, Vita, Marchesani, Chiaravalle, & Iammarino, 2020).

Meat and meat products are highly perishable due to the high water activity, pH, and abundant nutrients. Therefore, controlling and preventing the spoilage of these products, are critical (Bohrer, 2017). The main spoilage bacteria in meat include *Pseudomonas* and *Enterobacter.* Furthermore, meat can also get contaminated with pathogens, like *E. coli*, *S. aureus*, *L. monocytogenes* and *Bacillus*. It is, thus, a priority of the meat industry to apply preservation techniques that can maintain the microbial quality and safety, while improving, or without altering, the chemical and sensory aspects of the product (Kaveh, Hashemi, Abedi, Amiri, & Conte, 2023). In Boerewors, a traditional South African fresh sausage, the main preservative is SO_2 at an inclusion level of 450 mg/kg (Hugo & Hugo, 2015; South African Department of Health, 2020).

Plant-derived products as preservatives, have been used for years, but gained interest, since consumers demanded more natural ingredients in the products. Blends of rosemary with other natural extracts have opened new possibilities for natural preservation (Lorenzo, Munekata, Pateiro, Domínguez, Alaghbari, & Tomasevic, 2021). Rosemary extract and blends have antimicrobial and antioxidant properties, that extends the shelf-life of food products like fresh meat (Kaur, Gupta, Bronlund, & Kaur, 2023).

Biopreservation of meat is a newer trend in the food industry. One method of biopreservation is using protective cultures, which essentially consist of bacteria that have been selected for the specific ability to inhibit the growth of spoilage microorganisms and pathogens (Silva, Silva, & Ribeiro, 2018). Many studies on fresh meat preservation have included protective cultures, such as *Lactobacillus sakei*, *Lactobacillus curvatus*, and *Staphylococcus carnosus* (Castellano, Belfiore, Fadda, & Vignolo, 2008; Xu, Kaur, Pillidge, & Torley, 2022)

The purpose of this part of the study was to determine whether it will be possible to replace the SO_2 in Boerewors with natural preservatives in the form of plant extracts (Kalsec products) or protective cultures (Chr. Hansen products). The specific objectives of this chapter were to:

1. Determine the microbial quality of the raw material, which was used to manufacture the Boerewors models.
2. Determine the effect of the natural preservatives on the pH, a_w and lipid oxidative stability of the Boerewors over a 6-day storage period at 4 °C, and a 90-day storage period at -18 °C for the latter.
3. Determine the effect of the natural preservatives on the microbial quality of Boerewors over a 6-day storage period at 4 °C; and

4. Determine the effect of the natural preservatives on sensory acceptance and colour of Boerewors.

3.2 Materials and Methods

3.2.1 Sourcing of natural preservatives

The natural preservatives used to replace SO_2 in this study, were commercially available. Two types of natural preservatives, indicated in Table 3.1, were used in this study. The first type was protective cultures (PrC) which were obtained from Chr. Hansen® (Sales and Administration Support – Middle East & Africa, Dubai). The second type of natural preservative was obtained from Kalsec®, Inc. (Kalamazoo, Michigan, United States of America) and consisted of natural plant extracts. The bioactive compounds in the preservatives were discussed in Chapter 2 and are given in Table 3.1. The cost of each preservative used, is also indicated in Table 3.1.

3.2.2 Sourcing of lean meat, fat, and spices

The lean meat and back fat, used in this study, was sourced from a butchery in Bloemfontein, South Africa. Pork, made up of a minimum of 70.0% lean meat and 30.0% fat (70/30); lean beef (80/20) and high-quality pork back fat was used in the manufacturing of the Boerewors batters. The meat was collected and transported to the meat processing facility of the University of the Free State, for processing and kept refrigerated (4 °C) until used within 24 h. The additives and spices were obtained from Crown National (Bloemfontein, South Africa).

3.2.3 Formulation of Boerewors

Six different treatment formulations were used. Treatment 1 (Tables 3.2 and 3.3), the negative control (NC), was formulated with 0% SO_2. Treatments 2 (Tables 3.4 and 3.5) and 3 (Tables 3.6 and 3.7), were formulated with protective cultures, which replaced SO_2 in the conventional formulation, with 0.025% BLC 20 culture (PrC1) and 0.0125% BLC 48 culture (PrC2), respectively. Treatments 4 (Tables 3.8 and 3.9) and 5 (Tables 3.10 and 3.11), were formulated with plant extracts, which replaced SO_2 in the conventional formulation, with 0.35% Duralox NR20 (KD1) and 0.55% Duralox NV + 0.5% RD30 (KD2), respectively. The formulation of treatment 6, the positive control (PC), was based on the South African DoH's set regulations (2020) regarding the maximum levels of SO_2 in products: 450 mg/kg sausage. Treatment 6 (Tables 3.12 and 3.13) contained 0.0682% sodium metabisulphite ($Na_2S_2O_5$), which was approximately 0.035% SO_2 in the final meat product.

Table 3.1. The preservatives used in this study. *The cost given is excluding of VAT and import costs. On 28 July 2023 the Rand to Dollar exchange rate was R 1.00 ZAR = 0.056 United States Dollar.

Preservative	Abbreviation used	Company	Product name	Product cost (/kg meat)*	Active ingredient	Mode of action
Protective Culture 1	PrC1	Chr. Hansen	SafePro® B-LC-20	R 9.70	*Pediococcus acidilactici*	Anti-listerial Extend shelf life
Protective Culture 2	PrC2	Chr. Hansen	SafePro® B-LC-48	R 4.80	*Lactobacillus curvatus*	Suppress growth of spoilage and pathogenic bacteria such as indigenous lactic acid bacteria and *Listeria monocytogenes*. The culture performs within a wide temperature range down to 4 °C and survives freezing
Plant extract 1	KD1	Kalsec	Duralox® NR-20 (62-120-08)	R 0.36 – R 1.42	Synergistic liquid blend of rosemary extract, and acerola oil	Antioxidant Colour stability
Plant extract 2	KD2	Kalsec	Duralox® NV-3 (62.510.02) + Duralox® RD-30 (62.91.002)	R 0.36 – R 1.42	Synergistic powder blend of vinegar and rosemary extract, and acerola extract	Antimicrobial
Sulphur dioxide	PC	Crossmill Chemicals; Brenntag; Bragan	-	R 13.90	Sodium metabisulphite	Antimicrobial Antioxidant Colour stability

Table 3.2. Treatment 1 (NC) spice formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Coriander Ground	0.1620	1.620	4.860
Coriander Coarse	0.3918	3.918	11.755
MSG	0.1755	1.755	5.266
Black Pepper Ground	0.1620	1.620	4.860
Nutmeg Ground	0.1350	1.350	4.050
Clove Grounds	0.0400	0.400	1.200
Thyme rubbed	0.0270	0.270	0.810
Fine Salt	1.5000	15.000	45.000
Ascorbic acid	0.0062	0.062	0.186
Dextrose	0.1004	1.004	3.013
Maltodextrin	0.3850	3.850	11.550
Cereal binder	0.1650	1.650	4.950
BLC48	**0.0000**	**0.000**	**0.000**
BLC20	**0.0000**	**0.000**	**0.000**
Kalsec Duralox NV3 + Kalsec Duralox RD30	**0.0000**	**0.000**	**0.000**
Sodium Metabisulphite	**0.0000**	**0.000**	**0.000**
Total	**3.2500**	**32.500**	**97.500**

Table 3.3. Treatment 1 (NC) sausage formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Beef 90/10	60.00	600.00	1800.00
Pork 50/50	30.00	300.00	900.00
Vinegar	1.48	14.78	44.34
Water	3.40	34.00	102.00
Rusk	1.47	14.72	44.16
Worcester sauce	0.40	4.00	12.00
Spice mixture	3.25	32.50	97.50
Total	**100.00**	**1000.00**	**3000.00**

Table 3.4. Treatment 2 (PrC1) spice formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Coriander Ground	0.1620	1.620	4.860
Coriander Coarse	0.3918	3.918	11.755
MSG	0.1755	1.755	5.266
Black Pepper Ground	0.1620	1.620	4.860
Nutmeg Ground	0.1350	1.350	4.050
Clove Grounds	0.0400	0.400	1.200
Thyme rubbed	0.0270	0.270	0.810
Fine Salt	1.5000	15.000	45.000
Ascorbic acid	0.0062	0.062	0.186
Dextrose	0.1004	1.004	3.013
Maltodextrin	0.3675	3.675	11.025
Cereal binder	0.1575	1.575	4.725
BLC48	**0.0000**	**0.000**	**0.000**
BLC20	**0.0250**	**0.250**	**0.750**
Kalsec Duralox NV3 + Kalsec Duralox RD30	**0.0000**	**0.000**	**0.000**
Sodium Metabisulphite	**0.0000**	**0.000**	**0.000**
Total	**3.2500**	**32.500**	**97.500**

Table 3.5. Treatment 2 (PrC1) sausage formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Beef 90/10	60.00	600.00	1800.00
Pork 50/50	30.00	300.00	900.00
Vinegar	1.48	14.78	44.34
Water	3.40	34.00	102.00
Rusk	1.47	14.72	44.16
Worcester sauce	0.40	4.00	12.00
Spice mixture	3.25	32.50	97.50
Total	**100.00**	**1000.00**	**3000.00**

Table 3.6. Treatment 3 (PrC2) spice formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Coriander Ground	0.1620	1.620	4.860
Coriander Coarse	0.3918	3.918	11.755
MSG	0.1755	1.755	5.266
Black Pepper Ground	0.1620	1.620	4.860
Nutmeg Ground	0.1350	1.350	4.050
Clove Grounds	0.0400	0.400	1.200
Thyme rubbed	0.0270	0.270	0.810
Fine Salt	1.5000	15.000	45.000
Ascorbic acid	0.0062	0.062	0.186
Dextrose	0.1004	1.004	3.013
Maltodextrin	0.3763	3.763	11.289
Cereal binder	0.1612	1.612	4.836
BLC48	**0.0125**	**0.125**	**0.375**
BLC20	**0.0000**	**0.000**	**0.000**
Kalsec Duralox NV3 + Kalsec Duralox RD30	**0.0000**	**0.000**	**0.000**
Sodium Metabisulphite	**0.0000**	**0.000**	**0.000**
Total	**3.2500**	**32.500**	**97.500**

Table 3.7. Treatment 3 (PrC2) sausage formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Beef 90/10	60.00	600.00	1800.00
Pork 50/50	30.00	300.00	900.00
Vinegar	1.48	14.78	44.34
Water	3.40	34.00	102.00
Rusk	1.47	14.72	44.16
Worcester sauce	0.40	4.00	12.00
Spice mixture	3.25	32.50	97.50
Total	**100.00**	**1000.00**	**3000.00**

Table 3.8. Treatment 4 (KD1) spice formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Coriander Ground	0.1620	1.620	4.860
Coriander Coarse	0.3918	3.918	11.755
MSG	0.1755	1.755	5.266
Black Pepper Ground	0.1620	1.620	4.860
Nutmeg Ground	0.1350	1.350	4.050
Clove Grounds	0.0400	0.400	1.200
Thyme rubbed	0.0270	0.270	0.810
Fine Salt	1.5000	15.000	45.000
Ascorbic acid	0.0062	0.062	0.186
Dextrose	0.1004	1.004	3.013
Maltodextrin	0.3850	3.850	11.550
Cereal binder	0.1650	1.650	4.950
BLC48	**0.0000**	**0.000**	**0.000**
BLC20	**0.0000**	**0.000**	**0.000**
Kalsec Duralox NV3 + Kalsec Duralox RD30	**0.0000**	**0.000**	**0.000**
Sodium Metabisulphite	**0.0000**	**0.000**	**0.000**
Total	**3.2500**	**32.500**	**97.500**

Table 3.9. Treatment 4 (KD1) sausage formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Beef 90/10	60.00	600.00	1800.00
Pork 50/50	30.00	300.00	900.00
Vinegar	1.48	14.78	44.34
Water	3.05	30.50	91.50
Kalsec Duralox NR20	**0.35**	**3.50**	**10.50**
Rusk	1.47	14.72	44.16
Worcester sauce	0.40	4.00	12.00
Spice mixture	3.25	32.50	97.50
Total	**100.00**	**1000.00**	**3000.00**

Table 3.10. Treatment 5 (KD2) spice formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Coriander Ground	0.1620	1.620	4.860
Coriander Coarse	0.3918	3.918	11.755
MSG	0.1755	1.755	5.266
Black Pepper Ground	0.1620	1.620	4.860
Nutmeg Ground	0.1350	1.350	4.050
Clove Grounds	0.0400	0.400	1.200
Thyme rubbed	0.0270	0.270	0.810
Fine Salt	1.5000	15.000	45.000
Ascorbic acid	0.0062	0.062	0.186
Dextrose	0.1004	1.004	3.013
Maltodextrin	0.0000	0.000	0.000
Cereal binder	0.0000	0.000	0.000
BLC48	**0.0000**	**0.000**	**0.000**
BLC20	**0.0000**	**0.000**	**0.000**
Kalsec Duralox NV3 + Kalsec Duralox RD30	**0.5500**	**5.500**	**16.500**
Sodium Metabisulphite	**0.0000**	**0.000**	**0.000**
Total	**3.2500**	**32.500**	**97.500**

Table 3.11. Treatment 5 (KD2) sausage formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Beef 90/10	60.00	600.00	1800.00
Pork 50/50	30.00	300.00	900.00
Vinegar	1.48	14.78	44.34
Water	3.40	34.00	102.00
Rusk	1.47	14.72	44.16
Worcester sauce	0.40	4.00	12.00
Spice mixture	3.25	32.50	97.50
Total	**100.00**	**1000.00**	**3000.00**

Table 3.12. Treatment 6 (PC) spice formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Coriander Ground	0.1620	1.620	4.860
Coriander Coarse	0.3918	3.918	11.755
MSG	0.1755	1.755	5.266
Black Pepper Ground	0.1620	1.620	4.860
Nutmeg Ground	0.1350	1.350	4.050
Clove Grounds	0.0400	0.400	1.200
Thyme rubbed	0.0270	0.270	0.810
Fine Salt	1.5000	15.000	45.000
Ascorbic acid	0.0062	0.062	0.186
Dextrose	0.1004	1.004	3.013
Maltodextrin	0.3373	3.373	10.119
Cereal binder	0.1445	1.445	4.335
BLC48	**0.0000**	**0.000**	**0.000**
BLC20	**0.0000**	**0.000**	**0.000**
Kalsec Duralox NV3 + Kalsec Duralox RD30	**0.0000**	**0.000**	**0.000**
Sodium Metabisulphite	**0.0682**	**0.682**	**2.046**
Total	**3.2500**	**32.500**	**97.500**

Table 3.13. Treatment 6 (PC) sausage formulation

Component	Final product inclusion (%)	Final product inclusion (g/kg)	Final product inclusion (g/3 kg)
Beef 90/10	60.00	600.00	1800.00
Pork 50/50	30.00	300.00	900.00
Vinegar	1.48	14.78	44.34
Water	3.40	34.00	102.00
Rusk	1.47	14.72	44.16
Worcester sauce	0.40	4.00	12.00
Spice mixture	3.25	32.50	97.50
Total	**100.00**	**1000.00**	**3000.00**

3.2.4 Manufacturing of Boerewors

Three replicates of six Boerewors treatments were performed at different times of the year, to compensate for variations in raw materials, processing, and environmental conditions. A single replicate consisted of 3 kg batches, manufactured for each of the six treatments.

The Boerewors models were manufactured by using representative industrial procedures (Hugo, Roberts, & Smith, 1993), and in compliance with the South African regulations for Boerewors (Government Notice No. R.2718 of November 1990; Foodstuffs, Cosmetics and Disinfectants Act No. 54 of 1972). Fresh meat [beef (80/20) and pork (70/30)] were minced through a 13 mm mincing plate, fitted to a number 32 Okto mincer. The minced meat was thoroughly mixed, to obtain a homogenous raw material mixture. The preservatives with the spices and additives were mixed with ice water and then thoroughly mixed with the meat mixture, before being minced through a 4.5 mm mincing plate. Natural hog casings with a diameter of 28-32 mm were filled with the sausage mixture using a manual sausage filler (Trespade, Crown National, Johannesburg, South Africa). This resulted in a single, continuous roll of sausage that had a weight of 3 kg per treatment. Individual sausages were cut from each roll. Each treatment's roll of sausage was cut in 150 g pieces for days 0, 3 and 6, and 50 g pieces for the 90-day samples.

Each individual sausage was placed in an expanded polystyrene (EPS) tray containing an absorbent pad and was then over-wrapped with polyvinyl chloride (PVC) film. The sausages that were sampled on days 0, 3 and 6, were stored at 4 °C under retail refrigeration-type conditions, including fluorescent lighting, for fresh product shelf-life determination (20 sausages per treatment for each day of sampling). The other sausages for the 90-day analysis, were also over-wrapped with PVC, and stored at -18 °C for frozen product lipid stability determination. For the fourth replicate, a whole 3 kg sausage roll of all 6 treatment groups were stored in plastic bags at -18 °C until sensory analysis was carried out shortly after the sausages were made.

3.2.5 Sample preparation

In South Africa, Boerewors are typically sold as a fresh sausage. It is also common practice for fresh Boerewors to be bought and frozen at home. To this end, both a fresh product shelf-life evaluation of up to 6 days as well as a frozen product shelf-life evaluation of up to 90 days were used during which sampling took place. The fresh product shelf-life evaluation sampling took place on days 0, 3 and 6 of refrigerated storage at 4 °C, and the frozen product lipid oxidation shelf-life evaluation took place on day 90. For each sampling interval, four sausages

per treatment group per replicate were collected for quadruplicate physico-chemical and microbial analyses.

3.2.6 Physico-chemical analyses

Various physico-chemical analysis techniques were used across all six treatments. After samples for microbial analyses were taken aseptically, the remaining sausage sample of day zero was used to fill two plastic cuvettes with tight fitting lids with sample. One cuvette was frozen at –18 °C for chemical analyses that were conducted on days following the sampling day of days 0 and 6. The other cuvette was used for same-day physico-chemical analyses. The same process was repeated for each of the four samples, per treatment group, per time interval, per replicate. A 100 g sample of each Boerewors treatment were packaged in a Styrofoam tray, wrapped with plastic cling film, and frozen at –18 °C for 90 days for lipid oxidative stability analysis.

3.2.6.1 pH measurements

Measurements for pH were done directly by using a suitable 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, South Africa) to record quadruple pH measurements per treatment group per replicate at room temperature. Each day before use, the pH meter was calibrated with standardized buffers (Merck, Johannesburg, South Africa) with pH values of 4.01 and 7.00, respectively.

3.2.6.2 Water activity (a_w)

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

3.2.6.3 Lipid oxidative stability and moisture content

A 5 g sample was taken at each sampling interval per treatment group per replicate and used for thiobarbituric acid reactive substance (TBARS) analysis using the aqueous acid extraction method of Raharjo et al. (1993) to determine the effect of the different treatments on lipid oxidation. Frozen samples were defrosted overnight at 4 °C. The TBARS results were quantified in terms of milliequivalents (mEq) malondialdehyde (MDA) per kg of sample as other TBARS may also be present (Beltran et al., 2003). Analysis took place after days 0 and 6 of storage at 4 °C as well as after 90 days of storage at -18 °C. Moisture content (%) was determined by oven drying overnight at 121 °C (AOAC, 2005) and used as a second establishing parameter as it is required in the calculation of TBARS.

3.2.7 Microbial analyses

For the microbial analysis, first the natural casing around the sausage was removed aseptically with a pair of flame-sterilised scissors and tweezers. A 10 g sample from each product was aseptically weighed and placed into a sterile 207 ml WhirlPak™ bag (Lasec, Bloemfontein, South Africa) after which 90 ml of sterile 0.1 M buffered peptone water (BPW) solution was added to create a 10^{-1} dilution. It was stomached (AME Stomacher Lab-Blender 400, JHB) for 1 minute. Further dilutions (10^{-2} to 10^{-6}) were made by adding 1 ml of the 100 ml sample (10^{-1} dilution) in McCartney bottles containing 9 ml sterile 0.1 M phosphate buffer solution (Harrigan, 1998).

One millilitre volumes of each dilution was pour-plated, using SPCA (Oxoid CM0463) for the determination of the total bacterial count (TBC); violet red bile agar + 4-methylumbelliferyl-β-D-glucuronide (VRBM; Oxoid CM0978) was used for total coliform and *E. coli* counts, and fluorescence under ultraviolet light (366 nm, CAMAG Universal UV Lamp) was used as indication of the presence of *E. coli*; Baird Parker (BP) agar (Oxoid CM025), containing egg yolk tellurite emulsion (Oxoid SR054C) was used for *S. aureus* determination; violet bile glucose agar (VRBGA) (Oxoid CM0485) was used for *Enterobacteriaceae* determination; rose-bengal chloramphenicol agar (Oxoid CM0549), with chloramphenicol supplement (Oxoid SR0078), was used for yeasts and moulds determination; and MRS agar, consisting of MRS Broth (Oxoid CM0359) with 1.50% w/v agar bacteriological (Oxoid Agar No.1, LP0011), was used for the enumeration of lactic acid bacteria (LAB; Harrigan,1998). The above-mentioned media were sourced from ThermoFisher (Pty) Ltd (Randburg, South Africa).

Plates for TBC enumeration were incubated at 32 °C for 48 h; plates for coliforms, *E. coli*, *S. aureus* and *Enterobacteriaceae* enumeration were incubated at 37 °C for 24 h; plates for

yeasts and moulds enumeration were incubated at 25 °C for 4 days and plates for LAB enumeration were incubated at 35 °C for 48 h. The enumeration of colonies was performed, using a manual colony counter (Harrigan, 1998).

The sausage samples were also analysed for *Listeria monocytogenes*; 0.5 ml of the 10^{-1} dilutions were spread plated on two pre-poured RAPID'*L.mono* (Bio-Rad 356-4293) agar containing Supplements 1 (Bio-Rad 356-4294) and 2 (Bio-Rad 356-4746). The media were supplied by AEC Amersham (Johannesburg, South Africa). Incubation was at 37 °C for 24 h. Blue colonies on the red agar was a positive indicator for *Listeria monocytogenes* presence.

3.2.8 Sensory analyses

3.2.8.1 Colour

Colour measurements were performed on the refrigerated Boerewors samples on days 0, 3 and 6 of storage at 4 °C after the sample for microbial analysis were taken and samples were left to stand for 30 min. Four sausage samples of each of the six treatment groups were used and measurements per sausage were done in sextuplicate. Colour measurements were made using a Minolta CR 400 chromometer (8 mm measuring area). The *CIE L*a*b** colour scale was used for comparison, where *L** represented lightness, *a** represented redness, and *b** represented yellowness. The chroma ($\sqrt{a^{*2}+b^{*2}}$) and hue angle ($\tan^{-1}(b^*/a^*)$) were also calculated (Ripoll, Joy, & Muñoz, 2011; Tapp, Yancey, & Apple, 2011).

3.2.8.2 Consumer sensory evaluation

Samples of each treatment group were defrosted overnight at 4 °C. The sausages were then dry cooked in a convection oven pre-heated to 160 °C until an internal temperature of 72 °C was reached. During cooking the baking tray was rotated 90° every 2 min for even cooking conditions. The cooked sausages were cut into pieces each with a length of ~ 2 cm and placed individually in small glass bowls that were covered with squares of aluminium foil. The bowls were kept warm at 55 °C until just before serving. Each container was marked with a randomized, three-digit code unique to each sample. Six glass bowls, each representing one of the six treatment groups, were arranged from left to right on a serving tray, in ascending order of the three-digit codes, ensuring that the samples were evaluated in a random order from one consumer to the next.

A 100-member consumer panel of staff and students from the Agriculture Building of the University of the Free State was used. The panel consisted of 70% females and 30% males:

ranging from 17 to 61 years of age. The sensory evaluation was performed in individual booths of the sensory laboratory, and the booths were fitted with three overhead light fittings with three red coloured bulbs, emitting only red light, to mask any possible colour variations between different samples.

Each respondent received a printed, 5-page questionnaire consisting of five nine-point hedonic rankings per page, ranging from 1 = dislike extremely to 9 = like extremely (Table 3.14). The respondents were then expected to rank each sample individually for the following attributes: aroma, taste, aftertaste, texture, and general acceptability. Bottled water was presented at 20 °C as a palate cleanser between samples. Consumers would be excited about new, healthier products. Although, consumers might also react negatively when a supposedly healthier product is regarded as unpalatable (Guerrero et al., 2011). For these reasons, the members of this consumer panel were not specifically informed of the differences in preservatives of the six models, prior to sensory evaluation.

Table 3.14. Simplified example of the hedonic ranking used for consumer sensory analysis.

Nine-point ranking scale for aroma, taste, aftertaste, texture, and general acceptability								
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	2	3	4	5	6	7	8	9

Ethical clearance for the sensory analysis was obtained from the General/Health Ethics Committee of the University of the Free State with number UFS-HSD2020/0539/0809/21/22.

3.2.9 Statistical analyses

This analysis was performed to uncover key insights and differences between the different treatments and days 0, 3, and 6. Two primary statistical techniques were used: Analysis of Variance (ANOVA) and the Kruskal-Wallis (KW) test. The level of significance was set at $\alpha = 0.05$. Means and standard deviations were calculated for each Treatment, Day, and Treatment/Day combination. Before applying the ANOVA test, the data had to meet the underlying assumptions of this test. In instances where these assumptions were violated, non-parametric alternatives like the KW test were employed. The ANOVA test was employed to determine whether there were any statistically significant differences between the means of three or more independent groups. Post-hoc tests (Tukey HSD) were conducted in cases

where ANOVA resulted in a significant effect, to determine which specific groups differed from each other. Where the assumptions of the ANOVA were not met, the KW test, a non-parametric alternative to the ANOVA, was used. This test was used to determine whether there were statistically significant differences between the medians of three or more independent groups. The Dunns' procedure was used to determine which groups differed specifically.

3.3 Results and discussion

3.3.1 Physico-chemical analyses

3.3.1.1 pH, a_w, and moisture content

The Boerewors treatment influenced the pH (Table 3.15). On day 0, KD2 had a significantly ($p = 0.006$) higher pH than that of treatment groups PrC1, KD1 and PC. On day 3, the differences were not statistically significant enough with post-hoc analysis. At the end of the shelf-life, KD2 had a significantly ($p = 0.019$) higher pH value than KD1. The buffered vinegar in the KD2 treatment could be the reason for the pH difference because the pH of buffered vinegar can range from 4.75 up to 6.75 (Younes, Aquilina, Degen, Engel, Fowler, Frutos Fernandez, Fürst, Gundert-Remy, Gürtler, & Husøy, 2022). PrC1 had the same pH value as the PC on day 0. All the pH values, regardless of treatment and time interval, were slightly below the average pH of fresh meat, which is 5.50–5.60 (Jay et al., 2005; Arias, 2012). This was attributed to the addition of vinegar in the Boerewors formulation, which has a pH of 2 to 3 (Cosmulescu, Stoenescu, Trandafir, & Tuțulescu, 2022), to all the Boerewors formulations.

Storage time significantly affected the pH of the four treatment groups: NC, PrC1, PrC2, and KD1 (Table 3.16). Fluctuations in the pH were observed for all the treatments over the 6-day shelf-life. The pH of NC and PrC2 were significantly ($p = 0.006$ and $p = 0.017$, respectively) higher on day 3, compared to day 0. The pH of PrC1 on day 0 was higher ($p = 0.003$) than the pH values on day 3 and 6. KD1 had a significantly ($p = 0.002$) higher pH on day 3, compared to day 0 and 6. This increase in pH from day 0 to day 3 for most of the treatments in this study, just to decrease again on day 6 could not be explained. Generally, the KD2 and PC treatments resisted changes in pH better than the other four formulations.

The effect of added preservative on a_w, was observed, on day 0, with the water activity of the KD1 group significantly lower ($p = 0.046$) than that of PrC1 formulation (Table 3.15). The low a_w of KD1 could be due to the difference in the water added during the manufacturing process of each Boerewors formulation type. The formulation for the KD1 Boerewors had a water inclusion level of 3.05%, because it was the only group with a liquid preservative, whereas the inclusion level for the other treatments were 3.40% (Table 3.9). On days 3 and 6, there were no significant differences in a_w between the treatment groups.

The storage time had an effect on the water activity of three treatment groups: PrC1, PrC2, and KD1. The a_w of PrC1 was higher ($p = 0.036$) on day 3 than on day 6, and the a_w of PrC2 was the highest ($p = 0.005$) on day 3, compared to both days 0 and 6. However, the a_w of KD1 on day 3, was only higher ($p = 0.004$) than the a_w measured on day 0. A possible reason for this could be the change in the solubility of the solutes, with or without the influence of bacterial growth in the KD1 treatment, as it adapted to the environment and then decreased again due to the low storage temperature (Scott, & Bernard, 1983).

No significant differences in moisture content were found between any of the six treatment groups after any of the time intervals (Table 3.15). Similarly, storage time had no significant effect on the moisture content of any of the six treatment groups (Table 3.16). Even though freezing and thawing changes the moisture content of meat products (Wierbicki, & Deatherage, 1958), and many factors influence these changes, it was not statistically significant over time.

Table 3.15. The treatment effect of pH, a_w and moisture content of six Boerewors formulations with different preservatives at 4 °C, over a 6-day shelf-life, and frozen at -18 °C, for a 90-day period. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). a_w = water activity. Means with different superscripts differed significantly. NS = Not significant; NA = Not applicable. *ANOVA/KW significant, but post-hoc tests not significant enough to show a specific difference.

Day	Treatment	pH	Sign. level (n = 24)	a_w	Sign. level (n = 24)	Moisture content (%)	Sign. level (n = 12)
0	NC	$5.39^{ab} \pm 0.03$	p = 0.006	$0.93^{ab} \pm 0.02$	p = 0.046	63.92 ± 1.61	NS
	PrC1	$5.38^{b} \pm 0.03$		$0.93^{a} \pm 0.02$		63.65 ± 1.94	
	PrC2	$5.39^{ab} \pm 0.04$		$0.93^{ab} \pm 0.01$		63.40 ± 1.57	
	KD1	$5.36^{b} \pm 0.12$		$0.91^{b} \pm 0.02$		63.64 ± 1.80	
	KD2	$5.45^{a} \pm 0.04$		$0.93^{ab} \pm 0.01$		63.67 ± 1.86	
	PC	$5.38^{b} \pm 0.05$		$0.93^{ab} \pm 0.01$		63.40 ± 2.58	
3	NC	5.44 ± 0.04	p = 0.018*	0.93 ± 0.02	NS	NA	NA
	PrC1	5.43 ± 0.04		0.94 ± 0.01			
	PrC2	5.45 ± 0.09		0.94 ± 0.01			
	KD1	5.54 ± 0.20		0.94 ± 0.01			
	KD2	5.49 ± 0.06		0.94 ± 0.02			
	PC	5.41 ± 0.07		0.94 ± 0.02			
6	NC	$5.41^{ab} \pm 0.03$	p = 0.019	0.92 ± 0.02	NS	63.08 ± 1.37	NS
	PrC1	$5.42^{ab} \pm 0.03$		0.93 ± 0.01		63.45 ± 1.04	
	PrC2	$5.41^{ab} \pm 0.04$		0.93 ± 0.01		63.65 ± 1.31	
	KD1	$5.40^{b} \pm 0.05$		0.93 ± 0.01		63.66 ± 1.65	
	KD2	$5.47^{a} \pm 0.05$		0.93 ± 0.01		63.71 ± 1.24	
	PC	$5.43^{ab} \pm 0.05$		0.93 ± 0.01		62.97 ± 1.90	
90	NC	NA	NA	NA	NA	64.69 ± 1.85	NS
	PrC1					64.55 ± 2.06	
	PrC2					64.22 ± 1.98	
	KD1					64.83 ± 2.06	
	KD2					64.66 ± 1.27	
	PC					64.75 ± 2.62	

Table 3.16. The storage time effect on pH and a_w over a 6-day shelf-life, at 4 °C, and on moisture content, frozen at -18 °C, after a 90-day period, of six Boerewors formulations with different preservatives. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). a_w = water activity. Means with different superscripts in the same column differed significantly. NS = Not significant; NA = Not applicable.

Treatment	Day	pH	Sign. level (n = 24)	a_w	Sign. level (n = 24)	Moisture content (%)	Sign. level (n = 12)
NC	0	$5.39^{b} \pm 0.03$	p = 0.006	0.93 ± 0.02	NS	63.92 ± 1.61	NS
	3	$5.44^{a} \pm 0.04$		0.93 ± 0.02		NA	
	6	$5.41^{ab} \pm 0.03$		0.92 ± 0.02		63.08 ± 1.37	
	90	NA		NA		64.69 ± 1.85	
PrC1	0	$5.38^{b} \pm 0.03$	p = 0.003	$0.93^{ab} \pm 0.02$	p = 0.036	63.65 ± 1.94	NS
	3	$5.43^{a} \pm 0.04$		$0.94^{a} \pm 0.01$		NA	
	6	$5.42^{a} \pm 0.03$		$0.93^{b} \pm 0.01$		63.45 ± 1.04	
	90	NA		NA		64.55 ± 2.06	
PrC2	0	$5.39^{b} \pm 0.04$	p = 0.017	$0.93^{b} \pm 0.01$	p = 0.005	63.40 ± 1.57	NS
	3	$5.45^{a} \pm 0.09$		$0.94^{a} \pm 0.01$		NA	
	6	$5.41^{ab} \pm 0.04$		$0.93^{b} \pm 0.01$		63.65 ± 1.31	
	90	NA		NA		64.22 ± 1.98	
KD1	0	$5.36^{b} \pm 0.12$	p = 0.002	$0.91^{a} \pm 0.02$	p = 0.004	63.64 ± 1.80	NS
	3	$5.54^{a} \pm 0.20$		$0.94^{b} \pm 0.01$		NA	
	6	$5.40^{b} \pm 0.05$		$0.93^{ab} \pm 0.01$		63.66 ± 1.65	
	90	NA		NA		64.83 ± 2.06	
KD2	0	5.45 ± 0.04	NS	0.93 ± 0.01	NS	63.67 ± 1.86	NS
	3	5.49 ± 0.06		0.94 ± 0.02		NA	
	6	5.47 ± 0.05		0.93 ± 0.01		63.71 ± 1.24	
	90	NA		NA		64.66 ± 1.27	
PC	0	5.38 ± 0.05	NS	0.93 ± 0.01	NS	63.40 ± 2.58	NS
	3	5.41 ± 0.07		0.94 ± 0.02		NA	
	6	5.43 ± 0.05		0.93 ± 0.01		62.97 ± 1.90	
	90	NA		NA		64.75 ± 2.62	

3.3.1.2 Lipid oxidative stability

The lipid oxidative stability of the Boerewors was determined by the quantification of TBARS, using MDA as an indicator of secondary lipid oxidation levels. The lipid oxidative stability of all six Boerewors treatments were acceptable at the beginning and end of the 6-day shelf-life (Figure 3.1), with secondary lipid oxidation products (SLOP) levels far below 0.50 mEq MDA/kg, which is the primary detection threshold for rancidity (Wood, Enser, Fisher, Nute, Sheard, Richardson, Hughes, & Whittington, 2008). On day 0, the KD2 group had a significantly lower ($p < 0.0001$) TBARS value than both control groups, while PC had a significantly ($p < 0.0001$) higher value than all the natural preservative treatment groups (PrC1, PrC2, KD1 and KD2). The PC group continued to have the highest TBARS on day 6, and the plant extract treatment groups, KD1 and KD2, had significantly ($p < 0.0001$) lower TBARS than the control groups, NC, and PC, on day 6. After 90 days of storage at -18 °C, antioxidant effects of the added preservatives were clear; NC, with no added preservatives, had a significantly ($p < 0.0001$) higher TBARS value than KD1, KD2, and PC (Figure 3.1). KD1 and KD2 also had lower ($p < 0.0001$) TBARS than the protective culture treatment groups, PrC1 and PrC2. Although, after 90 days of frozen storage, all treatment groups were below the secondary rancidity detection threshold of 1.00 mEq MDA/kg (Gray & Pearson, 1987), and five of the treatment groups, PrC1, PrC2, KD1, KD2, and PC, were still below the primary threshold of detecting rancidity. This indicated that all the natural preservatives and the conventional SO_2 were effective in controlling the lipid oxidation during a 6-day shelf life at 4 °C and until 90 days storage at -18 °C. The bioactive compounds in plant extracts played an important role in preventing the lipid oxidation of the Boerewors due to their high antioxidant activity (Awad, Kumar, Ismail-Fitry, Jusoh, Ab Aziz, & Sazili, 2022).

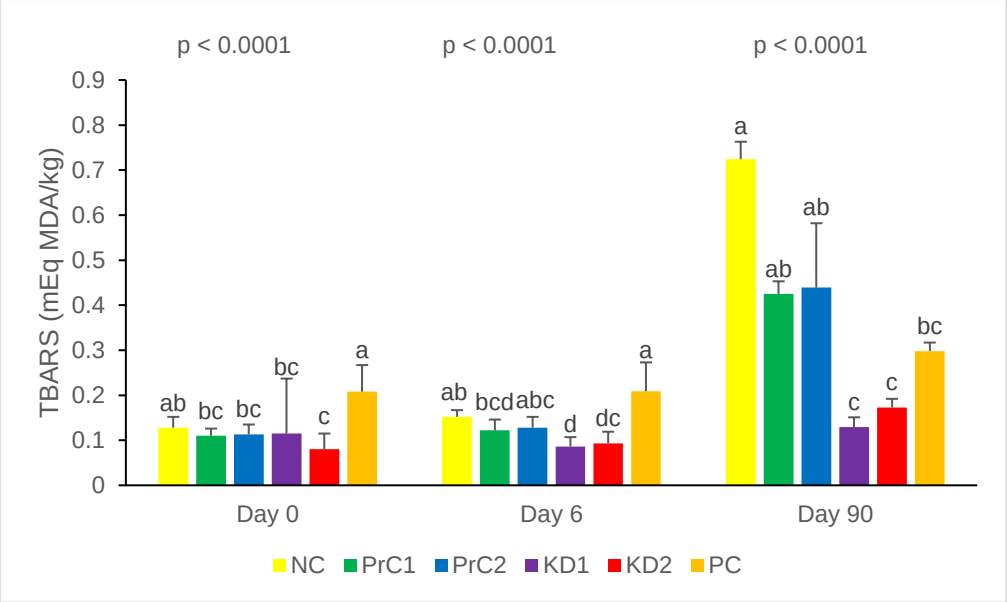

Figure 3.1. The effect of added preservative on the TBARS of Boerewors stored at -18 °C for 90 days (n = 12). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly.

When meat is frozen, the ester bonds in the fat are hydrolysed and the fatty acids get oxidised (Contini, Álvarez, O'Sullivan, Dowling, Gargan, & Monahan, 2014). Oxidation of meat lipids during freezing are difficult to control, because even small amounts easily react with other components, resulting in peroxide formation, which is the chemically unstable primary product of the process. Subsequentially, as secondary oxidative transformations occur, reactive hydroperoxides and other oxidation products are formed (Shaur, Siems, Bresgen, & Eckl, 2015; Wereńska, Okruszek, Haraf, Wołoszyn, & Goluch, 2022). As expected, storage time influenced the SLOP levels of all six treatment groups (Figure 3.2). The TBARS of all six Boerewors treatment groups remained quite stable during the first 6 days of storage at -18 °C, with levels below 0.30 mEq MDA/kg, but after 90 days, some treatment groups had substantially higher SLOP formations. NC showed the biggest difference ($p < 0.0001$), starting with 0.128 ± 0.024 mEq MDA/kg on day 0, to 0.724 ± 0.039 mEq MDA/kg on day 90. Even though the difference was still significant ($p = 0.001$), KD1 had the smallest difference from day 0 (0.115 ± 0.122 mEq MDA/kg) to a still very acceptable value (0.129 ± 0.022 mEq MDA/kg) on day 90. The plant extract treatment groups had the best antioxidant effects from start to finish, which could be attributed to the rosemary and acerola extract blends in KD1 and KD2. According to a study on soybean oil (Nedamani, Mahoonak, Ghorbani, &

Kashaninejad, 2015), rosemary showed high antioxidant activity but had an even greater effect when combined with other plant extracts. The rosemary extract contains caffeic acid derivates, and these compounds react with metal ions present in the environment, in this case meat, so chelates are formed, which consequently react with peroxide radicals and, therefore, stabilise the free radicals (Nieto, Ros, & Castillo, 2018).

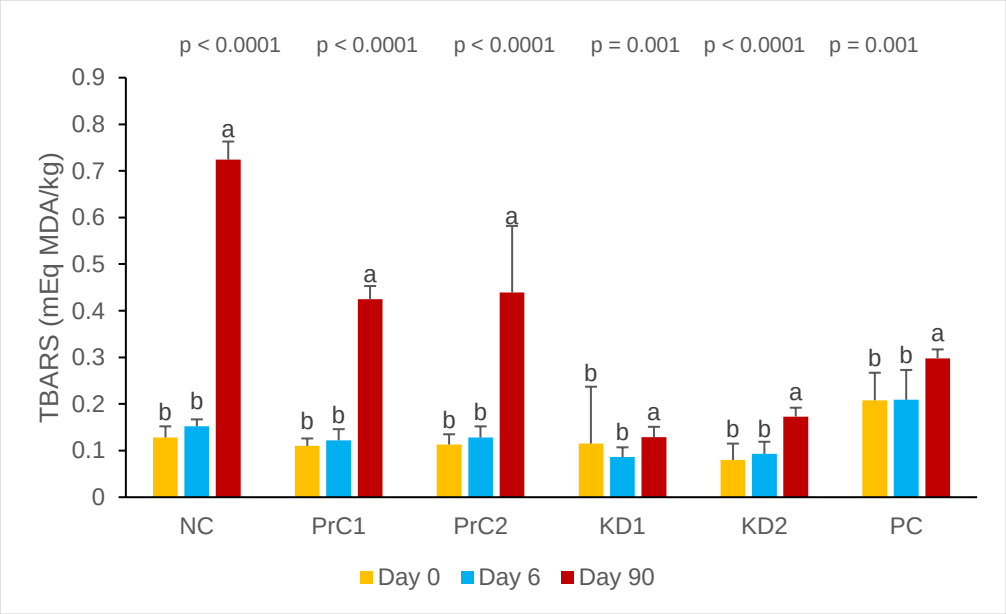

Figure 3.2. The storage time effect over a 90-day storage-life at -18 °C on the TBARS of six Boerewors formulations containing different preservatives (n = 12). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the inclusion level SO_2 recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly.

3.3.2 Microbial analyses

The microbial quality of the raw material, beef, pork, and fat was analysed in duplicate on all three occasions of meat sourcing, to determine, which pathogens, if any, were present and if microbiological standards were met (Table 3.17) before the different treatment models were manufactured. No *Listeria* was present in any of the samples, so it was excluded from the microbial analysis of the shelf-life of Boerewors treated with different preservatives.

The coliforms ranged from 1.848 ± 0.473 to 2.171 ± 1.430 log cfu/g, which was within the recommended limit of <3.00 log cfu/g (Shapton & Shapton, 1991). However, the TBC of all the raw materials were above the recommended limit for fresh meat, which is 4.48 log cfu/g

(Shapton & Shapton, 1991). *Staphylococcus aureus* and *E. coli* were mostly below the standard limits of 1.00 log cfu/g and 1.3 log cfu/g, respectively, except for the *E. coli* in the beef, which was 1.652 ± 0.200 log cfu/g, and was significantly (p = 0.005) higher than that of the pork and fat. The presence of *E. coli* could suggest possible faecal contamination during processing, either by evisceration during slaughter or unhygienic work surfaces and handling from the workers (Odonkor, & Ampofo, 2013). The yeast and mould counts were also within the standard limit of <3.00 log cfu/g (Shapton & Shapton, 1991). Although, the mould counts were significantly (p = 0.005) lower (not detected) in the fat than the 0.797 ± 0.320 log cfu/g in the beef and 0.947 ± 0.672 log cfu/g in the pork. Even though these counts were within the limits, the presence of yeasts and moulds indicate that the processing environment may be unsanitary with inadequate ventilation (Loureiro, & Querol, 1999).

Table 3.17. The microbiological quality (log cfu/g) of the raw materials, used in the manufacturing of the six Boerewors formulation types (n = 6).

	Beef (log cfu/g)	**Pork (log cfu/g)**	**Fat (log cfu/g)**	**Sign. level (n = 6)**
Coliforms	2.105 ± 0.555	1.848 ± 0.473	2.171 ± 1.430	NS
E. coli	1.652[a] ± 0.200	ND[b]	0.842[ab] ± 1.202	p = 0.005
S. aureus	0.696 ± 0.741	0.710 ± 0.714	0.887 ± 0.950	NS
TBC	5.002 ± 0.802	5.377 ± 0.445	5.489 ± 0.881	NS
Yeasts	2.295 ± 0.201	2.418 ± 0.363	1.798 ± 1.751	NS
Moulds	0.797[a] ± 0.320	0.947[a] ± 0.672	ND[b]	p = 0.005

There was an immediate effect on the TBC on day 0 by Boerewors treatment (Table 3.18 and Figure 3.3). The TBC of PrC1, at 6.66 ± 0.51 log cfu/g, was significantly higher (p < 0.0001) than that of the other treatments, except PrC2 (5.93 ± 0.95 log cfu/g). This result was most probably affected by the microbial load of the PrC1 and PrC2 treatments, which contained viable protective microorganisms, namely *Pediococcus acidilactici* and *Lactobacillus curvatus*, respectively. On day 3, the TBC of PC was significantly lower (p = 0.0002) than PrC1 and PrC2. The addition of viable bacteria from these treatments, were again attributed to improving the growth of the microorganisms, while the antimicrobial effect of the SO_2 in PC started to show. On the last day, the TBC of all the Boerewors treatment groups increased, and the trend continued with PrC1 and PrC2 having significantly higher (p = 0.001) TBC than the other treatments. The TBC of the Boerewors were all within the microbiological standards for comminuted meat products (Shapton, & Shapton, 1991), except for the PrC1 treatment on day 3.

Storage time had a significant effect ($p = 0.009$, $p = 0.016$, and $p = 0.017$, respectively) on the TBC of treatments NC, KD1 and KD2 (Table 3.20). The TBC of all treatment groups increased from day 0 to 6, but it was not significant in treatments PrC1, PrC2 and PC, which could mean that the preservatives in these treatments, in conjunction with the low storage temperature of 4 °C, at least slows the growth of microorganisms. For example, the protective culture in PrC1, *Pediococcus acidilactici*, has a minimum growth temperature of 15 °C, but can still survive below this temperature (Othman, Ariff, Kapri, Rios-Solis, & Halim, 2018), which could be a reason that the TBC did not increase significantly over the 6-day storage period.

The coliform counts of the Boerewors treatments were all within the microbiological standard limits of < 5.0 log cfu/g for comminuted meat products (Shapton & Shapton, 1991). No significant effects of the treatments on the coliform counts were observed during the first 3 days of shelf-life (Table 3.18). On day 6, the coliform count of PC was significantly ($p = 0.018$) lower than that of NC and PrC2. This indicated that the SO_2 had a better preservation effect on the coliforms after 6 days storage at 4 °C and that the PrC2 were not able to control the coliforms after 6 days. The plant extract treatments, KD1 and KD2, on day 6 clearly challenged the growth of coliforms when compared to the NC. According to Bouarab-Chibane, Ouled-Bouhedda, Leonard, Gemelas, Bouajila, Ferhout, Cottaz, Joly, Degraeve, & Oulahal (2017), preserving minced meat products with plant extracts had an inhibitory effect on coliform bacteria. The storage time also had no significant effect on the coliform counts (Table 3.20).

The *E. coli* and *Enterobacteriaceae* counts of the Boerewors treatments were all within the microbiological standards (1.7 and < 5.0 log cfu/g, respectively) for comminuted meat products (Shapton & Shapton, 1991). The only treatment that had any *E. coli*, was PC on day 0 with 0.03 ± 0.09 (Table 3.18), which was such a low amount, that it did not differ significantly from the other treatments with no *E. coli* detected. With no *E. coli* present to grow throughout the shelf-life, the storage time had no effect on the *E. coli* counts (Table 3.20). The growth of *Enterobacteriaceae* was not significantly affected by added preservative formulations at any time interval during the 6-day shelf-life (Tables 3.18 and 3.20). The *Enterobacteriaceae* count was included because, unlike coliforms, *Enterobacteriaceae* is a large family of Gram-negative bacteria, that produces acid from glucose, while coliforms use lactose as a substrate (Harrigan, 1998).

Some *S. aureus* were present at the beginning of the shelf-life, and there were no significant differences between the six treatments, but the counts declined quickly, to the point that no *S. aureus* were detected on the last day of the 6-day shelf-life. (Table 3.19). These counts were all within the microbiological standards (< 3.0 log cfu/g) for comminuted meat products (Shapton & Shapton, 1991). The storage time had a significant effect on the *S. aureus* counts

of NC, PrC1, and PrC2 (Table 3.21), reducing rapidly from day 0 to day 6. It is also clear that the plant extract preservatives, KD1 and KD2, inhibited the growth and survival of *S. aureus* more swiftly than the other treatments. Many studies have shown that plant extracts, like rosemary, green tea, and pomegranate plant extracts, have a bactericidal effect on *S. aureus* (Kwon, Apostolidis, Labbe, & Shetty, 2007; Imran, Khan, Khan, Saeed, Noor, Warsi, & Qadir, 2021; Awad et al., 2022).

The treatment had an influence on the yeast counts (Table 3.19 and Figure 3.4). There are no microbial limits for yeasts available for fresh sausages, however, the University of the Free State have been conducting research on Boerewors for a long time and have found the normal limit of yeasts to between 3 and 4 log cfu/g (Rautenbach et al., 2023). All the treatments in this study adhered to this limit on day 0. On day 0, PrC2 had a significantly higher ($p = 0.018$) yeast count than the PC. On day 3, yeasts continued to grow; PC had a significantly lower ($p = 0.004$) yeast count than NC, PrC1 and PrC2, but did not differ significantly from KD1 and KD2. On the last day, the yeast count of NC treatment group, with 4.48 ± 0.52 log cfu/g, was significantly higher ($p = 0.035$) than that of PC, with 3.63 ± 0.45 log cfu/g. These results are consistent with other studies that proved that adding SO_2 to food products, inhibited the growth of yeasts (Meat Industry Services, 2006; Sun, Zhang, Lü, Yu, & Li, 2016). These results also indicated that the two protective cultures (PrC1 and PrC2) were not able to control the growth of yeasts on day 3 of storage. At day 6, however, all four the natural treatments (PrC1, PrC2, KD1 and KD2) controlled the yeast growth equally well.

Storage time also affected the yeast counts (Table 3.21). With five of the six treatments (NC, PrC1, PrC2, KD1, and KD2), the yeast counts on day 6 were significantly higher ($p = 0.001$, $p = 0.002$, $p = 0.002$, $p = 0.0003$, and $p = 0.0001$, respectively) than the counts on both days 0 and 3. The PC yeast count did not increase as quickly as the other treatments, therefore, there were only a significant ($p = 0.004$) difference between day 0 and the last day.

No significant effect on mould counts were observed between treatments (Table 3.19) and the count was 1.07 log cfu/g for the NC on day, which was in accordance (1.17 log cfu/g) to other studies performed on Boerewors (Rautenbach et al., 2023). Mould is not usually found in fresh meat products but can be introduced to the meat during any part of processing (Habashy, Darwish, Hussein, & El-Dien, 2019). Over time, there was, however, a significant increase ($p = 0.032$ and $p = 0.009$, respectively) in the mould counts of NC and PrC2 (Table 3.21). This was an indication that the NC (as expected) and the PrC2 were not able to limit the growth of moulds during storage at 4 °C for 6 days.

There was an immediate effect on the LAB count, by Boerewors treatment (Table 3.19 and Figure 3.5). On day 0, the LAB of PrC1 at log 5.80 ± 1.27 cfu/g, was significantly ($p < 0.0001$) higher than all the other treatments, except PrC2, with 4.50 ± 0.74 log cfu/g. On the third day, the LAB of PrC1 and PrC2 were still the highest and differed significantly ($p < 0.0001$) from the control groups, NC, and PC. However, the control groups did not differ significantly from treatment groups KD1 and KD2. The trend continued on day 6, with PrC1 having a significantly higher ($p = 0.0002$) LAB count than all the other treatments, except PrC2. As mentioned before, PrC1 and PrC2 contained viable microorganisms, which mainly included lactic acid bacteria *Pediococcus acidilactici* and *Lactobacillus curvatus*. Although not significant, PrC1 had higher LAB counts than PrC2 throughout the storage period. A possible reason for this was because PrC1 had an inclusion level of 0.025% BLC 20, whereas PrC2 had 0.0125% BLC 48 added to the Boerewors. Storage time did not have a significant influence on the LAB (Table 3.21).

Table 3.18. Changes in the total bacteria count, coliform and *E. coli* count and *Enterobacteriaceae* count of six Boerewors formulations with different preservatives at 4 °C, on days 0, 3 and 6 (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). TBC = Total bacterial count; *E. coli* = *Escherichia coli.* Means with different superscripts differed significantly. NS = Not significant; ND = Not detected; NA = Not applicable. *ANOVA/KW significant, but post-hoc tests were not significant enough to show a specific difference.

Day	Treatment	TBC (log cfu/g)	Sign. Level	Coliforms (log cfu/g)	Sign. Level	*E. coli* (log cfu/g)	Sign. level	*Enterobacteriaceae* (log cfu/g)	Sign. level
0	NC	$5.74^{b} \pm 0.33$	$p < 0.0001$	2.58 ± 0.37	NS	ND	NS	2.31 ± 0.51	NS
	PrC1	$6.66^{a} \pm 0.57$		2.20 ± 0.61		ND		1.99 ± 0.64	
	PrC2	$5.93^{ab} \pm 0.95$		2.56 ± 0.43		ND		1.51 ± 1.28	
	KD1	$5.62^{b} \pm 0.48$		2.51 ± 0.35		ND		2.01 ± 1.01	
	KD2	$5.59^{b} \pm 0.46$		2.49 ± 0.57		ND		2.21 ± 0.50	
	PC	$5.35^{b} \pm 0.41$		2.15 ± 0.69		0.03 ± 0.09		2.05 ± 0.71	
3	NC	$5.09^{bc} \pm 1.16$	$p = 0.0002$	2.59 ± 0.38	NS	ND	NA	2.22 ± 0.39	NS
	PrC1	$7.02^{a} \pm 0.62$		2.34 ± 0.81				2.13 ± 0.58	
	PrC2	$6.42^{ab} \pm 0.38$		2.64 ± 0.40				2.28 ± 0.42	
	KD1	$5.71^{abc} \pm 1.77$		2.63 ± 0.32				2.15 ± 0.57	
	KD2	$5.74^{abc} \pm 1.77$		2.48 ± 0.47				2.18 ± 0.53	
	PC	$4.80^{c} \pm 1.08$		1.94 ± 0.88				1.41 ± 1.05	
6	NC	$6.27^{ab} \pm 0.74$	$p = 0.001$	$2.52^{a} \pm 0.24$	$p = 0.018$	ND	NA	1.92 ± 0.52	$p = 0.042^{*}$
	PrC1	$6.83^{a} \pm 0.80$		$2.28^{ab} \pm 0.31$				2.13 ± 0.30	
	PrC2	$6.57^{a} \pm 0.48$		$2.42^{a} \pm 0.30$				2.12 ± 0.37	
	KD1	$6.41^{ab} \pm 0.35$		$2.31^{ab} \pm 0.23$				1.97 ± 0.41	
	KD2	$6.17^{ab} \pm 0.24$		$2.35^{ab} \pm 0.21$				1.81 ± 0.37	
	PC	$5.76^{b} \pm 0.45$		$1.51^{b} \pm 1.02$				1.38 ± 0.82	

Table 3.19. Changes in *S. aureus* count, yeasts count, moulds count, and lactic acid bacteria count of six Boerewors formulations with different preservatives at 4 °C, over a 6-day shelf-life (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). *S. aureus* = *Staphylococcus aureus;* LAB = Lactic acid bacteria. Means with different superscripts differed significantly. NS = Not significant; ND = Not detected; NA = Not applicable. *ANOVA/KW significant, but post-hoc tests were not significant enough to show a specific difference.

Day	Treatment	*S. aureus* (log cfu/g)	Sign. level	Yeasts (log cfu/g)	Sign. Level	Moulds (log cfu/g)	Sign. level	LAB (log cfu/g)	Sign. level
0	NC	0.49 ± 0.63	NS	3.44^{ab} ± 0.55	p = 0.018	1.07 ± 1.04	NS	1.60^{c} ± 1.44	p < 0.0001
	PrC1	0.58 ± 0.76		3.38^{ab} ± 0.61		0.25 ± 0.62		5.80^{a} ± 1.27	
	PrC2	0.38 ± 0.57		3.58^{a} ± 0.20		0.57 ± 1.03		4.50^{ab} ± 0.74	
	KD1	0.19 ± 0.45		3.49^{ab} ± 0.23		0.17 ± 0.58		2.41^{c} ± 1.17	
	KD2	0.25 ± 0.45		3.30^{ab} ± 0.44		0.36 ± 0.84		2.98^{bc} ± 0.44	
	PC	0.30 ± 0.55		3.00^{b} ± 0.16		0.22 ± 0.51		2.08^{c} ± 1.02	
3	NC	0.08 ± 0.29	p = 0.04*	3.66^{a} ± 0.61	p = 0.004	0.44 ± 0.85	NS	2.18^{c} ± 1.67	p < 0.0001
	PrC1	ND		3.55^{a} ± 0.39		0.42 ± 0.79		6.48^{a} ± 0.68	
	PrC2	ND		3.61^{a} ± 0.30		0.33 ± 0.78		4.46^{ab} ± 0.81	
	KD1	ND		3.49^{ab} ± 0.33		0.53 ± 0.95		3.15^{bc} ± 0.38	
	KD2	ND		3.37^{ab} ± 0.27		0.36 ± 0.84		3.23^{bc} ± 0.36	
	PC	0.30 ± 0.55		3.10^{b} ± 0.91		0.11 ± 0.38		1.83^{c} ± 1.70	
6	NC	ND	NA	4.48^{a} ± 0.52	p = 0.035	1.87 ± 1.49	NS	2.58^{b} ± 2.06	p = 0.0002
	PrC1			4.38^{ab} ± 0.53		1.34 ± 1.71		5.79^{a} ± 1.25	
	PrC2			4.41^{ab} ± 0.58		1.89 ± 1.50		4.29^{ab} ± 0.40	
	KD1			4.41^{ab} ± 0.51		1.32 ± 1.68		3.45^{b} ± 1.05	
	KD2			4.37^{ab} ± 0.51		1.50 ± 1.63		3.59^{b} ± 0.84	
	PC			3.63^{b} ± 0.45		0.84 ± 1.07		3.21^{b} ± 1.07	

Table 3.20. The storage time effect over a 6-day shelf-life at 4 °C on the total bacteria count, coliform and *E. coli* count, and *Enterobacteriaceae* count of six Boerewors treatments with different preservatives (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly. NS = Not significant; ND = Not detected; NA = Not applicable.

Treatment	Storage day	TBC (log cfu/g)	Sign. Level	Coliforms (log cfu/g)	Sign. level	*E. coli* (log cfu/g)	Sign. level	*Enterobacteriaceae* (log cfu/g)	Sign. level
NC	0	$5.74^{ab} \pm 0.33$	p = 0.009	2.58 ± 0.37	NS	ND	NA	2.31 ± 0.51	NS
	3	$5.09^{b} \pm 1.16$		2.59 ± 0.38				2.22 ± 0.39	
	6	$6.27^{a} \pm 0.74$		2.52 ± 0.24				1.92 ± 0.52	
PrC1	0	6.66 ± 0.57	NS	2.20 ± 0.61	NS	ND	NA	1.99 ± 0.64	NS
	3	7.02 ± 0.62		2.34 ± 0.81				2.13 ± 0.58	
	6	6.83 ± 0.80		2.28 ± 0.31				2.13 ± 0.30	
PrC2	0	5.93 ± 0.95	NS	2.56 ± 0.43	NS	ND	NA	1.51 ± 1.28	NS
	3	6.42 ± 0.38		2.64 ± 0.40				2.28 ± 0.42	
	6	6.57 ± 0.48		2.42 ± 0.30				2.12 ± 0.37	
KD1	0	$5.62^{b} \pm 0.48$	p = 0.016	2.51 ± 0.35	NS	ND	NA	2.01 ± 1.01	NS
	3	$5.71^{ab} \pm 1.77$		2.63 ± 0.32				2.15 ± 0.57	
	6	$6.41^{a} \pm 0.35$		2.31 ± 0.23				1.97 ± 0.41	
KD2	0	$5.59^{b} \pm 0.46$	p = 0.017	2.49 ± 0.57	NS	ND	NA	2.21 ± 0.50	NS
	3	$5.74^{ab} \pm 1.77$		2.48 ± 0.47				2.18 ± 0.53	
	6	$6.17^{a} \pm 0.24$		2.35 ± 0.21				1.81 ± 0.37	
PC	0	5.35 ± 0.41	NS	2.15 ± 0.69	NS	0.03 ± 0.09	NS	2.05 ± 0.71	NS
	3	4.80 ± 1.08		1.94 ± 0.88		ND		1.41 ± 1.05	
	6	5.76 ± 0.45		1.51 ± 1.02		ND		1.38 ± 0.82	

Table 3.21. The storage time effect over a 6-day shelf-life at 4 °C on the *S. aureus* count, yeasts count, moulds count, and lactic acid bacteria count of six Boerewors treatments with different preservatives (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly. NS = Not significant; ND = Not detected.

Treatment	Storage day	*S. aureus* (log cfu/g)	Sign. level	Yeasts (log cfu/g)	Sign. level	Moulds (log cfu/g)	Sign. level	LAB (log cfu/g)	Sign. level
NC	0	$0.49^{a} \pm 0.63$	p = 0.015	$3.44^{b} \pm 0.55$	p = 0.001	$1.07^{ab} \pm 1.04$	p = 0.032	1.60 ± 1.44	NS
	3	$0.08^{ab} \pm 0.29$		$3.66^{b} \pm 0.61$		$0.44^{b} \pm 0.85$		2.18 ± 1.67	
	6	0 ± 0^{b}		$4.48^{a} \pm 0.52$		$1.87^{a} \pm 1.49$		2.58 ± 2.06	
PrC1	0	$0.58^{a} \pm 0.76$	p = 0.004	$3.38^{b} \pm 0.61$	p = 0.002	0.25 ± 0.62	NS	5.80 ± 1.27	NS
	3	0 ± 0^{b}		$3.55^{b} \pm 0.39$		0.42 ± 0.79		6.48 ± 0.68	
	6	0 ± 0^{b}		$4.38^{a} \pm 0.53$		1.34 ± 1.71		5.79 ± 1.25	
PrC2	0	$0.38^{a} \pm 0.57$	p = 0.013	$3.58^{b} \pm 0.20$	p = 0.002	$0.57^{ab} \pm 1.03$	p = 0.009	4.50 ± 0.74	NS
	3	0 ± 0^{b}		$3.61^{b} \pm 0.30$		$0.33^{b} \pm 0.78$		4.46 ± 0.81	
	6	0 ± 0^{b}		$4.41^{a} \pm 0.58$		$1.89^{a} \pm 1.50$		4.29 ± 0.40	
KD1	0	0.19 ± 0.45	NS	$3.49^{b} \pm 0.23$	p = 0.0003	0.17 ± 0.58	NS	2.41 ± 1.17	NS
	3	ND		$3.49^{b} \pm 0.33$		0.53 ± 0.95		3.15 ± 0.38	
	6	ND		$4.41^{a} \pm 0.51$		1.32 ± 1.68		3.45 ± 1.05	
KD2	0	0.25 ± 0.45	NS	$3.30^{b} \pm 0.44$	p = 0.0001	0.36 ± 0.84	NS	2.98 ± 0.44	NS
	3	ND		$3.37^{b} \pm 0.27$		0.36 ± 0.84		3.23 ± 0.36	
	6	ND		$4.37^{a} \pm 0.51$		1.50 ± 1.63		3.59 ± 0.84	
PC	0	0.30 ± 0.55	NS	$3.00^{b} \pm 0.16$	p = 0.004	0.22 ± 0.51	NS	2.08 ± 1.02	NS
	3	0.30 ± 0.55		$3.10^{ab} \pm 0.91$		0.11 ± 0.38		1.83 ± 1.70	
	6	ND		$3.63^{a} \pm 0.45$		0.84 ± 1.07		3.21 ± 1.07	

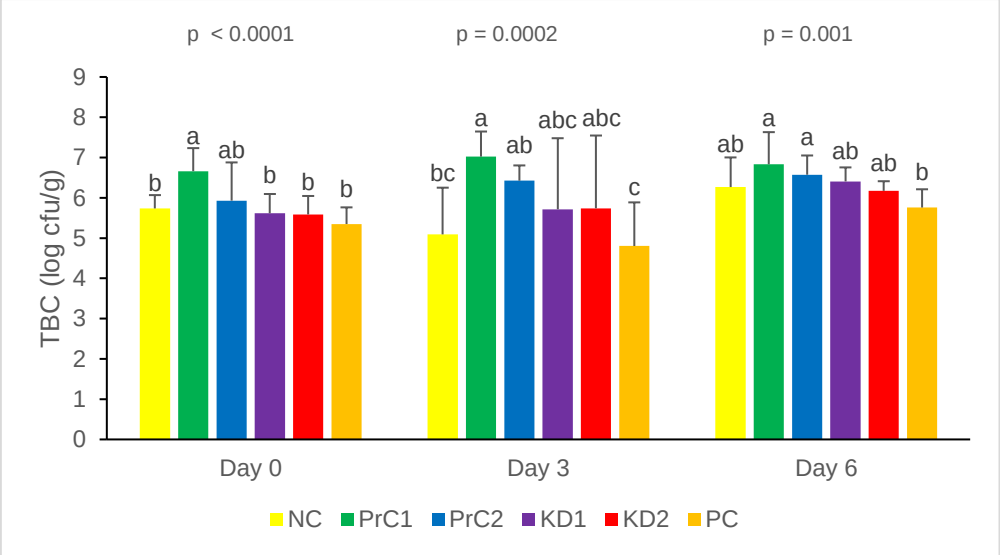

Figure 3.3. Total bacterial count (TBC)(log cfu/g) of six Boerewors treatments with different preservatives at 4 °C, over a 6-day shelf-life (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly.

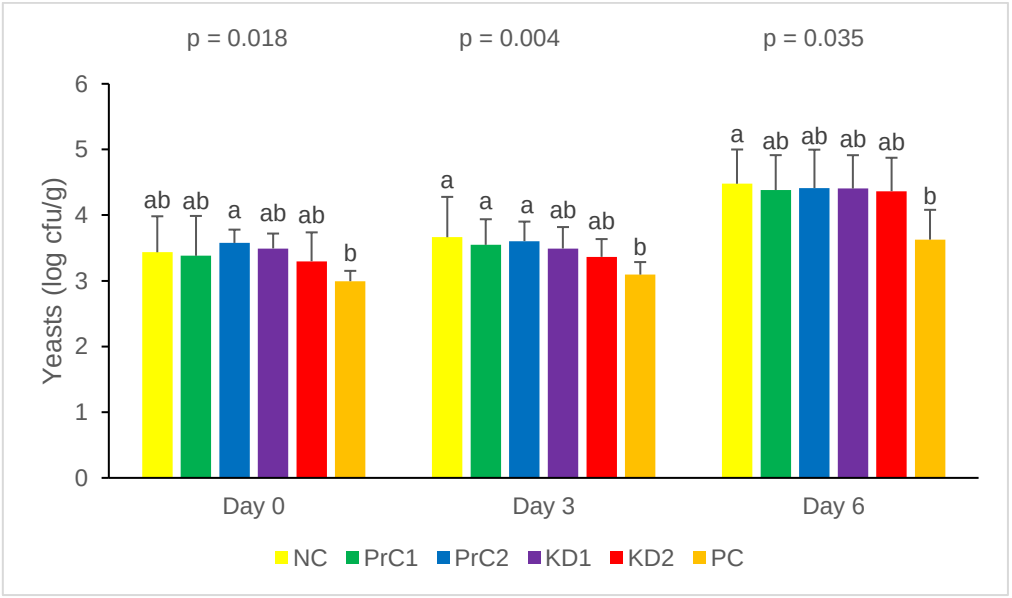

Figure 3.4. Yeasts count (log cfu/g) of six Boerewors treatments with different preservatives at 4 °C, over a 6-day shelf-life (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly.

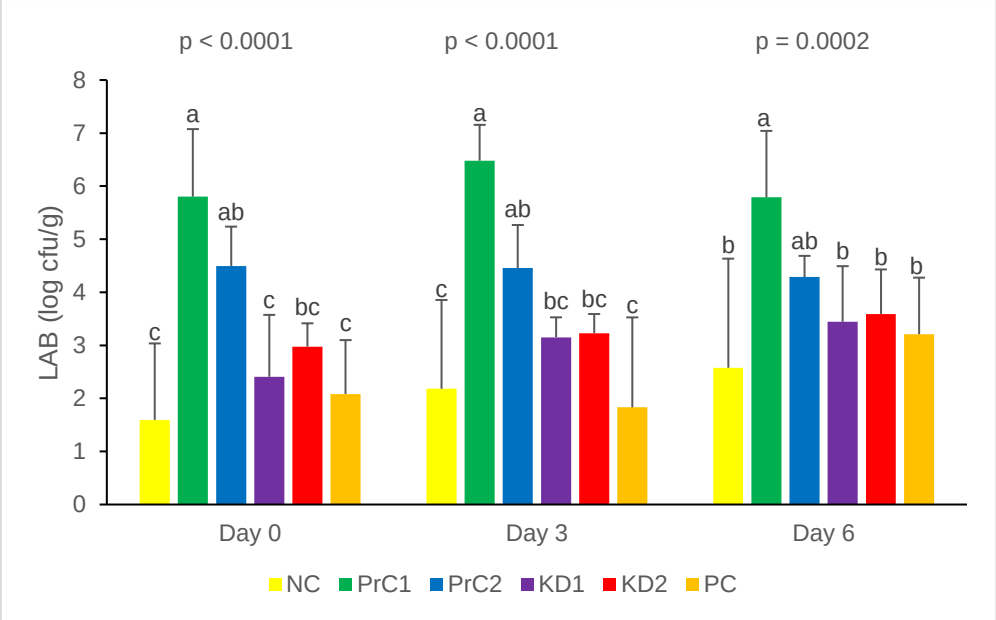

Figure 3.5. Lactic acid bacteria (log cfu/g) count of six Boerewors formulations with different preservatives at 4 °C, over a 6-day shelf-life (n = 24). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly.

3.3.3 Sensory analyses

3.3.3.1 Colour

Significant effects by treatment on colour parameters were observed after every time interval of days 0, 3 and 6 (Table 3.22). Lightness (*L**) values generally decreased over a 6-day shelf-life. According to Chin, Lee, & Chun (2004), the pork fat that is added to comminuted meat products, tend to increase the overall lightness. Figure 3.6 shows the *L** values of the Boerewors treatments over the 6-day storage period. On day 0, the *L** value of PC was significantly higher ($p < 0.001$) than three other treatments: NC, PrC1, and PrC2. On day 3, KD1 had the highest *L**-value, and with PC, it was significantly higher ($p = 0.0004$) than NC and PrC1. At the end of the shelf-life, day 6, the *L** of PC was higher ($p = 0.001$) than PrC1, PrC2, and KD2.

Storage time had a significant effect on the *L** of the different Boerewors treatments (Table 3.23), with most *L**-values decreasing from day 0 to 6. It is possible that the SO_2 in the PC, and the rosemary blend in KD1, could have influenced the meat lightness.

Table 3.22. Changes in the colour parameters of six Boerewors treatments with different preservatives, at 4 °C, over a 6-day shelf-life (n = 144).

NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). *L** = lightness; *a** = redness; *b** = yellowness; *C** = chroma; and *H** = hue angle. Means with different superscripts in the same column and for the same treatment differed significantly. NS = Not significant.

Day	Treatment	*L**	Sign. level	*a**	Sign. level	*b**	Sign. level	*C**	Sign. level	*H**	Sign. level
0	NC	50.99^{b} ± 5.17	p < 0.0001	17.18$^{\mathbf{bc}}$ ± 2.16	p < 0.0001	14.47 ± 1.45	NS	22.49^{b} ± 2.25	p < 0.0001	0.70^{b} ± 0.06	p < 0.0001
	PrC1	51.02^{b} ± 5.13		17.38$^{\mathbf{bc}}$ ± 2.06		14.61 ± 1.82		22.74^{b} ± 2.90		0.70^{b} ± 0.06	
	PrC2	51.07^{b} ± 5.81		17.82$^{\mathbf{ab}}$ ± 2.42		15.10 ± 1.77		23.41^{ab} ± 2.58		0.70^{b} ± 0.07	
	KD1	52.89^{ab} ± 4.65		16.38$^{\mathbf{c}}$ ± 2.80		15.16 ± 1.84		22.38^{b} ± 2.98		0.75^{a} ± 0.07	
	KD2	51.96^{ab} ± 4.15		17.05$^{\mathbf{bc}}$ ± 2.60		14.90 ± 1.80		22.69^{b} ± 2.82		0.72^{ab} ± 0.06	
	PC	53.49^{a} ± 7.18		19.38$^{\mathbf{a}}$ ± 3.23		15.09 ± 1.70		24.67^{a} ± 3.22		0.67^{c} ± 0.07	
3	NC	48.80^{c} ± 5.10	p = 0.0004	15.52^{c} ± 4.64	p < 0.0001	13.02^{a} ± 2.24	p = 0.001	20.40^{c} ± 4.57	p = 0.0003	0.70^{bc} ± 0.11	p = 0.0003
	PrC1	50.11^{bc} ± 4.34		15.36^{bc} ± 2.30		13.61^{a} ± 1.60		20.58^{bc} ± 2.37		0.73^{b} ± 0.07	
	PrC2	51.04^{abc} ± 4.07		15.39^{c} ± 2.50		13.82^{ab} ± 1.77		20.72^{bc} ± 2.77		0.73^{ab} ± 0.06	
	KD1	52.09^{a} ± 3.90		15.06^{c} ± 1.82		14.32^{b} ± 1.26		20.82^{abc} ± 1.81		0.76^{s} ± 0.06	
	KD2	50.97^{abc} ± 5.17		16.62^{ab} ± 2.58		14.02^{ab} ± 1.48		21.78^{ab} ± 2.63		0.70^{bc} ± 0.06	
	PC	51.89^{ab} ± 5.29		16.94^{a} ± 2.73		13.87^{ab} ± 1.69		21.94^{a} ± 2.85		0.69^{c} ± 0.07	
6	NC	50.06^{ab} ± 3.92	p = 0.001	12.91^{bc} ± 2.29	p < 0.0001	13.31 ± 1.78	NS	18.60^{bc} ± 2.46	p < 0.0001	0.80^{ab} ± 0.08	p < 0.0001
	PrC1	49.46^{b} ± 3.66		12.47^{c} ± 2.52		13.58 ± 1.42		18.52^{bc} ± 2.35		0.83^{a} ± 0.09	
	PrC2	49.69^{b} ± 6.57		12.17^{c} ± 1.92		13.08 ± 1.67		17.92^{c} ± 2.09		0.82^{ab} ± 0.08	
	KD1	50.48^{ab} ± 3.98		12.47^{bc} ± 1.63		13.42 ± 1.52		18.35^{bc} ± 1.95		0.82^{ab} ± 0.06	
	KD2	50.05^{b} ± 4.17		13.62^{b} ± 2.35		13.48 ± 2.40		19.26^{b} ± 2.76		0.78^{b} ± 0.11	
	PC	52.19^{a} ± 4.15		15.96^{a} ± 2.93		13.48 ± 1.11		20.96^{a} ± 2.62		0.71^{c} ± 0.8	

Table 3.23. The storage time effect over a 6-day shelf-life at 4 °C on the colour parameters of six Boerewors treatments with different preservatives (n = 144). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the inclusion level SO_2 recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). *L** = lightness; *a** = redness; *b** = yellowness; *C** = chroma; and *H** = hue angle. Means with different superscripts in the same column and for the same treatment differed significantly. NS = Not significant.

Treatment	Storage day	*L**	Sign. level	*a**	Sign. level	*b**	Sign. level	*C**	Sign. level	*H**	Sign. level
NC	0	50.99 ± 5.17	NS	17.18^{a} ± 2.16	p < 0.0001	14.47^{a} ± 1.45	p < 0.0001	22.49^{a} ± 2.25	p < 0.0001	0.70^{b} ± 0.06	p < 0.0001
	3	48.80 ± 5.10		15.07^{b} ± 2.45		13.02^{b} ± 2.24		20.00^{b} ± 2.82		0.71^{b} ± 0.10	
	6	50.06 ± 3.92		12.91^{c} ± 2.29		13.31^{b} ± 1.78		18.60^{c} ± 2.46		0.80^{a} ± 0.08	
PrC1	0	51.02 ± 5.13	NS	17.38^{a} ± 2.06	p < 0.0001	14.61^{a} ± 1.82	p = 0.0002	22.74^{a} ± 2.90	p < 0.0001	0.70^{b} ± 0.06	p < 0.0001
	3	50.11 ± 4.34		15.36^{b} ± 2.30		13.61^{b} ± 1.60		20.58^{b} ± 2.37		0.73^{b} ± 0.07	
	6	49.46 ± 3.66		12.47^{c} ± 2.52		13.58^{b} ± 1.42		18.52^{c} ± 2.35		0.83^{a} ± 0.09	
PrC2	0	51.07^{a} ± 5.81	p = 0.015	17.82^{a} ± 2.42	p < 0.0001	15.10^{a} ± 1.77	p < 0.0001	23.41^{a}± 2.58	p < 0.0001	0.70^{b} ± 0.07	p < 0.0001
	3	51.04^{ab} ± 4.07		15.39^{b} ± 2.50		13.82^{b} ± 1.77		20.72^{b} ± 2.77		0.73^{b} ± 0.06	
	6	49.69^{b} ± 6.57		12.17^{c} ± 1.92		13.08^{b} ± 1.67		17.92^{c} ± 2.09		0.82^{a} ± 0.08	
KD1	0	52.89^{a} ± 4.65	p = 0.009	16.38^{a} ± 2.80	p < 0.0001	15.16^{a} ± 1.84	p < 0.0001	22.38^{a} ± 2.98	p < 0.0001	0.75^{b} ± 0.07	p < 0.0001
	3	52.09^{ab} ± 3.90		15.06^{b} ± 1.82		14.32^{b} ± 1.26		20.82^{b} ± 1.81		0.76^{b} ± 0.06	
	6	50.48^{b} ± 3.98		12.47^{c} ± 1.63		13.42^{c} ± 1.52		18.35^{c} ± 1.95		0.82^{a} ± 0.06	
KD2	0	51.96^{a} ± 4.15	p = 0.021	17.05^{a} ± 2.60	p < 0.0001	14.90^{a} ± 1.80	p < 0.0001	22.69^{a} ± 2.82	p < 0.0001	0.72^{b} ± 0.06	p < 0.0001
	3	50.97^{ab} ± 5.17		16.62^{a} ± 2.58		14.02^{b} ± 1.48		21.78^{a} ± 2.63		0.70^{b} ± 0.06	
	6	50.05^{b} ± 4.17		13.62^{b} ± 2.35		13.48^{b} ± 2.40		19.26^{b} ± 2.76		0.78^{a} ± 0.11	
PC	0	53.49^{a} ± 7.18	p < 0.003	19.38^{a} ± 3.23	p < 0.0001	15.09^{a} ± 1.70	p < 0.0001	24.67^{a} ± 3.22	p < 0.0001	0.67^{b} ± 0.07	p = 0.003
	3	51.89^{b} ± 5.29		16.94^{b} ± 2.73		13.87^{b} ± 1.69		21.94^{b} ± 2.85		0.69^{ab} ± 0.07	
	6	52.19^{b} ± 4.15		15.96^{b} ± 2.93		13.48^{b} ± 1.11		20.96^{b} ± 2.62		0.71^{a} ± 0.8	

According to McKenna, Mies, Baird, Pfeiffer, Ellebracht, & Savell (2005), the oxidative condition of myoglobin does not affect the lightness, however, it is affected by the muscle structure and processing, which affects the water holding capacity (WHC; Ripoll, Joy, & Muñoz 2011). Due to their ability to expand and contract, myofibrils can scatter light, but mincing reduces WHC and, therefore, also reduces the ability to scatter light (Offer, & Trinick, 1983).

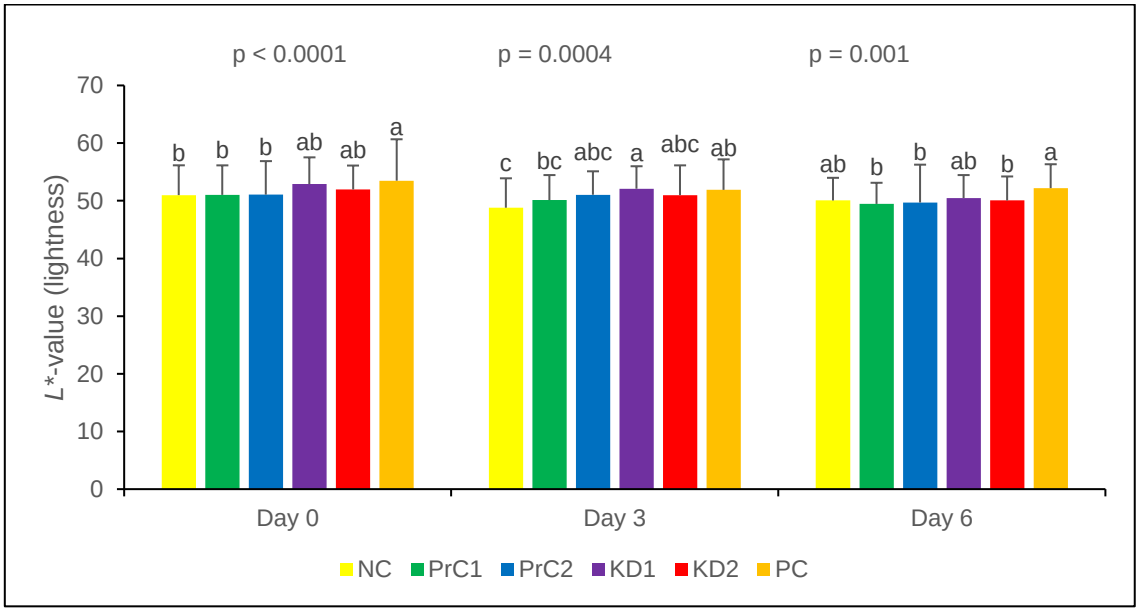

Figure 3.6. Lightness (*L**) of six Boerewors treatments with different preservatives, at 4 °C, over a 6-day shelf-life (n = 144). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same column and for the same treatment differed significantly. Error bars represented standard deviations of means.

The first impression consumers have of fresh meat products, is the red colour, thus, making it one of the most important aspects. Immediately after cutting, beef is quite dark, with a purplish-red colour, and as oxygen from the air meets the surface of the meat, it binds to the iron part. The meat blooms as the myoglobin are oxygenated. The pigment, oxymyoglobin, gives beef the bright red colour (Mancini, & Hunt, 2005). Throughout the 6-day shelf-life, PC had the highest *a**-values (Figure 3.7). On day 0, PrC2 did not differ significantly ($p < 0.0001$) from PC. On day 3, KD2 was the only treatment that did not have a significantly ($p < 0.0001$) lower a*-value than the PC, which could be ascribed to the acerola extract in the KD2 treatment. The ascorbic acid in the acerola extract prevents metmyoglobin pigment formation and allows it to rebind oxygen to produce a bright red colour (Suchoparova, Janoud, Rydlova, Beno, Pohunek, & Ševcik, 2022). On the last day, all the other treatments had significantly ($p <$

0.0001) less redness than PC. The SO_2 in PC, which is responsible for retaining the red colour in the sausage, does this by maintaining the heme iron in a reduced state, to prevent oxidation of the oxymyoglobin (Prabhakar, & Mallika, 2014).

Storage time had a significant effect on the redness (*a**) of the Boerewors treatments, as it gradually decreased from day 0 to 6 (Table 3.23). Fresh sausage ultimately turns brown due to the formation of metmyoglobin, which is caused by the oxidation of the oxymyoglobin (Tomasevic, Djekic, Font-i-Furnols, Terjung, & Lorenzo, 2021).

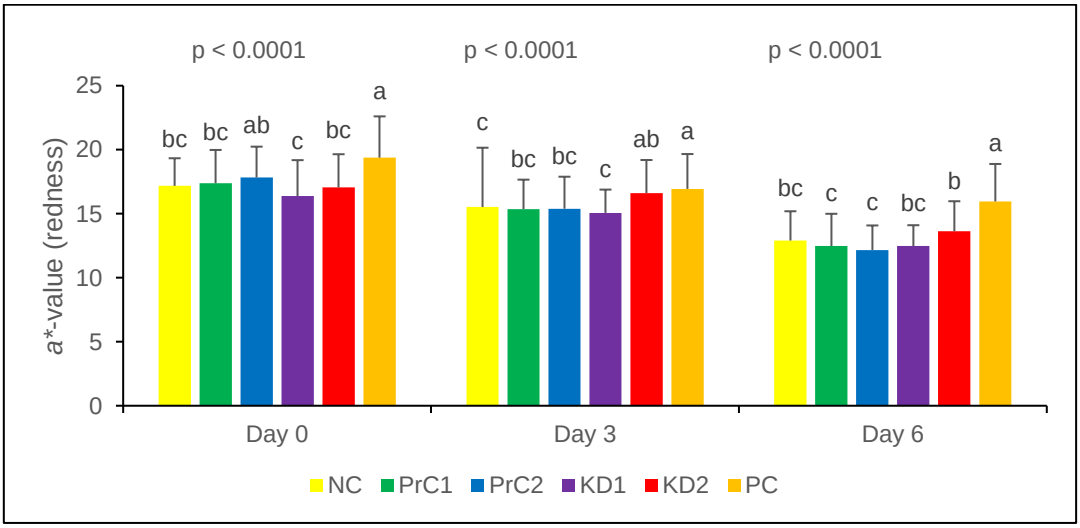

Figure 3.7. Redness (*a**) of six Boerewors treatments with different preservatives, at 4 °C, over a 6-day shelf-life (n = 144). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same column and for the same treatment differed significantly. Error bars represented standard deviations of means.

Yellowness (*b**) generally decreased over time, and the values of each treatment lowered significantly from day 0 to day 6 (Table 3.23). There were no significant differences between the Boerewors treatments on day 0 and day 6 (Figure 3.8). However, on day 3, KD1 had a significantly higher (p = 0.001) *b**-value than NC and PrC1. This could possibly be attributed to the rich brown-yellow colour of the added KD1 liquid solution. The oxidation of oxymyoglobin to metmyoglobin has been reported to cause an increase in *b**-values (Fernández-López, Pérez-Alvarez, Sayas-Barberá, & Aranda-Catalá, 2000), although in these models an almost reversed progression was found. In all probability, the decreases in *L**, *a**, and *b** could be attributed to the deterioration caused by the display lighting used over the course of the 6-day shelf-life.

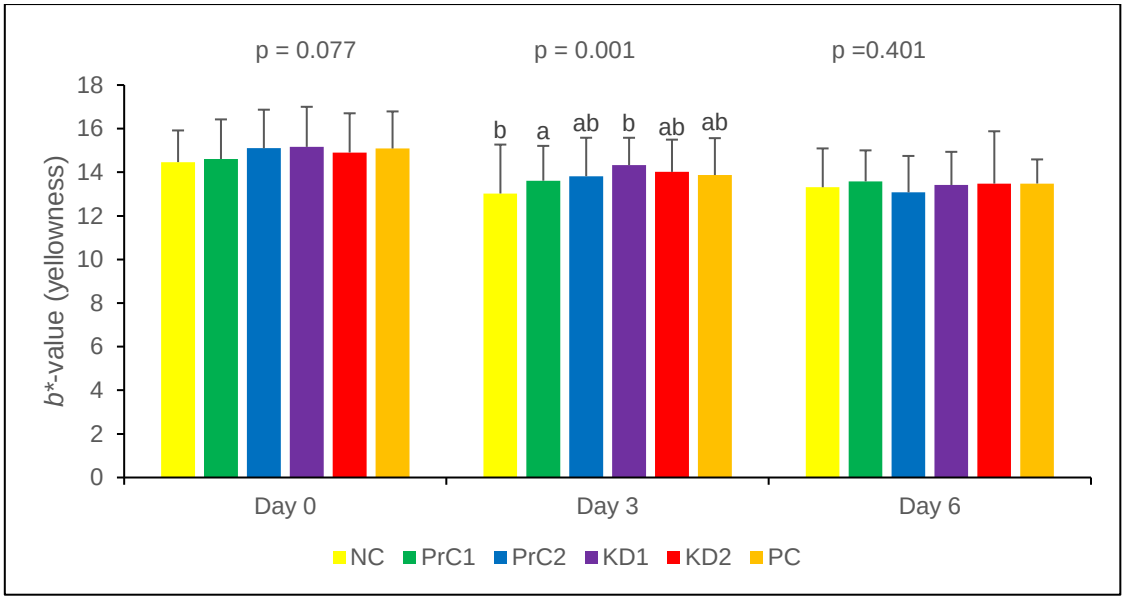

Figure 3.8. Yellowness (*b**) of six Boerewors treatments with different preservatives, at 4 °C, over a 6-day shelf-life (n = 144). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same column and for the same treatment differed significantly. Error bars represented standard deviations of means.

Colour brightness, indicated by Chroma (*C**), was the discolouration of the meat, with a progression to greying at lower Chroma. The *C**-values of all six Boerewors treatments decreased over the course of 6 days (Table 3.23 and Figure 3.9). On day 0, PC had a significantly higher ($p < 0.0001$) *C**-value than all the other treatments, except PrC2. On day 3, NC had a significantly lower ($p = 0.0003$) *C**-value than PC and KD2. On the last day, PC had a significantly higher ($p < 0.0001$) C*-value than all the other treatments. The significantly higher ($p < 0.0001$) *a** contributed to an overall increase in colour brightness for the PC Boerewors.

Hue angle (*H**) represents the purity of the colour; *H**-value nearer to 0° means it is closer to red and *H** nearer to 90° means it is closer to yellow. It was also greatly affected by the storage time (Table 3.23) and preservative treatment (Figure 3.10). The *H** remained stable up until day 3 but increased on day 6. On day 0, the PC had a significantly lower ($p < 0.0001$) H*-value than the other treatments, while KD1 had a significantly higher ($p < 0.0001$) *H** than all the other treatments. As mentioned before, the yellowness could be attributed to the brown-yellow colour of the added KD1 liquid solution. On day 3, the PC was significantly lower ($p < 0.0001$) than PrC1, PrC2 and KD1, and KD1 was significantly higher ($p < 0.0001$) than all the other

treatments. On the last day, PC remained the significantly lowest ($p < 0.0001$), while PrC1 had significantly higher ($p < 0.0001$) *H**-values than PC and KD2. Overall, the Boerewors remained closer to the red end of the spectrum throughout the 6-day shelf-life.

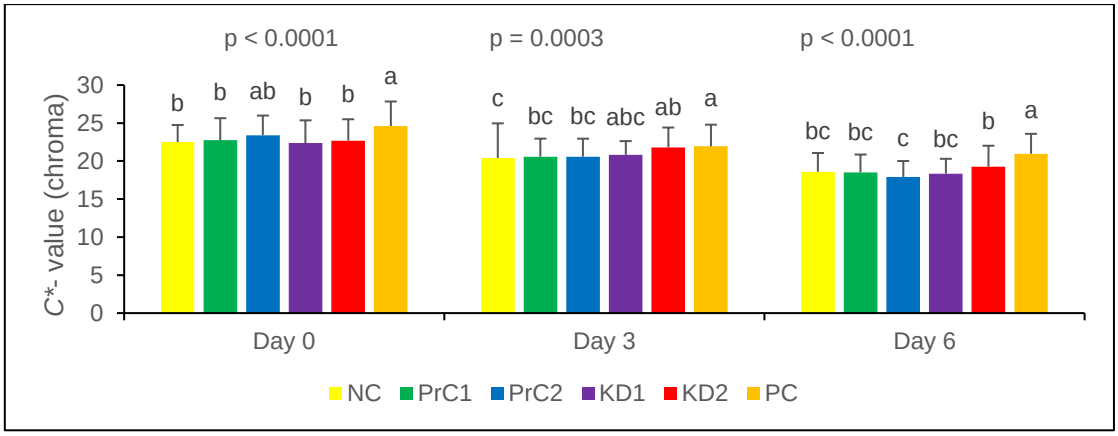

Figure 3.9. Chroma (brightness; *C**) of six Boerewors treatments with different preservatives, at 4 °C, over a 6-day shelf-life (n = 144). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same column and for the same treatment differed significantly. Error bars represented standard deviations of means.

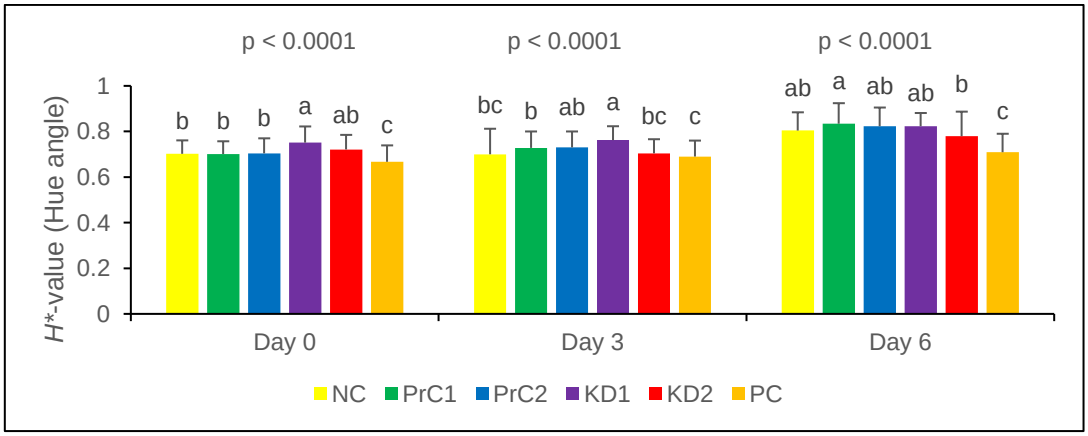

Figure 3.10. Hue angle (*H**) of six Boerewors treatments with different preservatives, at 4 °C, over a 6-day shelf-life (n = 144). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts differed significantly. Error bars represented standard deviations of means.

3.3.3.2 Consumer sensory evaluation

No significant differences between treatment groups could be identified for any of the sensory attributes (Figure 3.11). There were, however, small differences between treatment groups for these rankings. In general, the PrC2 group had the highest rankings for taste, aftertaste, texture, and overall acceptability, and the NC group had the lowest rankings for the aroma, aftertaste, texture, and overall acceptability attributes. Some sensory evaluation studies contradict these results, suggesting that consumers disliked the food products that contained protective cultures (Pedonese, Torracca, Mancini, Pisano, Turchi, Cerri, & Nuvoloni, 2020).

PC had the highest score for aroma, which could be due to the familiarity of the smell of what is found in store-bought Boerewors. KD1 had the lowest ranking in taste. The insignificant differences in liking scores, suggested that SO_2 could possibly be replaced by natural preservatives in Boerewors, as it was not noticeably different from the rest of the Boerewors treatment groups.

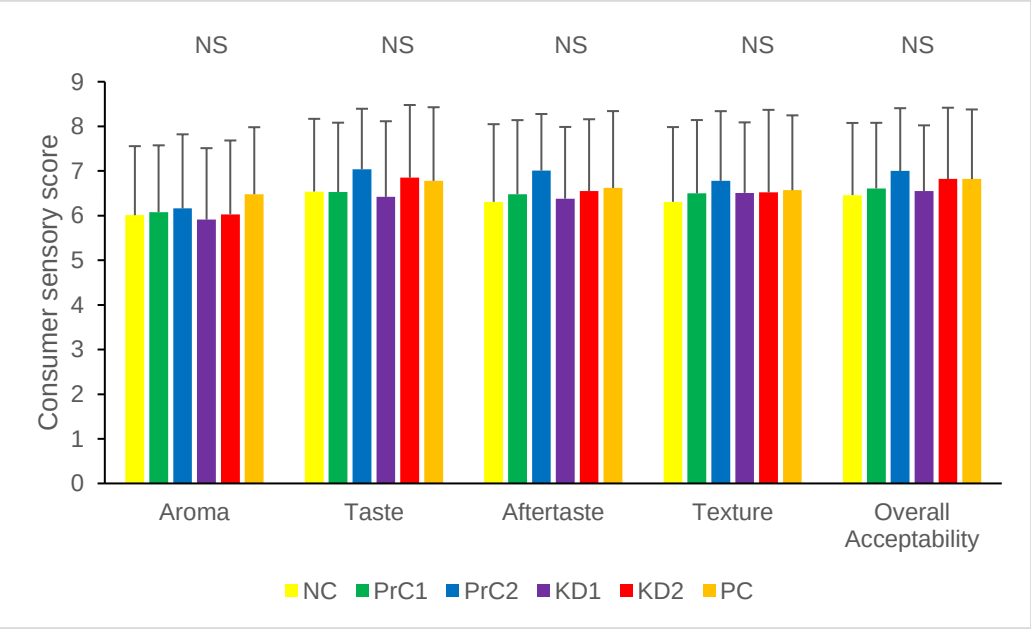

Figure 3.11. Consumer sensory rankings of six Boerewors formulations based on different added preservatives (n = 100). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). NS = Not significant. Error bars represented standard deviations of means.

Another interesting aspect of the consumer evaluation was the difference between attribute ranking means based on gender (Figure 3.12). Male individuals, on average, gave lower ranking scores for every attribute in the sensory test; however, three of these attributes, aroma, texture, and overall acceptability, were scored significantly ($p = 0.003$, $p = 0.002$, and $p = 0.026$, respectively) lower than the scores given by female individuals. Research have shown that gender differences in sensory acuity may affect food preferences (Weaver, & Brittin, 2001). According to Michon, O'Sullivan, Delahunty, & Kerry (2009), females, on average, obtained higher scores than their male counterparts for sensitivity to taste, odour, and texture. Women also have 43.2% more cells in the olfactory bulb than men, therefore, women have better odour memory and higher levels of acuity for some substances (Oliveira-Pinto, Santos, Coutinho, Oliveira, Santos, Alho, Leite, Farfel, Suemoto, Grinberg, & Pasqualucci, 2014).

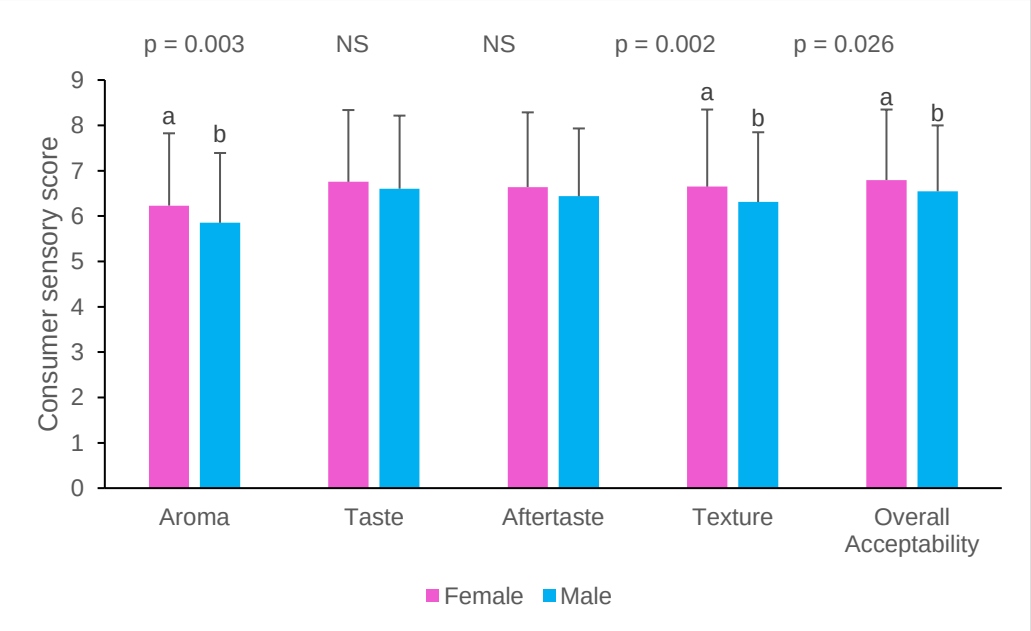

Figure 3.12. Consumer sensory rankings of Boerewors attributes, by gender. NS = Not significant. Means with different superscripts differed significantly. Error bars represented standard deviations of means.

3.4 Conclusions

The main focus area regarding the replacement of SO_2 in Boerewors, was the evaluation of the application of various natural preservatives in an effect to reduce dependency on SO_2 on maintaining product stability, safety, and quality. The end goal was to improve these parameters of Boerewors, using natural ingredients, for overall human health and satisfaction. In this study, the natural preservatives acted as SO_2 replacers for the most part.

The natural preservatives had limited effects on the physico-chemical parameters. Over a 6-day storage period at 4 °C, the pH, a_w and moisture values of all the natural preservative treatments were comparable to the PC.

It appeared as if TBC and LAB counts would not be the best measurement of microbial quality and shelf-life when protective cultures are used in the products, as it immediately increased the TBC and LAB counts after manufacturing, possibly indicating a shorter shelf-life according to microbial quality standards. Different microbiological standards should be used in cases where products contain protective cultures. The plant extract groups fared better and did not have significantly higher counts than PC. Another microbial parameter that was influenced by formulation type, was the yeast count. PC had the lowest yeast counts throughout the 6-day shelf-life. Although, none of the plant extract groups differed significantly from PC. However, the protective culture groups had significantly higher yeast counts than PC on day 3.

The plant extract groups significantly improved the lipid oxidative stability of the Boerewors compared to the rest of the treatment groups. KD1 and KD2 outperformed the other treatment groups at every time interval, and even had lower SLOP levels on day 90 than that of PC, which contained SO_2, on day 0. Nevertheless, all natural preservative Boerewors samples had TBARS values below the primary detection threshold of rancidity, from day 0 to day 90 of frozen storage.

The colour parameters were greatly influenced by the Boerewors treatment type. The PC and KD1 groups maintained the best and highest lightness values. The redness decreased from day 0 to 6, but PC had the highest redness values throughout the 6-day shelf-life. Yellowness was higher when KD1 was used in the formulation, but for the most part, there were no significant differences in the yellowness of the Boerewors. The PC treatment maintained colour brightness the best, followed by KD2. All the Boerewors treatments had great red colour purity values, but PC consistently had the values closest to red than the other treatments. The colour results prove how difficult it would be to replace SO_2 as a colour preservative in Boerewors.

Differences between treatments for the consumer sensory analysis were limited. The consumers did not have a preference to SO_2, and although not significant, PrC2 received the highest attribute scores and the natural preservatives could, therefore, replace the SO_2 in Boerewors in terms of consumer sensory analysis. In terms of gender preferences, women, on average, gave higher ranking scores than the men on this sensory panel.

Cost is a final consideration of the suitability of these replacers that will play a major part in the selection of replacers for commercial products. All of the natural preservatives used in this

study was cheaper than the conventional SO_2 preservative. Taking into account the results of this study in addition to cost, it is believed that both KD1 and KD2 remained the cheapest, simplest and most elegant replacers to be considered for the replacement of SO_2 in Boerewors.

CHAPTER 4

MICROBIAL COMMUNITY ANALYSIS OF BOEREWORS MANUFACTURED WITH NATURAL PRESERVATIVES USING BIOLOG™ ECOPLATE™

ABSTRACT

The replacement of SO_2, with natural preservatives, can affect the product safety and shelf-life of fresh meat products, such as Boerewors. The effect of the replacement on the microbial communities of Boerewors have not yet been investigated. Biolog™ Ecoplate™ have been used to physiologically profile bacterial communities from different preservative treated Boerewors samples. The Boerewors were sampled on day 0 and incubated at 25 °C, in an OmniLog® system, for 72.5 h. The growth and reaction of the microbial communities to the 31 carbon compounds were measured, based on the production of formazan. The results were statistically analysed, using two methods: 1) AWCD and Shannon indices, and 2) applying the Gompertz function to analyse growth parameters. The results of the first approach showed that KD1 had the lowest growth rate and a similar microbial diversity as the PC, while the NC had the highest overall growth and diversity. The results of the second approach showed that PrC1 had a much later midpoint of growth than the other treatments, while the microbial community in KD2 had the least explosive growth of all the treatments. Overall, a reduction in microbial functional diversity and explosiveness of growth was noted in Boerewors with added preservatives. In terms of community-level physiological profiling, SO_2 replacement can, therefore, affect the microbial quality and safety of Boerewors in mostly positive ways.

Keywords: microbial communities, SO_2 replacement, natural preservatives, Boerewors

4.1 Introduction

For many years, sulphur dioxide (SO_2) has been used as an effective preservative in a wide range of food products, including processed meat (Ough & Were, 2005). However, according to studies done by the World Health Organization (WHO) and International Program on Chemical Safety (IPCS; WHO, 2018), sulphites not only have a negative impact on asthmatics, but could also affect people that are not sensitive to sulphites. In 1986, the Food and Drug Administration (FDA) banned the use of sulphites in fresh fruit and vegetables after it resulted in 13 asthmatic deaths and many illnesses in the USA (Timbo, Koehler, Wolyniak, & Klontz, 2004).

The adverse effects caused by SO_2 made the food industry search for alternatives. However, reducing or replacing SO_2 in food is difficult, not only because of its multi-functionality, but also by finding an alternative that could deliver more or less the same benefits, without other negative effects (Roberts & McWeeny, 2007).

Replacing SO_2 can have an impact on food safety. Research have shown the efficacy of SO_2 against microbial spoilage and pathogenic bacteria in food (Usseglio-Tomasset, 1992). Therefore, changing the preservative can possibly lead to altered functional diversity of microbial communities in meat products. No research has previously been performed on the microbial profile, based on their capability to oxidize carbon substrates, of food in terms of SO_2 replacement. Analysing the microorganisms in processed meat, like Boerewors, can lead to more accurate predictions of how effective the change in a control system, like preservatives, can be on food quality and safety. Community-level physiological profiling (CLPP), using Biolog™ EcoPlate™, is one of the approaches to study microbial diversity. This technique was initially applied in soil studies at around 2001 (Rasmussen, & Sørensen, 2001; Biolog Inc., 2023).

When it comes to environmental changes, microorganisms provide helpful information. Microbial communities are usually the first to react to any environmental change and they are often the component that determines the viability of the whole environment. Therefore, Biolog™ EcoPlate™ is used to give a reliable index of environmental changes, by measuring the metabolism of 31 carbon sources, per assay, in triplicate. This is called community-level physiological profiling (Biolog Inc., 2023).

Community-level physiological profiling has been effective in determining spatial and temporal changes in bacterial communities. These changes can be compared and statistically analysed to provide knowledge on how populations change over time in the specific food environment (Weber & Legge, 2010; Ye, Line, Hinton, Gao, & Zhuang, 2019; Biolog Inc., 2023).

The aims of this chapter included:

1. Evaluating microbial communities in Boerewors with different preservative treatments, by determining the functional diversity of the bacterial communities from the data generated by CLPP (Biolog™ Ecoplate™), by using:
    a) Average well colour development (AWCD)
    b) Shannon diversity (*H*)
    c) Shannon richness (*S*)
    d) Shannon evenness (*E*)
    e) Heatmaps

f) Principal component analysis

2. Evaluating the growth of the microbial communities in Boerewors with different preservative treatments from the data generated by CLPP (Biolog™ Ecoplate™), with the Gompertz function, by using:
    a) Overall growth rate and Principal component analysis
    b) Position of growth
    c) Explosiveness of growth

4.2 Materials and methods

4.2.1 Sourcing of lean meat, fat, additives, and spices

The sourcing of the lean meat, fat, additives, and spices were performed as described in Chapter 3 section 3.2.2.

4.2.2 Formulation of Boerewors

The formulation of the Boerewors models was according to Chapter 3 section 3.2.3.

4.2.3 Manufacturing of Boerewors

The manufacturing of the Boerewors models was performed, according to Chapter 3 section 3.2.4.

4.2.4 Sample preparation for Biolog™ EcoPlate™

Samples from each treatment were collected by transferring a portion (± 12 g) of the Boerewors batter into a sterile Petri dish, then aseptically weighing 10 g into a sterile 207 ml WhirlPak™ bag (Lasec, Bloemfontein, South Africa). Ninety milliliters of sterile distilled water were added to create the first dilution (10^{-1}) point. It was stomached (AME Stomacher Lab-Blender 400, Johannesburg) for 1 minute. Further dilutions (10^{-2} to 10^{-4}) were made by adding 1 ml of the 100 ml sample (10^{-1} dilution) in McCartney bottles containing 9 ml sterile distilled water. The 10^{-2} to 10^{-4} dilutions were poured aseptically into separate sterile disposable reservoirs, after which the 10^{-2} to 10^{-4} dilutions were pipetted directly into the Biolog™ EcoPlate™ using a multichannel pipette. The 10^{-2} dilution was pipetted in the first set of 31 wells of the Biolog™ Ecoplate™ (Fig. 4.1), the 10^{-3} dilution was pipetted in the second set 31 wells, while the 10^{-4} dilution was pipetted in the third set of 31 wells. The Biolog™ EcoPlate™ with lids were incubated at 25 °C in the fully automated OmniLog® incubator/reader for 72.5

h. Each well contained tetrazolium dye that converted to insoluble violet formazan after bacterial oxidation (Figure 4.1). The concentration of the violet formazan correlated to the degree of respiration by the microbes in the communities (Ye et al., 2019). Biolog™ EcoPlate™ were obtained from Anatech Instruments (Pty) Ltd, Randburg, South Africa. Each Biolog™ EcoPlate™ contained three replicates of 31 carbon sources and one well for water as the control. The carbon sources in each well are indicated in Figure 4.2.

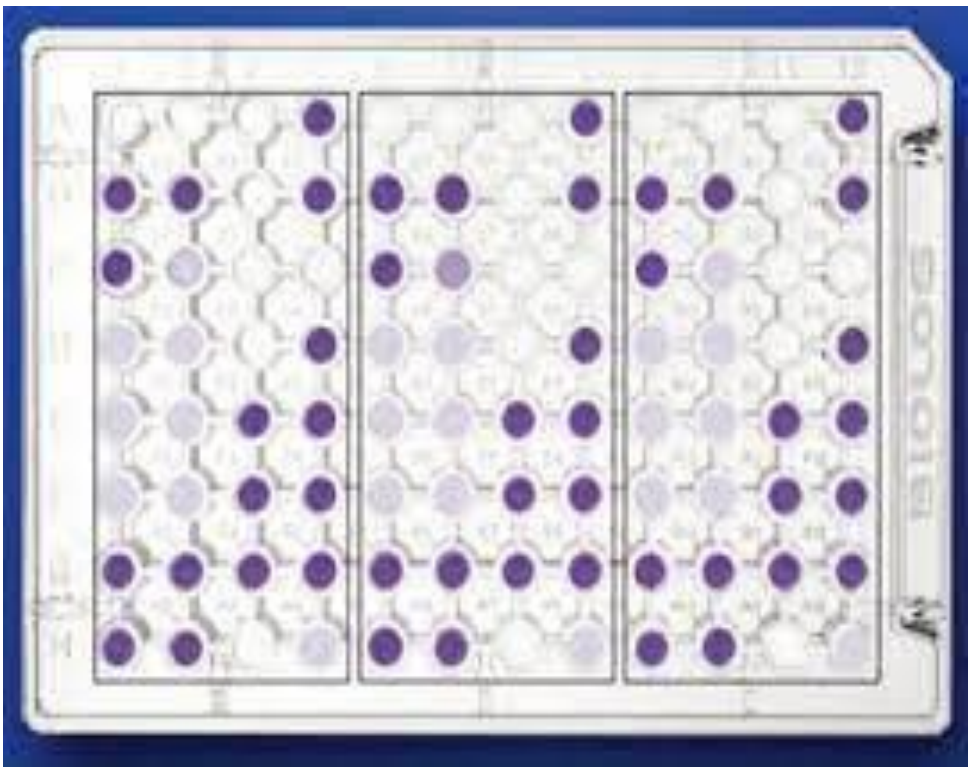

Figure 4.1. An example of a Biolog™ Ecoplate™ with 31 carbohydrates in triplicate (Biolog, Inc., 2023)

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A5 Water	A6 β-Methyl-D-Glucoside	A7 D-Galactonic Acid γ-Lactone	A8 L-Arginine	A9 Water	A10 β-Methyl-D-Glucoside	A11 D-Galactonic Acid γ-Lactone	A12 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B5 Pyruvic Acid Methyl Ester	B6 D-Xylose	B7 D-Galacturonic Acid	B8 L-Asparagine	B9 Pyruvic Acid Methyl Ester	B10 D-Xylose	B11 D-Galacturonic Acid	B12 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C5 Tween 40	C6 i-Erythritol	C7 2-Hydroxy Benzoic Acid	C8 L-Phenylalanine	C9 Tween 40	C10 i-Erythritol	C11 2-Hydroxy Benzoic Acid	C12 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D5 Tween 80	D6 D-Mannitol	D7 4-Hydroxy Benzoic Acid	D8 L-Serine	D9 Tween 80	D10 D-Mannitol	D11 4-Hydroxy Benzoic Acid	D12 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-DGlucosamine	E3 γ-Amino Butyric Acid	E4 L-Threonine	E5 α-Cyclodextrin	E6 N-Acetyl-Dglucosamine	E7 γ-Amino Butyric Acid	E8 L-Threonine	E9 α-Cyclodextrin	E10 N-Acetyl-Dglucosamine	E11 γ-Amino Butyric Acid	E12 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-Lglutamic Acid	F5 Glycogen	F6 D-Glucosaminic Acid	F7 Itaconic Acid	F8 Glycyl-Lglutamic Acid	F9 Glycogen	F10 D-Glucosaminic Acid	F11 Itaconic Acid	F12 Glycyl-Lglutamic Acid
G1 D-Cellobiose	G2 Glucose-1Phosphate	G3 α-Keto Butyric Acid	G4 Phenylethylamine	G5 D-Cellobiose	G6 Glucose-1Phosphate	G7 α-Keto Butyric Acid	G8 Phenylethylamine	G9 D-Cellobiose	G10 Glucose-1Phosphate	G11 α-Keto Butyric Acid	G12 Phenylethylamine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H5 α-D-Lactose	H6 D,L-α-Glycerol Phosphate	H7 D-Malic Acid	H8 Putrescine	H9 α-D-Lactose	H10 D,L-α-Glycerol Phosphate	H11 D-Malic Acid	H12 Putrescine

Figure 4.2. Biolog™ EcoPlate™ with 31 carbon sources in triplicate (Biolog, 2023).

4.2.5 Data collection and statistical analysis

The respiration rate of the substrates in the Biolog™ EcoPlate™ as a sole carbon source was measured by the production of formazan at a 590 nm wavelength. The readings, measured in optical density (OD), were recorded every 15 min by the Biolog™ OmniLog plate reader and exported as an Excel file for statistical analysis.

The water well was important because it served as a control (Figure 4.3), which represented the bacterial growth in the different treatments, regardless of the substrates. Before subtracting the control, the raw data was drawn first, in order to better understand the patterns. To visualize any dimensions of choice, the data of all the dilutions were combined in a single long form sheet, considering one substrate at a time. Because of the large number of carbon sources (31) that were evaluated in this study, pyruvic acid methyl ester and Tween 40 were chosen as examples and the remaining results of all the data in this study, will be given in Annexures. The results of the oxidation of the pyruvic acid and Tween 40 substrates are given in Figure 4.4 and Figure 4.5, respectively. The Biolog OD results, with all the dilutions, of the remaining substrates, are given in Annexure 1.

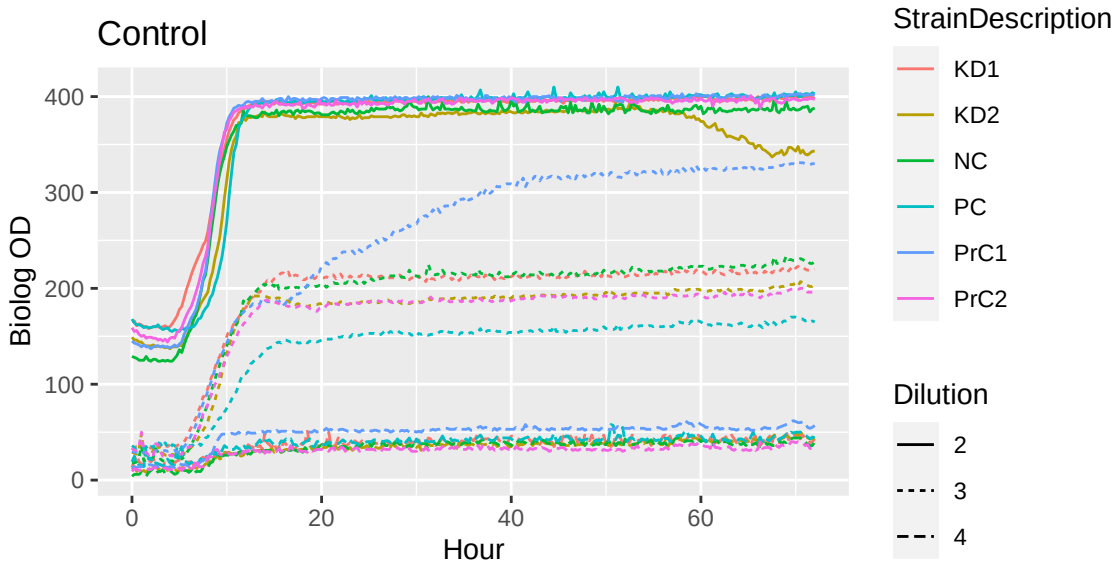

Figure 4.3. Control well comparing the six treatments and dilutions over 72.5 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

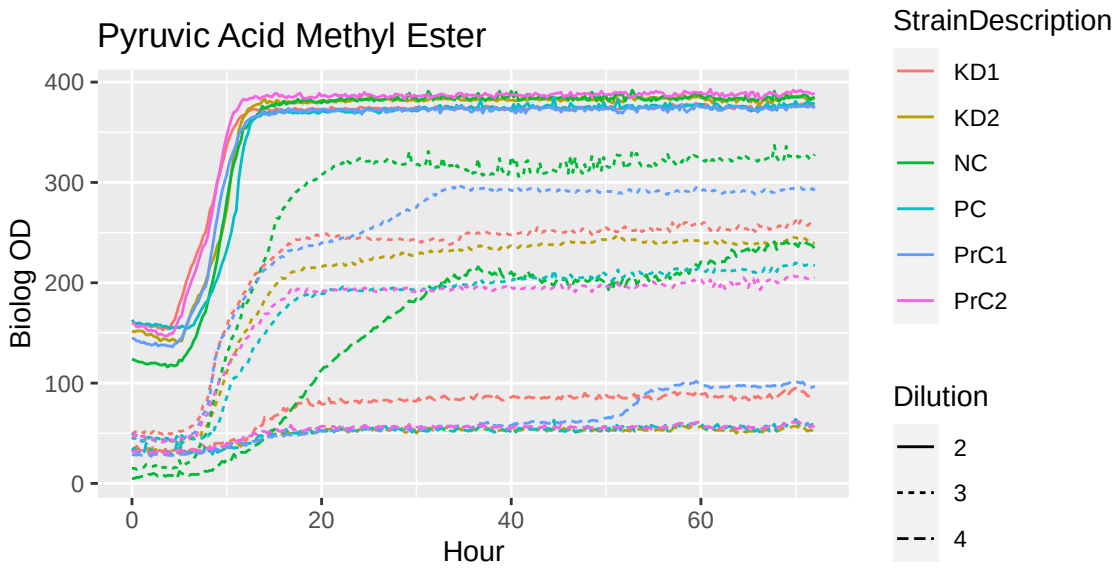

Figure 4.4. Pyruvic acid methyl ester substrate comparing the six treatments and dilutions over 72.5 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

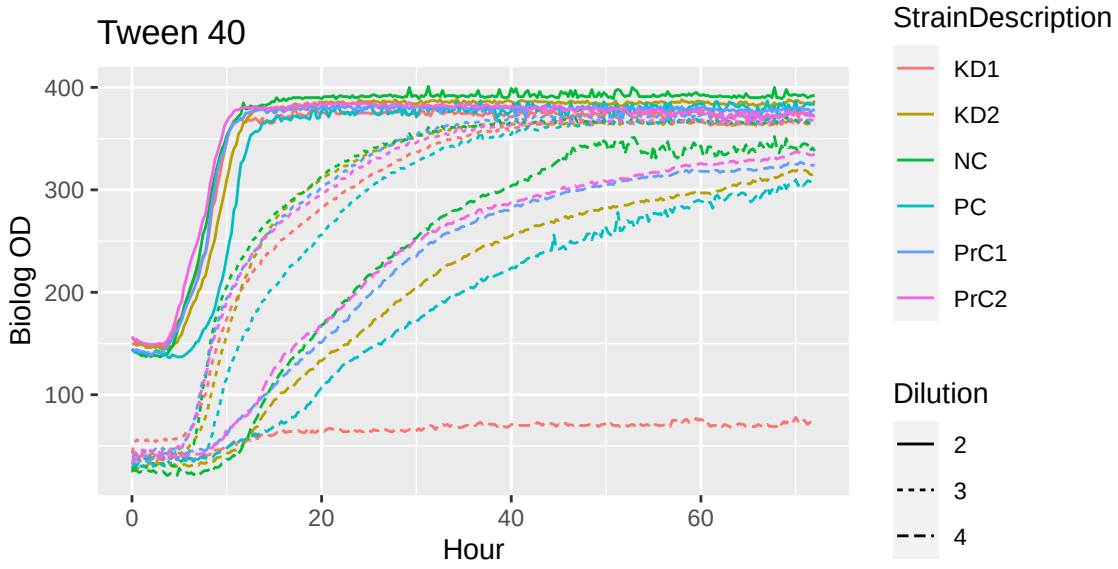

Figure 4.5. Tween 40 substrate comparing the six treatments and dilutions over 72.5 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The experiment ended at time *T* = 72.5 h, but the following additional statistics were calculated, using data at time *T* = 48 h: Average well colour development (AWCD), substrate diversity (*H*), substrate evenness (*E*), and richness (*S*). These indices were calculated using an algorithm. This time slot of 48 h was chosen because it included all the microbial growth phases in most cases, recommended by Weber & Legge (2010). Dilution 10^{-4} was used for the analysis in this study, because the growth patterns in this dilution were more stable than in the other dilutions.

Some external factors could have caused irregular patterns (Figures 4.3, 4.4 and 4.5) that were not related to microbial growth. Thus, where the data was not as expected, the curve was classified as flat. The statistics additionally required that all colour developments (Biolog OD values) below 0 were artificially replaced with 0 (Garland, 1997; Weber & Legge, 2010). *E, S,* and further calculations required specification of a lower detection limit that was reasonable for the equipment used; a value of 20 was assumed. Due to limited resources and circumstances concerning the apparatus, the experiment was done once. Typically, reported statistics do not account for measurement error and error between plates, and if the experiment were repeated, then the results could differ markedly. For error calculation, in this case, resampling standard deviations were used. Resampling, also known as bootstrapping, is sampling with the replacement from the data set to estimate variability in statistics; it allows for the calculation of standard errors, construct intervals, and perform hypothesis testing for sample statistics (Efron, 2000). The resampling of this data set was done by considering the wells to be equally important and interchangeable, in terms of uncertainty.

Thus, the algorithm for calculating the statistics was as follows:

1. Isolate the colour development (CD) values at time $T = 48$ by subtracting the control $(j = 0)$ from each substrate $j = 1 \ldots J$, where $J = 31$ is the number of substrates.
2. Calculate the standard deviation of the control over a stable period (σ_c), then replace CD values within roughly $2\sigma_c$ of 0 with 0 itself.
3. Calculate the sum over substrates and store that value $\left(G = \sum_{j=1}^{J} C\ D_j\right)$.
4. Calculate AWCD as G/J.
5. Count the number of non-zero CD values as S, so formally $S = \sum_{j=1}^{J} I\left(CD_j > 0\right)$ where I is the indicator function.
6. Calculate the proportional CD values $p_j = CD_j/G$.

7. Calculate the information values of each substrate as $-p_j/\log(p_j)$ and sum them to obtain H. Note that $\log$ refers to the natural logarithm and where $CD_j = p_j = 0$ is taken $-p_j/\log(p_j)$ to be 0.
8. Calculate $E = H/\log(S)$.

As a next step, the CD values were repeatedly resampled, by sampling with replacement, to obtain new replicate samples CD^k where $k = 1 \ldots M = 2000$. For each replicate sample, the above algorithm was repeated, and a replicate set of statistics was obtained. The standard error of each statistic was calculated based on the standard deviation of the replicate statistics. These errors incorporated the uncertainty within tray, across wells, only. They did not account for uncertainty across trays.

A heat map and principal component analysis (PCA) plot were used to further show the microbial growth in the Boerewors treatments with the substrates, but also the guilds in which the substrates were grouped; carbohydrates, carboxylic/ketonic acids, amino acids, polymers, and amines/amides. The substrates had equal weights within the guilds. The amino acids guild would be regarded as the most important in this study, as sausages, like Boerewors, are good sources of complete protein, and amino acids are the building blocks of protein (Damodaran, 2008).

Sometimes it is meaningful to collapse sections into reduced numbers of latent dimensions. Principal component analysis seeks to find a linear combination of responses that captures as much as possible variation in as few dimensions as possible. A heatmap was used to show the intensity of colour development at 48 h over the different substrates. It also gave an average linkage hierarchical clustering in each direction to show some of the groupings that appeared in the data.

All the analyses done on the raw and adjusted data were repeated based on the Gompertz curve fits, to smooth out illogical patterns. The Gompertz results will not be completely re-discussed, only how it differed from the raw data. The Gompertz curve is a type of mathematical model for a time series and is widely used in many aspects of biology. It is a sigmoid function, which describes growth as being the slowest at the beginning and end of a selected time period (Tjørve & Tjørve, 2017). The basic Gompertz function is $a\exp(-be^{-ct})$, but to fit the data, the variation around the curve must also be accounted for. With the Gompertz function, each curve was summarized into three numbers instead of many: j denoted the curve number, i denoted the observation number along the curve, while corresponding with time t_i. In this experiment $t_i = i/4/48$ h.

The Gompertz curve formal definition was as follows:

$$\begin{aligned} y_{ij} &\sim Student\ t(\nu_j,\ \mu_{ij},\ \sigma_j) \\ \mu_{ij} &= a_j \exp(-b_j e^{-c_j t_{ij}}) \\ \log\pi(a_j, b_j, c_j, \nu_j, \sigma_j) &= -a_j - b_j - c_j - 2\log\sigma_j + \log\nu_j - 3\log(\nu_j + 0.75) + k \end{aligned}$$

Then

- a_j denoted the scale of the overall growth, or the maximum growth.
- b_j denoted the rough position of the growth.
- c_j denoted the explosiveness of the growth.
- μ_{ij} denoted the expected position of the Gompertz curve, created from the Gompertz parameters above.
- σ_j denoted the scale of the variation around the curve.
- ν_j denoted the tail index of the variation around the curve, controlling its shape. Small values indicated the presence of extreme variations in places.

The discussions of the Gompertz curves were focused on the overall growth, rough position, and explosiveness of the growth (a_j, b_j and c_j, respectively) in each substrate.

Before each fit, the following steps were taken:

1. The intercepts were zeroed by taking the median of the first 5 h and subtracting that from each series.
2. The data was restricted to the first 48 h of the experiment.
3. If the last value at 48 h was less than the cut-off point, then the curve was assumed flat and not fitted.

The model was specified in Bayesian hierarchical form. The Bayesian approach was particularly useful in this context as it allowed one to propagate the uncertainty in the curve fitting process into further analyses. The Gompertz data model was implemented using the STAN interface (mc-stan.org; R Core Team, 2022).

4.3 Results and discussion

4.3.1 Microbial community analysis of the raw data (72.5 h) and adjusted data (48 h) of the Boerewors treatments

The raw data of the two substrates, pyruvic acid methyl ester (Figure 4.6) and Tween 40 (Figure 4.7), visualized the growth of the microbial communities from the Boerewors samples with different preservative treatments, for the 72.5 h incubation time. In both substrate wells, the NC had the highest CD readings at the end of the incubation period. This result was expected since the NC did not contain any preservatives. The dips and spikes in the growth pattern could have been caused by many factors; nutrition concentration, pH, water activity and salt concentration and other ions (Hamad, 2012). The 10^{-4} dilution colour development (CD) results of the remaining substrates are given in Annexure 2.

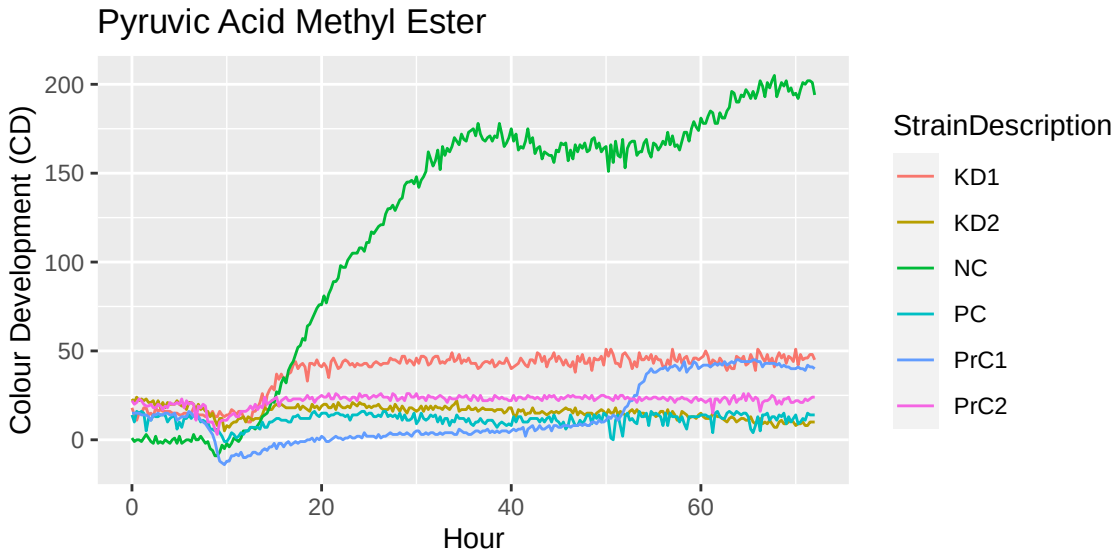

Figure 4.6. Microbial community growth with pyruvic acid methyl ester in dilution 10^{-4} of the Boerewors treatments up to 72.5 hours. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

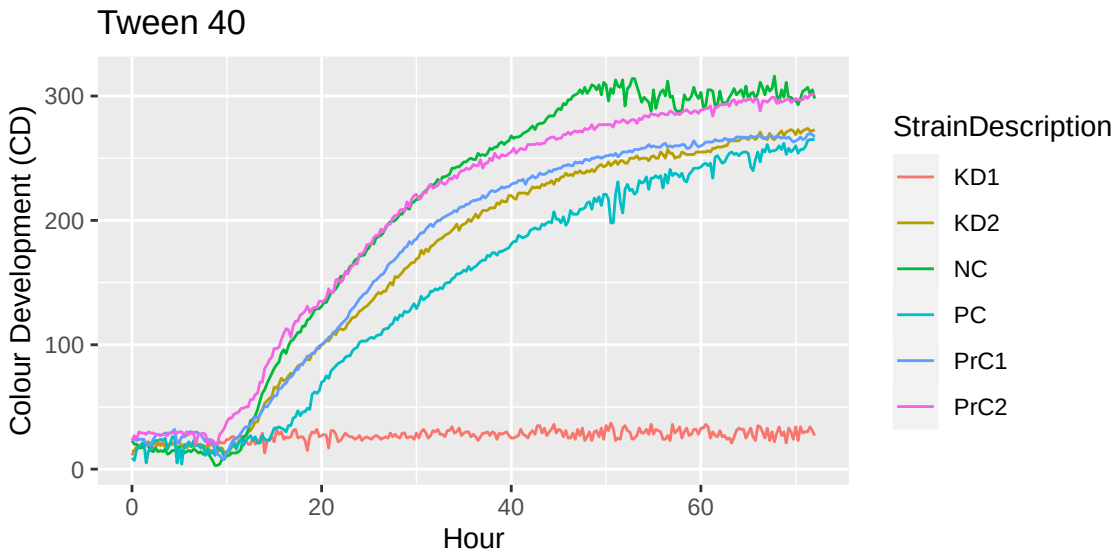

Figure 4.7. Microbial community growth with Tween 40 in dilution 10^{-4} of the Boerewors treatments up to 72.5 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The AWCD and Shannon indices of the adjusted data, of all the substrates, were calculated (Table 4.1). The AWCD and Shannon indices were included because they have become the conventional approach to analyse the microbial community profile of a given substance or product, and were used in many other studies (Garland, 1997; Weber, & Legge, 2010; Stoops, Ruyters, Busschaert, Spaepen, Verreth, Claes, Lievens, & Van Campenhout, 2015; Furtak, Grządziel, Gałązka, & Niedźwiecki, 2019; Yeh, Line, Hinton Jr, Gao, & Zhuang, 2019). As expected, the AWCD and Shannon diversity of the NC (142.39 ± 18.05 and 3.130 ± 0.078, respectively) were the highest compared to the other treatments, suggesting a higher metabolism, most likely due to no preserving agents added to this treatment, which allowed more microorganisms to survive, and even thrive, in the Boerewors (Rawat, 2015). The Boerewors with the KD1 as preservative, had the lowest AWCD (74.68 ± 16.63) and second lowest *H* value (2.624 ± 0.179), which were slightly lower than the AWCD of the PC (76.26 ± 17.68) and the PC had the lowest *H* with 2.593 ± 0.179. The *H* value indicated the diversity of microbes within the community, which is useful to determine if a few of the same type of microorganisms were causing the colour development, or if it was caused by many different species of microorganisms. This implicated that the KD1 preservative controlled the growth of the microbial communities better than the PC but had slightly more diversity. Plant extracts,

like those used in KD1 (rosemary and acerola extracts), possess multiple modes of action for the inhibition of microbes; two of these mechanisms include the interference with intermediate metabolism, and disruption of the cell membrane (Bouarab-Chibane, Ouled-Bouhedda, Leonard, Gemelas, Bouajila, Ferhout, Cottaz, Joly, Degraeve, & Oulahal, 2017). Both of these mechanisms could be the reason for the low growth rate and minimal diversity shown in the KD1 treatment. The growth and diversity of KD2 were slightly higher than KD1, but still lower than most other treatments.

The protective culture 2 (PrC2) treatment had the highest richness index with 28.000 ± 1.656, and KD1 and PC had the lowest with 17.000 ± 2.780. The richness (*S*) represented the number of wells with colour development, which meant that even though the NC had the highest growth rate and species diversity, the microbial community in the PrC2 treatment oxidised the most substrates. The PrC2 contained *Lactobacillus curvatus*, and can ferment carbohydrates and hydrolyse polymers (Wang, Wu, Lv, Shao, Hungwe, Wang, Bai, Xie, Wang, & Geng, 2021), which included a large group of the 31 carbon compounds in the Biolog™ EcoPlate™. These results supported the results shown in chapter 3 and other studies (Hugo & Hugo, 2015; Stoops, Ruyters, Busschaert, Spaepen, Verreth, Claes, Lievens, & Van Campenhout, 2015), that the addition of protective cultures can influence the results and not accurately represent their preservation abilities. It also showed that the added preservatives, like SO_2 and KD1, which consisted of rosemary and acerola extracts, reduced the total viable count in fresh ground meat products.

Table 4.1. Growth kinetics of the microbial communities from Boerewors samples, with different treatments, from the Biolog™ EcoPlate™ assay, at 48 h (n = 31). AWCD = average well colour development; *H* = Shannon diversity; *S* = Shannon richness; *E* = Shannon evenness; NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

Treatment	AWCD	*H*	*S*	*E*
NC	142.39 ± 18.05	3.130 ± 0.078	28.000 ± 1.654	0.939 ± 0.015
PrC1	92.97 ± 16.22	2.882 ± 0.129	22.000 ± 2.505	0.933 ± 0.018
PrC2	122.29 ± 16.52	3.120 ± 0.071	28.000 ± 1.656	0.936 ± 0.012
KD1	74.68 ± 16.63	2.624 ± 0.179	17.000 ± 2.780	0.926 ± 0.021
KD2	83.13 ± 14.84	2.891 ± 0.115	23.000 ± 2.420	0.922 ± 0.015
PC	76.26 ± 17.68	2.593 ± 0.179	17.000 ± 2.780	0.915 ± 0.025

The adjusted data of the single carbon source utilization, by microbial communities from the Boerewors treatments, were further visualized, using a heatmap and PCA biplot. The heatmap (Figure 4.8) showed that the substrates were oxidised at various degrees; overall, L-asparagine was used the most. Asparagine is a non-essential amino acid found mostly in plant proteins. It is involved in protein synthesis in humans and animals (Lomelino, Andring, McKenna, & Kilberg, 2017) which explained the reason for being present in the Boerewors treatments, since the Boerewors contained beef meat. Microorganisms such as *Bacillus*, *Pseudomonas* and *E. coli*, commonly found in food products, can produce asparaginase, which is the enzyme that breaks down L-asparagine to aspartic acid and ammonia, which could explain the high utilization rate (Ebrahiminezhad, Rasoul-Amini, & Ghasemi, 2011). *Bacillus* and *Pseudomonas* are psychrotolerant microorganisms, which means that they can survive and grow in refrigerated conditions, making refrigerated food products, like Boerewors, more susceptible to spoilage. *Pseudomonas* spoils meat by breaking down proteins, which can produce odour and flavour defects, as well as discolouration (Wickramasinghe, Ravensdale, Coorey, Chandry, & Dykes, 2019).

On the heatmap (Figure 4.8), 2-hydroxybenzoic acid was used the least. 2-Hydroxybenzoic acid, more commonly known as salicylic acid, has anti-microbial properties (Kubo, Muroi, Himejima, Yamagiwa, Mera, Tokushima, Ohta, & Kamikawa, 1993), which could explain the low utilization rate by the microbial communities in the Boerewors samples, regardless of treatment.

The dendrogram on the top of the heatmap (Figure 4.8) showed that utilization of the different substrates could be divided into three groups: 1) most oxidised substrates, 2) moderately oxidised substrates, and 3) least oxidised substrates. The first group consisted of Tween 40, D-mannitol, itaconic acid, D-glucosaminic acid, L-asparagine, L-arginine, Tween 80, D-xylose, and D-galacturonic acid. The moderately oxidised group included D-galactonic acid γ-lactone, D-malic acid, L-serine, putrescine, and γ-amino butyric acid. The last group consisted of N-acetyl-D-glucosamine, 4-hydroxy benzoic acid, 2-hydroxy benzoic acid, α-D-lactose, D-cellobiose, i-erythritol, α-cyclodextrin, L-threonine, pyruvic acid methyl ester, glucose-1-phosphate, β-methyl-D-glucoside, α-keto butyric acid, L-phenylalanine, phenylethylamine, glycyl-L-glutamic acid, D, L-α-glycerol phosphate, and glycogen.

The dendrogram on the top of the heatmap (Figure 4.8) also gave an indication of the guilds that each individual substrate belonged to, i.e., carbohydrates, carboxylic/ketonic acids, amino acids, polymers, and amines/amides. However, it was clear that there was no correlation between the groupings by the dendrogram and the guilds that the individual substrates belonged to.

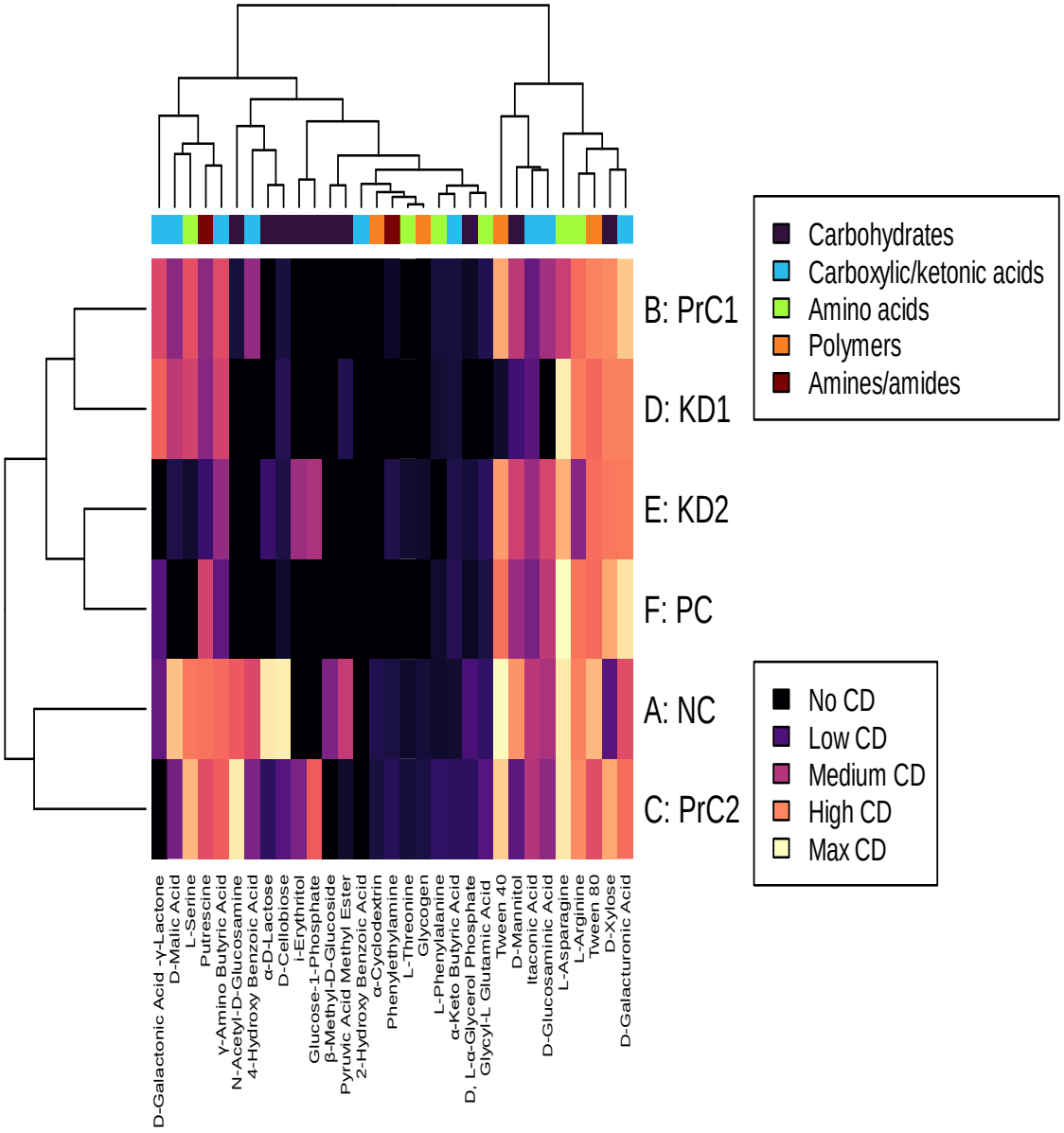

Figure 4.8. Heatmap diagram of the oxidation of the chemical compounds by the microbial communities from Boerewors samples, with different preservative treatments. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The dendrogram on the left of the heatmap (Figure 4.8), grouped the PrC1, KD1, KD2 and PC together, while the NC and PrC2 were further related. PrC2 contained a high amount of lactic acid bacteria, which could be the reason for relating to the NC in terms of colour development.

As illustrated in Figure 4.9, the PCA biplot of the substrate profiles showed the separation of the microbial communities from the different treatments; PC, KD1, KD2 and PrC1 clustered

together, while the NC was separate from these treatments, the PrC2 treatment was in the same quadrant as the NC. The clustering of the treatments (PC, KD1, KD2 and PrC1) showed that they were functionally more similar than the NC and PrC2 treatments. Five of the chemical compounds, γ-aminobutyric acid, D-mannitol, putrescine, Tween 40, and L-serine, associated with the NC and PrC2 treatments, and five chemical compounds, Tween 80, L-arginine, L-asparagine, D-galacturonic acid, and D-xylose, clustered with the other treatments. The dendrogram of the treatments on the heatmap (Figure 4.8), correlated with the PCA results in Figure 4.9. The chemicals that associated with PrC2 are all part of the metabolism of lactic acid bacteria, whether it is degradation, synthesisation, or other metabolic involvements (Wang et al., 2021), which could be the reason for the association. The compounds that associated with the plant extract treatments, KD1 and KD2, and with the PC, are mostly found in plants, including vegetables, grains, and wood (Bode-Böger, Scalera, & Ignarro, 2007; Cheng, Zheng, Chen, & Zhang, 2019) and would, therefore, not be negatively affected by the bioactive compounds in the treatments.

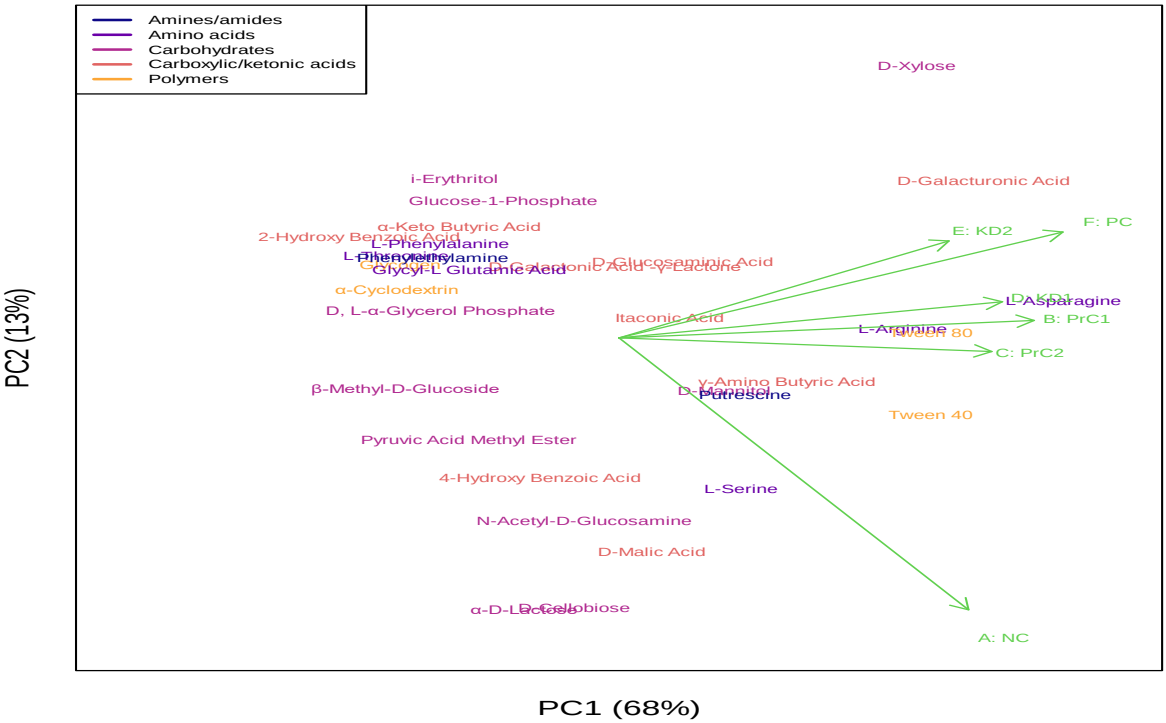

Figure 4.9. Principal component analysis (PCA) biplot of the profile of oxidation of carbon compounds by the microbial communities in the Boerewors samples, with different treatments. Axes PC1 and PC2: 81%. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The substrate oxidation data was then collapsed over five guilds (carbohydrates, carboxylic/ketonic acids, amino acids, polymers, and amines/amides) to see if there were any group-specific trends or correlations. Although some groups contained more substrates, each guild carried equal weight in the data sets. Table 4.2 and Figure 4.10 show the guild colour development at 48 h; the polymers group had the highest colour development with a CD value of 158.5 in the NC treatment, while the carbohydrates were oxidised the least, with a CD value of 37.8 in the KD1 treatment. The polymers evaluated in this study included Tween 40, Tween 80, glycogen, and α-cyclodextrin. Polymers are sometimes considered as food additives, used to emulsify, or stabilize (Rayner, Östbring, & Purhagen, 2016; Brovč, E.V., Mravljak, J., Šink, R., & Pajk, S. (2020). Some spoilage causing microorganisms can degrade polymers. These microorganisms include *Bacillus, Corynebacterium, Staphylococcus, Streptococcus, Shigella, Alcaligenes, Acinetobacter, Escherichia, Klebsiella* and *Enterobacter* (Joutey, Bahafid, Sayel, & El Ghachtouli, 2013). According to a study based on biofilm formation, *S. aureus* was especially efficient in utilizing Tween 80 (Nielsen, Kjems, Mygind, Snabe, & Meyer, 2016).

Table 4.2. Guild colour development (CD) from the Boerewors samples, with different treatments, from the EcoPlate™ assay, at 48 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

	Carbohydrates (CD)	**Carboxylic/ketonic acids (CD)**	**Amino acids (CD)**	**Polymers (CD)**	**Amines/amides (CD)**
NC	144.0	139.444	139.000	158.50	125.5
PrC1	47.5	127.222	107.167	118.75	72.0
PrC2	114.1	105.333	156.667	132.25	116.5
KD1	37.8	96.889	119.167	58.25	58.5
KD2	79.0	81.222	79.500	119.50	50.5
PC	42.2	84.889	97.167	105.75	86.0

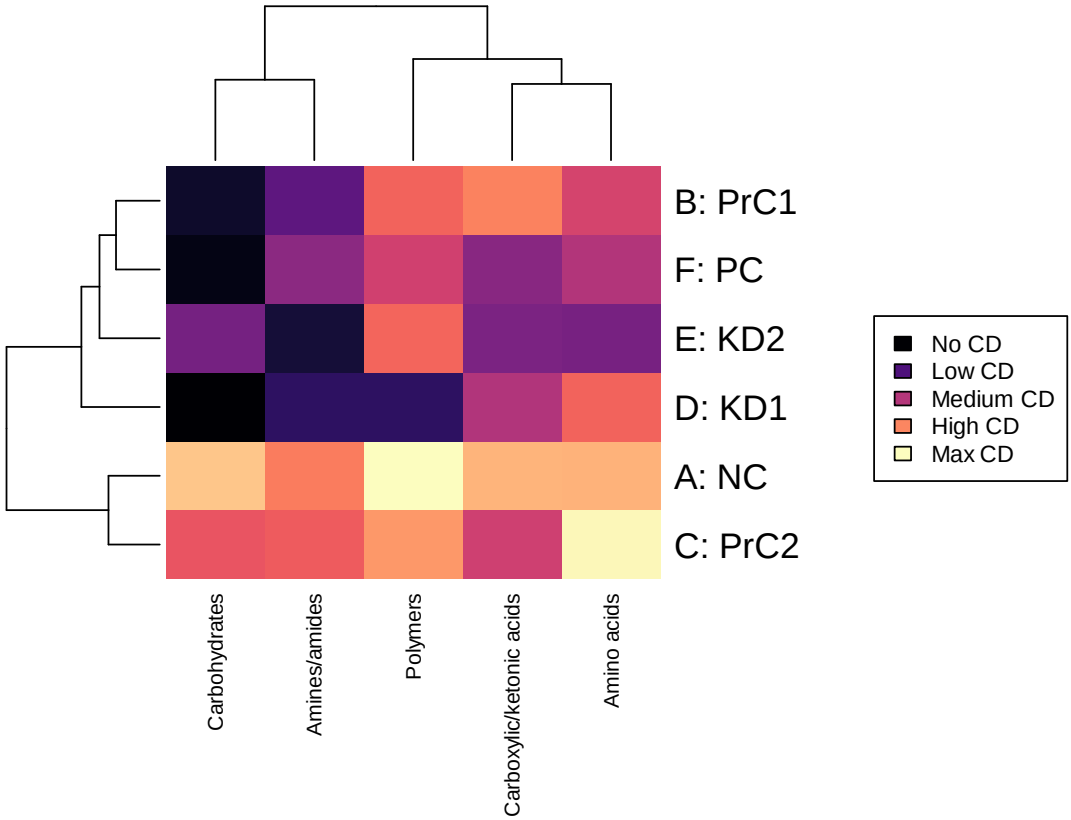

Figure 4.10. Heatmap of the guild colour development (CD) from Boerewors samples with different treatments, from the EcoPlate™ assay, at 48 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

4.3.2 Microbial community analysis of the Boerewors treatments based on the Gompertz curve data (48 h)

The Gompertz curve is often used to describe the growth of animal, plants, or microorganisms, which makes it a useful tool in survival studies (Tjørve, & Tjørve, 2017). The Gompertz growth curves of pyruvic acid methyl ester and Tween 40 (Figures 4.11 and 4.12, respectively) were again used as examples and had fewer irregularities and more stable growth patterns than the raw data growth curves (Figures 4.6 and 4.7). In almost all cases, it appeared that the start was stable, followed by a growth period that curved upwards, then growth in a stable fashion for a while, and the final period tapered off back to a stable pattern at the end. With the data summarised in only three numbers, the Gompertz curve showed an approximation of what

was happening in the wells. To show the similarities and differences of the Gompertz data, compared to the raw data results, the overall growth (a) of the microbial communities in the six Boerewors formulations were also presented in the form of a PCA biplot. The CD results, based on the Gompertz curves, of the remaining substrates, are given in Annexure 3.

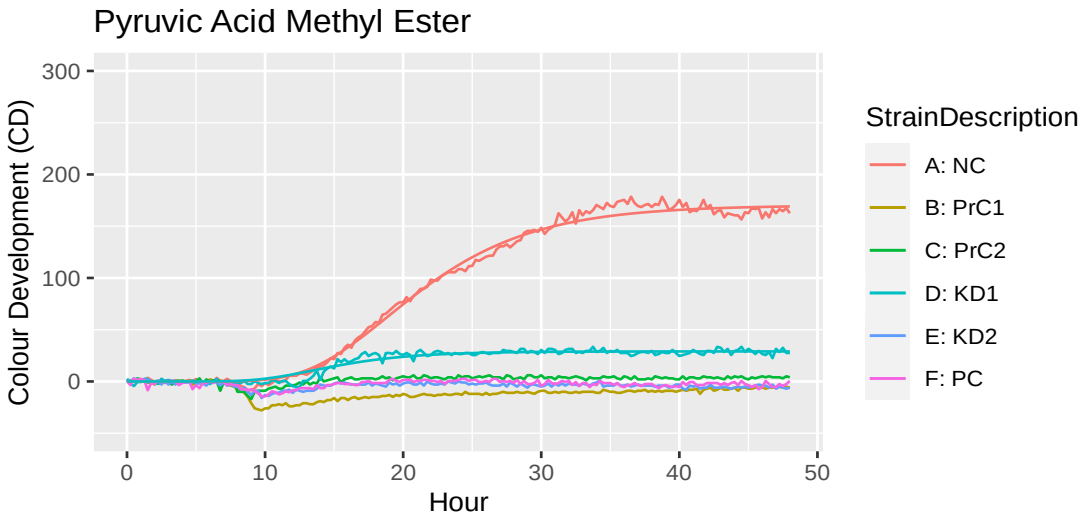

Figure 4.11. Gompertz fit of microbial community growth with pyruvic acid methyl ester in dilution 10^{-4} of the Boerewors treatments up to 48 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

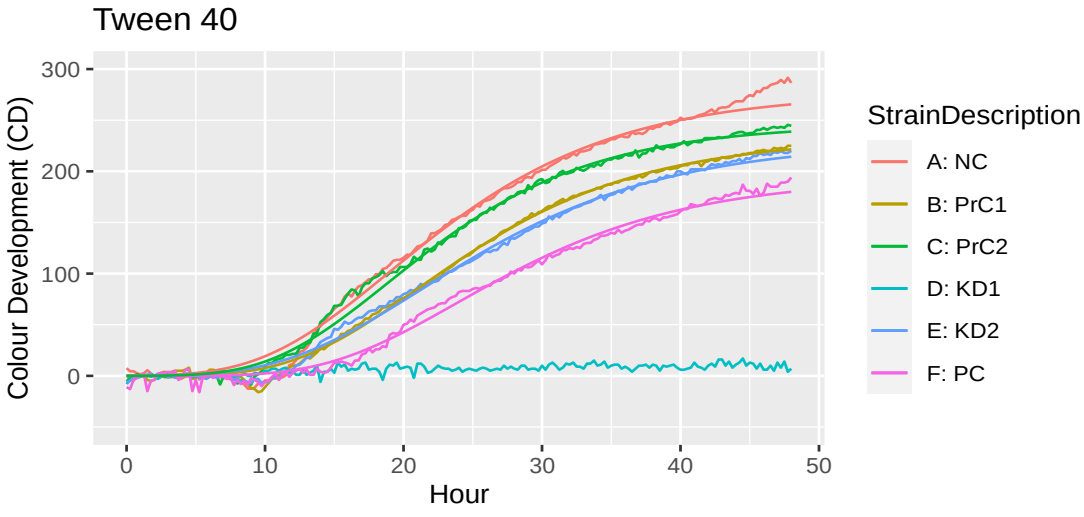

Figure 4.12. Gompertz fit of microbial community growth with Tween 40 in dilution 10^{-4} of the Boerewors treatments up to 48 h. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The Gompertz model PCA biplot (Figure 4.13), showing the oxidation of substrates by microbial communities, also had slightly different results than the original PCA biplot (Figure 4.9). In this model, the KD2 treatment clustered with the PC, PrC1 and PrC2 treatments, and the KD1 treatment associated with the NC, whereas it was vice versa in the original PCA biplot. More carbon compounds (7) clustered with the PC, PrC1, PrC2 and KD2 treatments in the Gompertz model, which is two more than in the adjusted raw data PCA biplot. Only three carbon compounds associated with the NC and KD1 treatments, including the L-asparagine, the most utilized substrate. The reason for the differences between the raw data and the Gompertz data was because the Gompertz data only used three data points instead of all of the values.

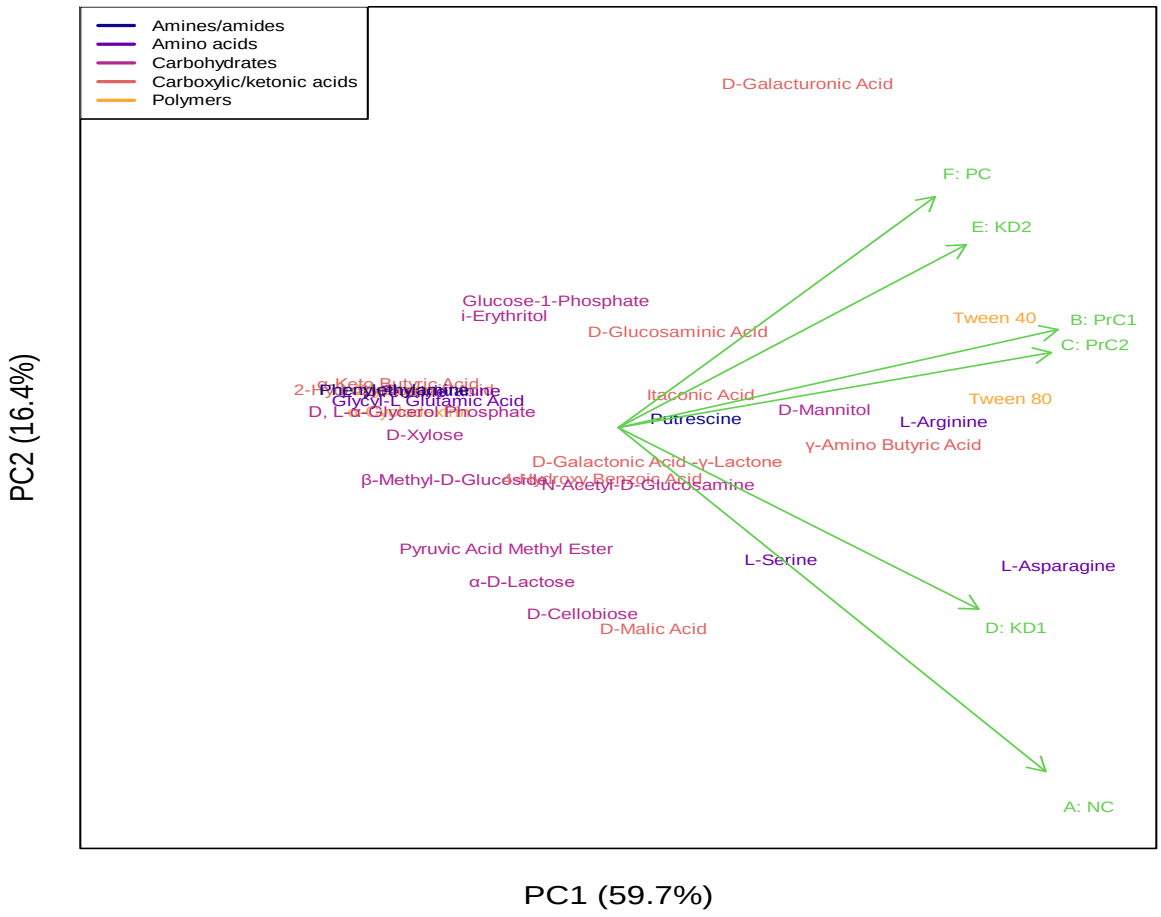

Figure 4.13. Principal component analysis (PCA) biplot of the profile of oxidation of chemical compounds by microbial communities in Boerewors samples, with different treatments, based on the Gompertz data. Axes PC1 and PC2: 76.1%. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The colour development in guilds based on the Gompertz model (Table 4.3) were also lower than the original data (Table 4.2). The polymers guild in treatment NC had the most colour development in both cases, with 130.178 and 158.50, respectively. However, in the Gompertz model, there was no colour development in the amines/amides guild in the KD2 treatment, whereas the carbohydrates guild in KD1 had the least colour development (37.8) in the adjusted raw data set. By only using three data points instead of all of the values, it changed the results when the Gompertz function was applied.

Table 4.3. Guild colour development from Boerewors samples, with different treatments, from the Biolog™ EcoPlate™ assay, at 48 h, based on the Gompertz data. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

	Carbohydrates (CD)	**Carboxylic/ketonic acids (CD)**	**Amino acids (CD)**	**Polymers (CD)**	**Amines/amides (CD)**
NC	123.376	96.084	116.297	130.178	64.265
PrC1	16.513	112.884	80.237	109.035	45.509
PrC2	65.378	69.377	123.139	97.007	55.203
KD1	12.495	68.565	111.927	50.436	49.020
KD2	40.708	45.953	52.527	98.887	0.000
PC	14.934	73.482	32.793	95.967	73.057

To be consistent, pyruvic acid methyl ester (Table 4.4) and Tween 40 (Table 4.5) were the two substrates used as examples to explain the other parameters of the Gompertz function. The Gompertz curve parameters of the remaining substrates are given in Annexure 4. As mentioned in the fitting rules of the Gompertz function, if the last value (at 48 h) was less than the cut-off point, the curve was assumed flat and the value was, therefore, 0. The maximum growth values of these tables will not be discussed, as it was discussed previously. The rough position was the estimated time at which bacteria started to grow.

In both substrates, the NC treatment group was the first to start to grow at 25.7 h and 7.9 h, respectively. This could be explained by the absence of any preservative. In pyruvic acid methyl ester, when the microbial community in KD2 started to grow, it was slightly faster than that of the NC, with explosiveness values of 12.0 and 8.2, respectively. The possible reasons

for the early growth in KD2 could be threefold; the preservative itself, the substrate or the microorganisms present in the Boerewors, because these factors have a direct influence on each other. In Tween 40, PrC2 had the highest growth explosiveness (5.7), and KD2 had the lowest (4.8). This could be due to the viable microorganisms (*Pediococcus acidicilactici* and *Lactobacillus curvatus*) present in the protective culture treatments (PrC1 and PrC2) added to the Boerewors, as PrC1 had the second highest value of 5.3.

Table 4.4. Gompertz parameter summary of six Boerewors treatments in pyruvic acid methyl ester as substrate. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).a_hat = maximum growth; b_hat = rough position of growth; c_hat = explosiveness of growth; sigma_hat = estimates scale of variation around curve; nu_hat = degrees of freedom; f_hat = expected value at 48 h.

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
NC	170.2	25.7	8.2	5.2	9.0	169.8	157.9	182.2
PrC1	0.0	0.0	0.0	6.5	100.0	0.0	-23.0	1.0
PrC2	0.0	0.0	0.0	4.0	100.0	0.0	-9.0	6.0
KD1	29.0	28.9	12.0	2.6	4.7	28.9	22.1	35.7
KD2	0.0	0.0	0.0	3.1	100.0	0.0	-11.0	1.0
PC	0.0	0.0	0.0	3.1	100.0	0.0	-9.5	2.5

Table 4.5. Gompertz parameter summary of six Boerewors treatments in Tween 40 as substrate. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). a_hat = maximum growth; b_hat = rough position of growth; c_hat = explosiveness of growth; sigma_hat = estimates scale of variation around curve; nu_hat = degrees of freedom; f_hat = expected value at 48 h.

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
NC	277.1	7.9	5.2	5.2	1.6	265.0	238.4	293.7
PrC1	233.1	10.3	5.3	2.7	2.5	221.4	211.9	231.3
PrC2	246.4	9.4	5.7	2.9	1.8	238.8	225.6	253.8
KD1	0.0	0.0	0.0	4.7	100.0	0.0	-4.0	13.0
KD2	229.5	8.5	4.8	5.0	7.0	214.5	202.3	226.5
PC	194.1	12.8	5.1	5.8	29.1	180.2	168.4	191.8

By using the above-mentioned parameter values, f_hat was calculated, which was the expected colour development of the well, at 48 h, with a 95% prediction interval. In pyruvic

acid methyl ester, the f_hat values of all the treatments were closer to the maximum growth values, than that of Tween 40, and in both cases, NC had the highest predicted colour development compared to the other treatments. This implied that the maximum growth was closer to the 48 h mark in pyruvic acid methyl ester, than in Tween 40, and adding any preservative to the Boerewors, would lower the maximum growth of the microbial communities expected after 48 h. Sigma_hat was the scale of variation around the curve, which showed that all the treatments, in both substrates, had similar amounts of variation around the curve. Nu_hat was the degrees of freedom, with 100.0 as a placeholder for 'large value'. A small value indicated a heavy tail (more disturbances in the pattern than expected), while larger values suggested normal variation. In pyruvic acid methyl ester, NC and KD1 had very low values, showing more unexpected pattern behaviour than that of the other treatments. In Tween 40, four of the six treatments, NC, PrC1, PrC2 and KD2, had very low values, whereas PC was somewhat higher, with 29.1, and KD1 had normal variation. This could possibly be attributed to flat curves in the other treatments, as only non-flat curves were shown in these results.

The position (Figure 4.14) and explosiveness (Figure 4.15) of the Gompertz curves were summarised across all substrates. Critically, for this analysis to make sense, only the non-flat curves were considered, as these parameters would not make sense if there was no development. This greatly affected the interpretation of the results. The NC group had a midpoint positional growth average across all substrates, at 15.3 h. KD2 was the first to show growth at 8.1 h and had a midpoint position growth average at 12.1 h. PrC2 and KD1 had very similar patterns throughout the entire incubation period, with the midpoint positional growth average at 14.9 and 17.6 h, respectively. The PC group had a positional growth average at roughly 15.8 h, and PrC1 had an irregular pattern, with a late start at 10.8 h and the latest midpoint growth average was at 24.4 h of incubation. Most treatments had a median position growth of about 12 to 17 h incubation, but PrC1 had a completely different midpoint of growth at 24.4 h. The explosiveness of growth had no unit and was expressed with numbers that suggested a measured statistical. PC had a limited variety of explosiveness of growth, with an average of 5.6, whereas KD1 had the most variety in explosiveness of growth with an average of 6.5. The microbial communities in the NC, PrC1, and PrC2 groups also had more variety in explosiveness of growth with average values of 6.0, 5.3, and 5.9, respectively. KD2 had the least variety of explosiveness, with an average of 4.8, which means that in the cases where growth was present, it was mostly gradual growth and no sudden or explosive growth. Unfortunately, there were no statistically significant differences between the treatments in this analysis. The differences between treatments seem subtle and may be different if the experiment were repeated.

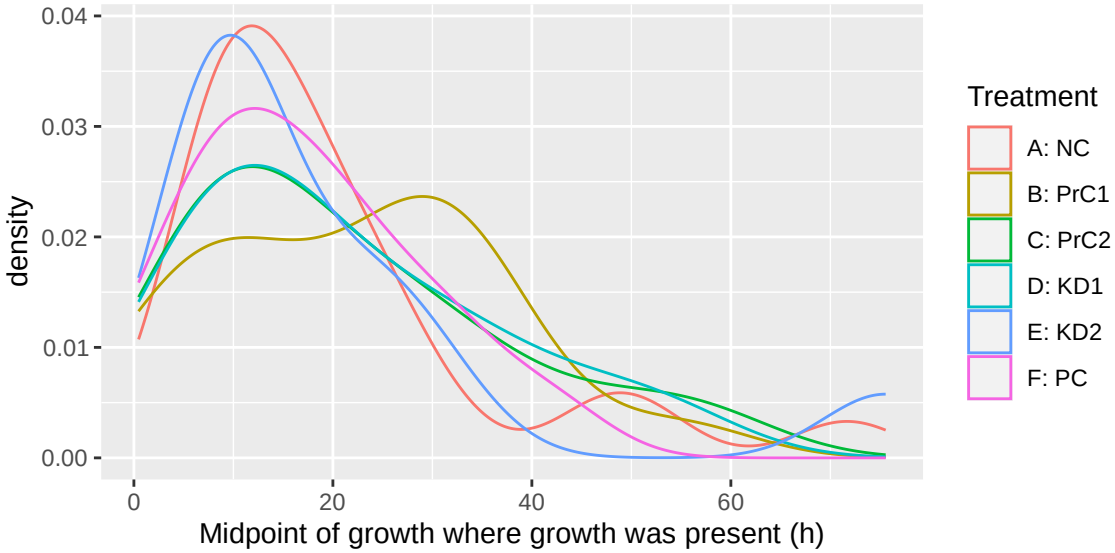

Figure 4.14. Gompertz position of growth parameter (b), of six Boerewors treatments, summarised across all substrates (72.5 h). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

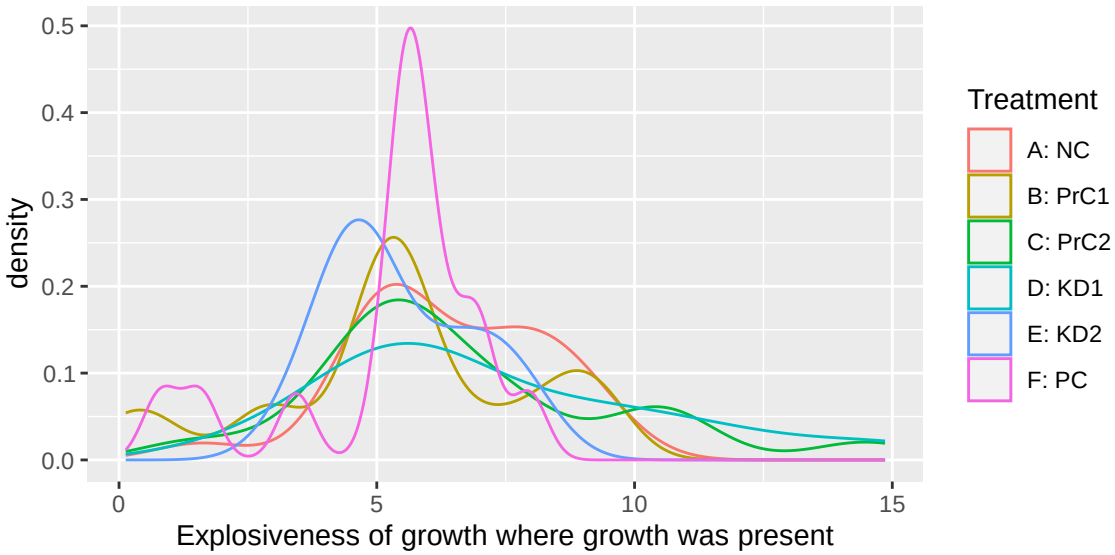

Figure 4.15. Gompertz explosiveness of the growth parameter (c), of six Boerewors treatments, summarised across all substrates. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

The heatmap of the rough position of growth of the Gompertz curve (Figure 4.16), showed which substrates, in each treatment, had no CD, early growth, middle growth, late growth, and end growth. Two substrates immediately stood out, with end growth in certain treatments: β-methyl-D-glucoside in NC, and D-galacturonic acid in KD2. The dendrogram above the guilds also showed that, across all treatment groups, some substrates showed 1) middle to end growth, while others showed 2) early to middle growth, and a lot of substrates had no CD, to early CD. The first group consisted of D-galacturonic acid, D-malic acid, D-galactonic acid $_{\gamma}$-lactone, itaconic acid, and L-arginine. The second group consisted of 4-hydroxy benzoic acid, L-phenylalanine, L-asparagine, D-mannitol, D-glucosaminic acid, and putrescine. The rest of the substrates had no growth or very early growth, with the exclusion of a few in the NC and PrC2 groups.

The different reactions from the microbial communities in each Boerewors treatment showed that the mode of action or contents of each preservative can affect the substrate utilization. For example, PrC1 and PrC2 had sucrose in their culture mixture to facilitate the growth of the cultures (www.chr-hansen.com). The protective cultures also have the ability to inhibit other bacteria by competitive exclusion, by binding to substrates and using the available nutrients (Engstrom, Anderson, & Glass, 2021), which could also attribute to the variety in the growth position and explosiveness of these treatment groups. The plant extracts groups also showed variety in growth position and explosiveness. Some bacteria were more severely affected by the bactericidal effects of the carnosic and rosmarinic acid in the preservatives, by altering the transport of electrons and damaging the functionality and structure of the cell membranes of the bacteria (Nieto, Ros, & Castillo, 2018), which changed the microbial community and therefore, the type of substrates used by the microorganisms still growing in the Boerewors.

The heatmap of explosiveness of growth of the Gompertz curve (Figure 4.17), indicated which substrates, in each treatment group, had no CD, gradual growth, moderate growth, sudden growth, and explosive growth. In this case, there were also two substrates that stood out, showing explosive microbial growth: N-acetyl-D-glucosamine in PrC2, and D-galactonic acid $_{\gamma}$-lactone in KD1. In the NC and KD1 treatment groups, D-malic acid, pyruvic acid methyl ester, and D-galactonic acid $_{\gamma}$-lactone had moderate to explosive growth. The dendrogram also showed that across all Boerewors treatments, D-cellobiose, putrescine, D-mannitol, itaconic acid, D-galacturonic acid, D-glucosaminic acid, Tween 40, $_{\gamma}$-amino butyric acid, L-arginine, and Tween 80 had the most growth, ranging from gradual to sudden. The rest of the substrates varied in growth, but most had no CD. Both of these heatmaps showed many cases of no CD,

which would probably look different if the experiment was repeated more than once. Even though not significant, these results indicated that, in terms of substrates used, the microbial community in the KD1 Boerewors had preferences and growth patterns more similar to the microbial communities in NC, which contradicts the findings in the raw data analysis which used the AWCD and Shannon indices.

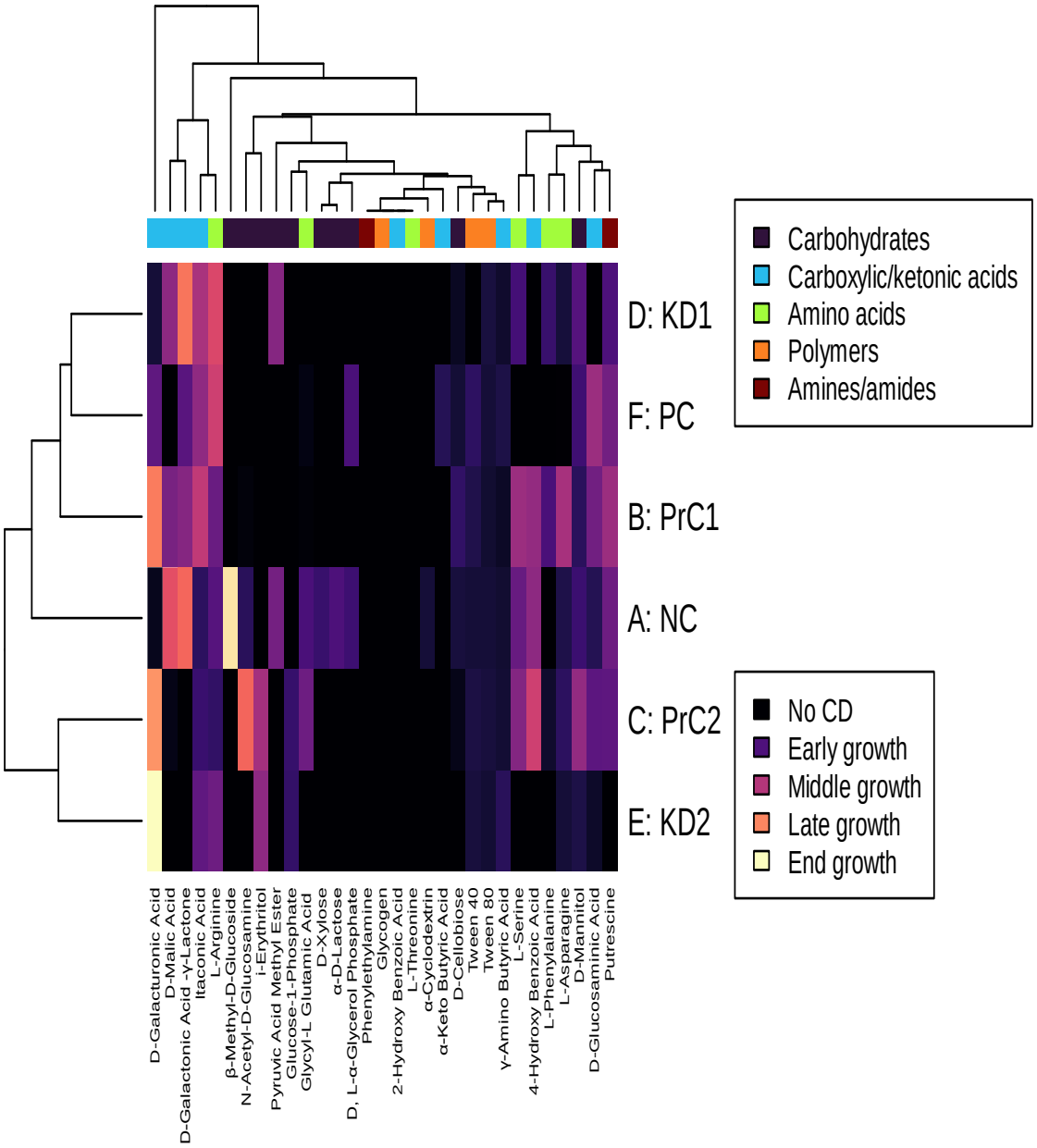

Figure 4.16. Heatmap of the rough position of growth (b) parameter of the Gompertz curve, from Boerewors samples with different treatments. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

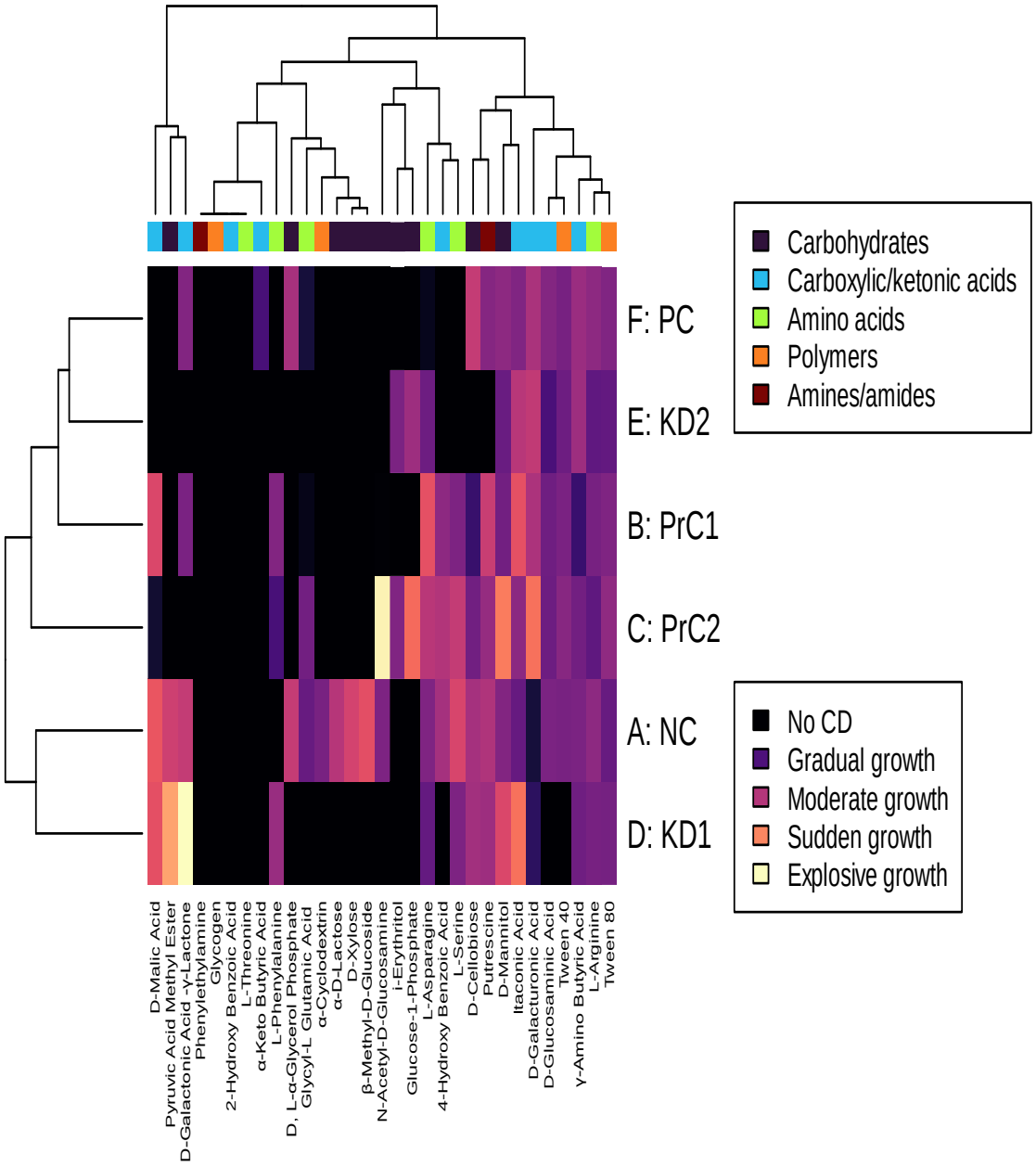

Figure 4.17. Heatmap of the explosiveness of growth (c) parameter of the Gompertz curve, from Boerewors samples with different treatments. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

4.4 Conclusions

The microbial communities in Boerewors treated with different preservatives were successfully analysed by CLPP using Biolog™ EcoPlate™. Using the Shannon indices, the results showed that the microbial communities in each Boerewors sample metabolized the substrates at different rates, with NC mostly having the biggest microbial community and highest diversity. The heatmaps and PCA biplot corroborated these findings. The microbial communities in all of the Boerewors treatments, and both data sets (AWCD + Shannon indices and Gompertz), utilized 2-hydroxybenzoic acid the least, and L–asparagine was metabolized at the highest rate.

The results in this chapter suggested that all the preservatives evaluated in this study, restricted microbial growth, and diversity to some point. Although, the use of protective cultures affected the results and the higher growth rates could be due to the lactic acid bacteria, and not spoilage or pathogenic bacteria. The parameters used may have not accurately shown the positive effects of the protective cultures and, therefore, could not be fairly compared as potential replacers for SO_2. The plant extracts, especially KD1, which consisted of a rosemary and acerola extract blend, had the lowest growth rate, diversity, and richness in the original data showing the best potential as an alternative preservative to SO_2 in Boerewors.

The Gompertz curve analysis showed no statistically significant differences, however, the microbial communities had subtle changes in the Boerewors treatments, over time, in terms of position of growth and explosiveness of growth, across all substrates. KD2 was the first treatment to show microbial growth, and PrC1 showed more late-stage growth. If Boerewors had a shelf-life of 24 h, the PrC1 would have been a strong candidate as a potential replacement for SO_2, as it only had a midpoint of growth after 24 h of incubation. Even though KD2 was the first to grow, this treatment had the least explosive growth, with an average of 4.8, while KD1 had the most explosive growth, with 6.5. Explosiveness of growth would have a bigger impact on the quality of the Boerewors, as most treatment groups showed a midpoint of growth within 48 h, which means, in this case, KD2 would be the best option as a SO_2 replacer in Boerewors.

Both plant extracts, KD1 and KD2, showed promising results in different aspects of analysing the microbial communities in Boerewors. Other methods or new protocols should be used when products with added protective cultures are compared to products without added cultures, to have accurate results on their preservation abilities. These results validated the microbial quality findings in Chapter 3.

This was the first study where data from the Biolog™ Ecoplate™ was used to do CLPP on Boerewors to determine whether the chemical preservative in Boerewors, SO_2, can be successfully replaced by natural preservatives. The data generated by the Biolog™ Ecoplate™ system can in future be used to evaluate the effectiveness of natural preservatives in fresh meat products such as Boerewors. Both of these statistical methods, analysing the raw data with AWCD and Shannon indices, and implementing the Gompertz curve to show other growth parameters, were useful. However, using the AWCD and Shannon indices gave the option to compare the results with the findings of other studies, while using all of the data points. The Gompertz curve data only used three data points, which impacted the overall results, but the parameters showed other sides of the microbial communities in the Boerewors. The Gompertz data would be a better method if the experiment was repeated more than once, but in the case of this study, it seemed that the first method was more useful.

CHAPTER 5

THE GROWTH AND SURVIVAL OF *Escherichia coli* AND *Staphylococcus aureus* IN BOEREWORS MANUFACTURED WITH NATURAL PRESERVATIVES AS SULPHUR DIOXIDE REPLACERS

ABSTRACT

In this study, SO2 replacement with plant extract preservatives containing rosemary and acerola extract (KD1) and rosemary and acerola extract with buffered vinegar (KD2), were evaluated for effects on the growth and survival of Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) reference strains. The bacterial strains were inoculated separately into Boerewors batters that were stored at 4 °C for up to 6 days. The purpose of this study was to determine if these potential pathogens could use natural preservatives to their advantage in Boerewors. The inoculated E. coli struggled to survive both of the natural preservatives, especially KD1, without a significant difference to the PC. Survival decreased substantially from day 0 to 6, with the low storage temperature having a big influence on the counts. The inoculated S. aureus decreased from day 0 to 6, but the natural preservatives did not affect the counts as much as the SO2, except on the last day of storage. For the most part, natural preservatives and an ideal storage temperature exhibited antimicrobial potential.

Keywords: Escherichia coli, Staphylococcus aureus, growth and survival, SO2 replacement, plant extracts, Boerewors

5.1 Introduction

Fresh meat and processed meat products are highly susceptible to spoilage and exploitation of pathogens. Meat spoilage is almost always caused by microbial growth because microorganisms prefer foods that are moist, high in protein and low in acid (Iulietto et al., 2015). Ready-to-eat products and fresh meat products for international trade have become a major part of the modern diet, and these products are required to be high quality and meet all food safety standards (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Many studies have focused on methods to prevent undesirable changes during the storage of meat products; however, some spoilage and pathogenic bacteria are persistent (Iulietto et al., 2015).

Pathogens that dominate in raw meat and processed meat products, include *Escherichia coli* (*E. coli*), *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus* (*S. aureus*) (Li, Sun, Liao, & Gänzle, 2020). Meat can easily get contaminated with *S. aureus* during handling and processing. *Staphylococcus aureus* is also halophilic, which means that it can survive and grow in an environment with more than 5% sodium chloride levels. This is a major reason why the contamination of *S. aureus*, in fresh processed meat, cannot be eradicated (Feng, Ming, Zhou, Lu, Wang, & Su, 2022). *Escherichia coli* strains, including the O175:H7 strain, have been found in minced meat products. It has been the cause of many foodborne illness outbreaks around the world, and some cases have resulted in serious illness and death, especially young children. Therefore, meat should be cooked thoroughly to destroy the pathogen (Kornacki, & Marth, 1982). Some preservation methods, like irradiation and adding organic acids, have also been used to control *E. coli* in food products (Puligundla, & Lim, 2022).

Even though current preservation methods are quite effective, some of them can have intrinsic disadvantages, such as toxicity and altering sensory properties. Biopreservation and plant-derived products have gained attention and are rapidly emerging as effective, safe for human consumption, and environmentally friendly alternatives (Puligundla, & Lim, 2022). When fresh meat is well-packaged and stored at a refrigeration temperature of 4 °C, it can have a good shelf-life of 3 to 5 days. Adding effective preservatives can extend this shelf-life and inhibit the growth of pathogens past this storage period (Nethra, Sunooj, Aaliya, Navaf, Akhila, Sudheesh, Mir, Shijin, & George, 2023). Many studies have claimed that *E. coli* and *S. aureus* are particularly resistant, which highlights the importance of preservation and antimicrobial efficacy (Tornuk, Cankurt, Ozturk, Sagdic, Bayram, & Yetim, 2011; Teixeira, Marques, Ramos, Neng, Nogueira, Saraiva, & Nunes, 2013; Nieto, Ros, & Castillo, 2018).

The purpose of this part of the project was to investigate the growth and survival of two reference bacteria strains, over 6 days, at 4 °C: *E. coli* as an example of Gram-negative bacteria, and *S. aureus* as an example of Gram-positive bacteria in closely simulated fresh sausage models formulated with natural preservatives as SO_2 replacement. Specific aims for this chapter included:

1. Determining if a replacement of SO_2 affected microbial survivability and growth of these two microorganisms; and
2. Determining whether a Gram-negative or Gram-positive bacterial strain proved to be more opportunistic at a replaced SO_2 content.

5.2 Materials and methods

5.2.1 Bacterial strains and treatment selection

The two bacterial control strains used in this study were *E. coli* and *S. aureus*, which were representative of a Gram-negative and a Gram-positive organism, respectively, and both are well-known food spoilers and pathogens. Specific reference strains of the above-mentioned bacteria, with low pathogenicity, were chosen, as a result of safety considerations. *Escherichia coli* ATCC 8739^{TM} (Microbiologics 0483E3) and *Staphylococcus aureus* ATCC 25923^{TM} (Microbiologics 03603E3) were obtained from ThermoFischer (Johannesburg, South Africa). The respective microorganism's pellets, contained 4.1 x 10^3 cfu/pellet (*E. coli*) and 8.7 x 10^3 cfu/pellet (*S. aureus*),

Four of the six treatments were identified to be used in this inoculation study: treatments 1 (NC), 4 (KD1), 5 (KD2) and 6 (PC) – see Chapter 3. The protective culture treatments, PrC1 and PrC2, were excluded because their major mode of action is targeted against *Listeria*, which was not used in this experiment.

5.2.2 Sourcing of lean meat, fat, additives, and spices

The sourcing of the lean meat, fat, additives, and spices were performed, as described in Chapter 3 section 3.2.2.

5.2.3 Formulation of Boerewors

The formulation of the Boerewors models was according to Chapter 3 section 3.2.3.

5.2.4 Manufacturing of Boerewors

The manufacturing of the Boerewors models was performed, according to Chapter 3 section 3.2.4.

5.2.5 Sample preparation for spiking

The left-over Boerewors (Chapter 3) batters of each replicate that were not stuffed in casings, were used for this chapter. For each treatment, eight x 99 g batter of Boerewors were prepared and kept frozen at -18°C, until needed.

Four x 99 g batter samples of each treatment were spiked with *E. coli* and sampled on days 0, 3, and 6. Four x 99 g batter bags were spiked with *S. aureus* and sampled on days 0, 3, and 6. All the spiked batters were microbiologically analysed on the three sampling days. This process was repeated for the four treatments per replicate and there were three replicates in this study.

5.2.6 Inoculation and incubation of the Boerewors batters

The concentration of the two strains, *S. aureus* (ATCC 25923™) with 7.0 x 10^3 cfu (log 3.85 cfu) per pellet and *E. coli* (ATCC 25922™) with 4.1 x 10^3 cfu (3.61 log cfu) per pellet, were tested and confirmed to contain these numbers, before inoculating the Boerewors batters. Approximately 24 h before the spiking of the batters, the frozen batter samples were defrosted at 4 °C and allowed to adjust. The inoculum was prepared by heating 2 x 9 ml phosphate buffers to 37 °C, using an incubator, after which two reference strain pellets of *S. aureus* (ATCC 25923™) were aseptically added to one heated phosphate buffer, and one reference strain pellet of *E. coli* (ATCC 25922™), was aseptically added to the other heated phosphate buffer, and incubated at 37 °C for 1 hour, to allow the pellets to suspend completely. In a laminar flow cabinet, a Whirl-Pak™ bag (Lasec, Bloemfontein, South Africa), containing the 99 g Boerewors batter, was opened and 1 ml of the suspended reference strain was added. This resulted in a 100-x dilution of both the *E. coli* and *S. aureus* counts. The bag was then flattened to remove the air and closed by folding the opening three times. The batter and the strain suspension were mixed thoroughly by using the hand mixing procedure (Figure 5.1) and stored momentarily at 4 °C, until all the samples were spiked and mixed. The day 0 samples were analysed within 30 minutes of inoculation. The samples for days 3 and 6 were placed in a low temperature incubator (Labcon; Vacuum Technologies, Johannesburg, South Africa) at 4 °C to mimic the storage temperature used for fresh Boerewors.

5.2.7 Hand mixing procedure

The 99 g batter portions were hand mixed, using the procedure as suggested by Cluff (2016) and as illustrated in Figure 5.1. Before mixing, the wire tabs were opened, and the bag was rolled open. On a flat countertop, with one hand open and firmly placed on the empty part of the bag (iv), the index, middle and ring fingers of the other hand was placed on top of the bag and used to flatten and spread the batter sample (ii) in a circular motion, for one minute. After the first 30 seconds and again after 1 minute, the batter was firmly pushed toward the bottom of the bag (i) from where it had occasionally spread to a larger area (iii). The batter sample

was not allowed to spread to the empty part of the bag (iv) as this would have limited the amount of sample that could be mixed at a time.

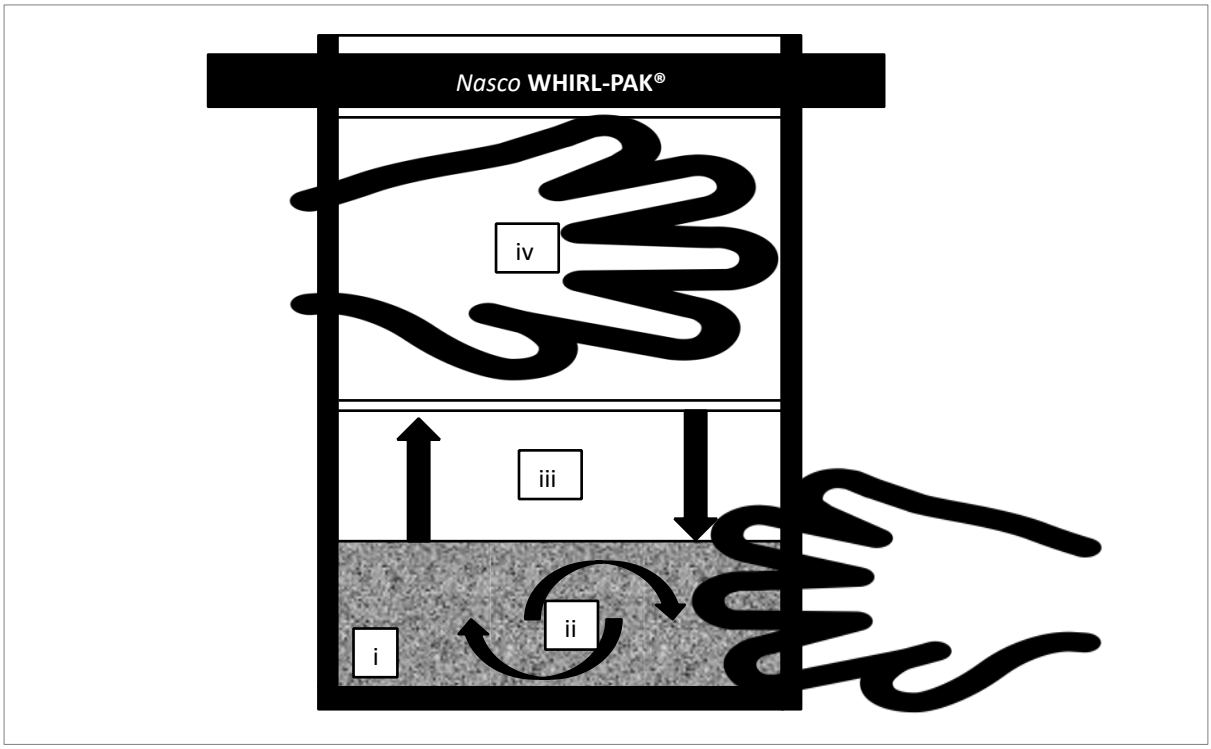

Figure 5.1. Illustration of the hand mixing procedure used to overcome the difficulty in mixing the 99 g batter samples (Cluff, 2016).

5.2.8 Microbial analyses

On days 0, 3 and 6 of storage at 4 °C, a 10 g sample of each treatment were aseptically weighed into a sterile 207 ml WhirlPak™ bag (Lasec, Bloemfontein, South Africa). Ninety millilitres of sterile 0.1 M buffered peptone water (BPW) solution were added to create a 10^{-1} dilution which was actually a further 10-x dilution of the original culture of both the *E. coli* and *S. aureus* cultures. It was stomached (AME Stomacher Lab-Blender 400, Johannesburg) for 1 min. A 1 ml volume of each 10^{-1} dilution was pour-plated in duplicate using Brilliance™ *E. coli*/coliform selective medium (Oxoid CM1046) for *E. coli* determination in the Boerewors samples inoculated with *E. coli*, and Baird-Parker agar (BP; Oxoid CM025) containing egg yolk tellurite emulsion (Oxoid SR054C) for *S. aureus* determination in the Boerewors samples inoculated with *S. aureus*.

5.2.9 Statistical analyses

Statistical analysis was performed to uncover key insights and differences between the different treatments and days 0, 3, and 6 of storage at 4 ˚C. The primary statistical technique, Analysis of Variance (ANOVA) was used. The level of significance was set at $\alpha = 0.05$. Means and standard deviations were calculated for each treatment, day, and treatment/day combination. Before applying the ANOVA test, the data had to meet the underlying assumptions of this test. The ANOVA test was employed to determine whether there were any statistically significant differences between the means of three or more independent groups. Post-hoc tests (Tukey HSD) were conducted in cases where ANOVA resulted in a significant effect, to determine which specific groups differed from each other.

5.3 Results and discussion

5.3.1 The growth and survival of *E. coli* in inoculated batters

Regardless of treatment, storing the inoculated Boerewors at 4 °C for 6 days, rapidly reduced the *E. coli* count (Table 5.1). The average *E. coli* counts across all treatments started at 0.828 ± 0.139 log cfu/g on day 0, reduced to 0.306 ± 0.285 log cfu/g on day 3 and was significantly ($p < 0.0001$) lower on day 6 with only 0.108 ± 0.182 log cfu/g. Most *E. coli* strains are harmless; however, some strains cause severe foodborne illness, which includes the Shiga toxin-producing *E. coli* (STEC). The STEC can grow at temperatures ranging from 7 °C to 50 °C, with an optimum of 37 °C (WHO, 2018). Thus, storing the Boerewors at 4 °C inhibited the growth of *E. coli*, and greatly impaired the survival thereof. Other additives used in the formulation of Boerewors, like salt (NaCl), could also be the reason for the rapid reduction in counts; generally, *E. coli* grows optimally at 0.5% NaCl concentration, however, levels higher than 0.5 % NaCl decreases the growth, and eventually the survivability, of *E. coli* in food (Zhang, Nakaura, Zhu, Zhang, & Yamamoto, 2020). The inclusion level in the Boerewors formulations were 1.5% NaCl. The a_w of all the Boerewors treatments (Chapter 3), were lower than the minimal a_w level of 0.95 for *E. coli* growth (WHO, 2018), which could also have affected the results of this experiment. The pH of the Boerewors would not have had a notable effect on the growth of *E. coli*, because it has a wide growth range of 4.5 to 9 (Wilks, & Slonczewski, 2007).

Table 5.1. Analysis of variance of the effects of storage time on the survival of *E. coli* in Boerewors batters, stored at 4 °C. Means with different superscripts differed significantly.

	Day 0	**Day 3**	**Day 6**	**Sign. Level (n = 8)**
E. coli **(log cfu/g)**	$0.828^{a} \pm 0.139$	$0.306^{a} \pm 0.285$	$0.108^{b} \pm 0.182$	$p < 0.0001$

The effect of preservative treatment on the growth and survival of *E. coli* over a 6-day storage time at 4 °C, is indicated in Table 5.2 and Figure 5.2. The NC (0.609 ± 0.348 log cfu/g) had a higher average count, over the six-day storage period, than the other treatments and differed significantly ($p = 0.041$) from the PC (0.260 ± 0.360 log cfu/g). This result confirmed that SO_2 had the best preservative effect on the growth and survival of the *E. coli* over a mean storage time of 6 days. The two plant extracts, KD1 and KD2 also inhibited *E. coli* growth, but it was not as significant as SO_2. Even though not significant, another observation was made; KD1, which contained a blend of rosemary and acerola extracts, was slightly better at inhibiting *E. coli* than KD2, which contained buffered vinegar with the rosemary and acerola extract blend.

The effect of preservative treatment on the growth and survival of *E. coli* were evaluated on the individual storage days 0, 3 and 6. In Table 5.3 and Figure 5.3, on day 0, the *E. coli* count of the NC after a 1000-x dilution (0.933 log cfu/g) correlated to the initial number of inoculation of the culture (3.61 log cfu/g). The *E. coli* counts of the KD1 (0.782 ± 0.092 log cfu/g) and KD2 (0.866 ± 0.099 log cfu/g) did not differ significantly from the NC and PC control treatments, however, NC (0.933 ± 0.124 log cfu/g) had a significantly ($p = 0.041$) higher *E. coli* count than PC (0.731 ± 0.160 log cfu/g). According to Meat Industry Services (2006), SO_2 slows the growth of bacteria commonly found in meat and meat products, by increasing their lag phase, which is the period before they start to grow in a new environment. On day 3, NC had significantly ($p < 0.0001$) higher counts than the rest of the treatments, with 0.665 ± 0.148 log cfu/g. The plant extract treatments, KD1 and KD2, did not differ from the PC, which indicated that the plant extracts had equal effectiveness to SO_2 to control the growth of *E. coli* during a 3-day storage time. There was no significant difference ($p = 0.119$) between the treatments on day 6; as the *E. coli* numbers declined over the storage period, the *E. coli* counts were too low to show a significant difference.

In the Boerewors inoculated with *E. coli*, there were no significant interactions between day and treatment. The repetitions were also analysed, but were not significant at all, which shows that the repetitions were independent and had no random effect on the data.

Table 5.2. Analysis of variance of the effects of the preservative treatments, on the survival of *E. coli* (log cfu/g) in Boerewors, over a storage time of 6 days at 4 °C. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same row differed significantly.

	NC	KD1	KD2	PC	Sign. Level (n = 8)
E. coli **(log cfu/g)**	$0.609^{a} \pm 0.348$	$0.337^{ab} \pm 0.357$	$0.449^{ab} \pm 0.348$	$0.260^{b} \pm 0.360$	p = 0.041

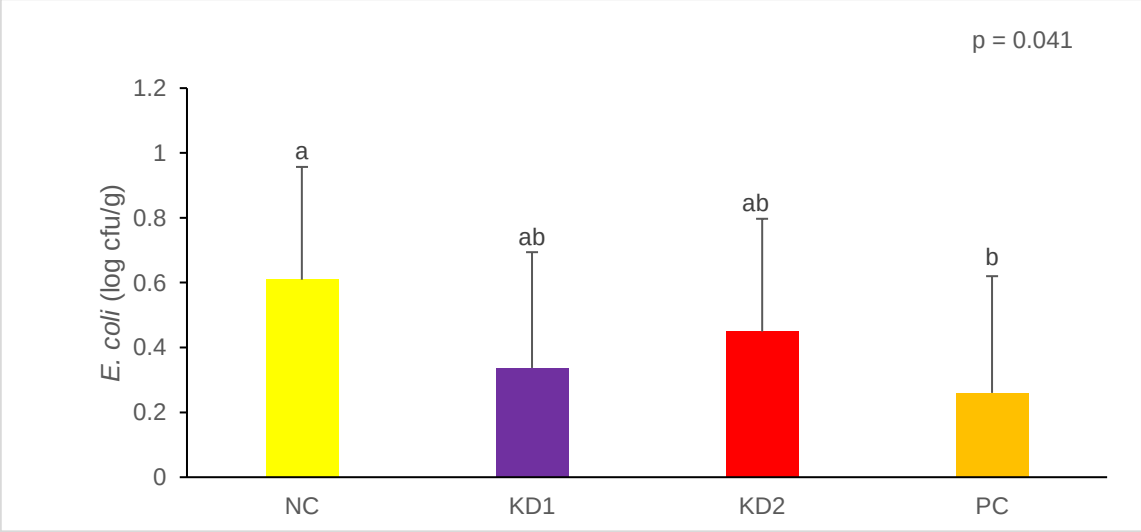

Figure 5.2. The effects of the preservative treatments, on the survival of *E. coli* (log cfu/g) in Boerewors, over a storage time of 6 days at 4 °C (n = 8).NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Treatment means with different superscripts differed significantly.

Table 5.3. Analysis of variance of the effects of the preservative treatments, on the survival of *E. coli* (log cfu/g) in Boerewors, on days 0, 3 and 6 at 4 °C. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same row differed significantly.

	NC (log cfu/g)	**KD1 (log cfu/g)**	**KD2 (log cfu/g)**	**PC (log cfu/g)**	**Sign. level (n = 8)**
Day 0	$0.933^{a} \pm 0.124$	$0.782^{ab} \pm 0.092$	$0.866^{ab} \pm 0.099$	$0.731^{b} \pm 0.160$	$p = 0.045$
Day 3	$0.665^{a} \pm 0.148$	$0.180^{b} \pm 0.207$	$0.330^{b} \pm 0.204$	$0.05^{b} \pm 0.123$	$p < 0.0001$
Day 6	0.23 ± 0.27	0.05 ± 0.123	0.151 ± 0.165	0 ± 0	NS ($p = 0.119$)

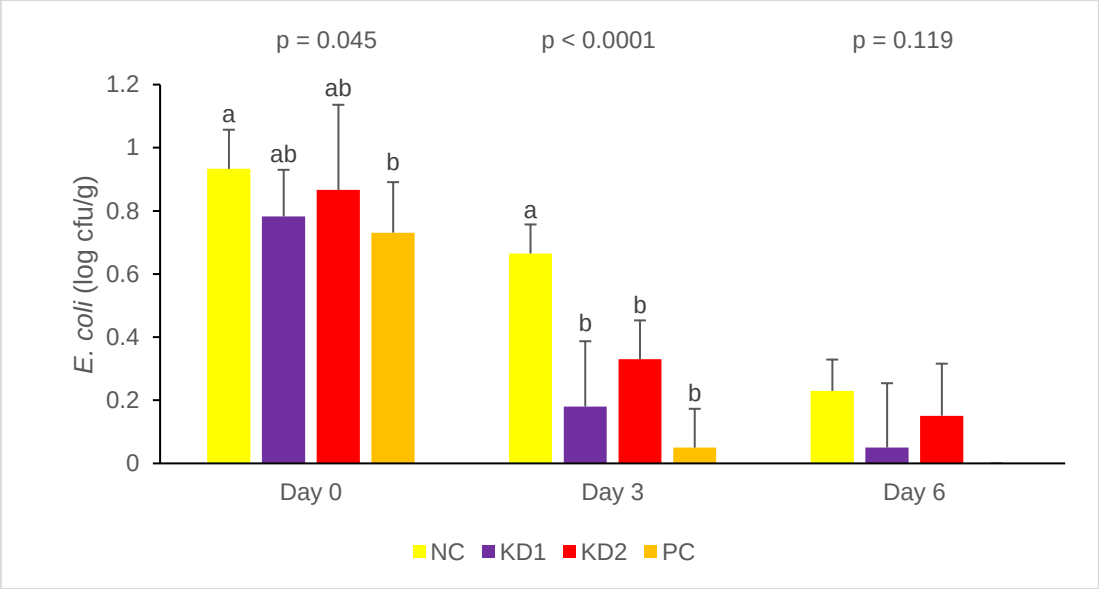

Figure 5.3. The effects of the preservative treatments, on the survival of *E. coli* (log cfu/g) in Boerewors, on days 0, 3, and 6 of storage at 4 °C (n = 8). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020).

5.3.2 Growth and survival of *S. aureus* in inoculated batters

The average *S. aureus* counts across all Boerewors treatments declined significantly ($p < 0.0001$) from days 0 to 6 of the shelf-life at 4 °C (Table 5.4). *Staphylococcus aureus* has a minimum growth temperature of 7 °C (Hennekinne, De Buyser, & Dragacci, 2012), which is evident in the results of this experiment; the counts declined rapidly from 0.952 ± 0.253 log cfu/g on day 0 to 0.692 ± 0.387 log cfu/g on day 3, and a significantly ($p < 0.0001$) lower count on day 6 (0.322 ± 0.335 log cfu/g).

Considering the average counts of all treatments, *S. aureus* counts (Table 5.4) were higher than the *E. coli* counts (Table 5.1) at every time interval because the Boerewors were inoculated with a higher number of *S. aureus* culture (3.85 log cfu/g).

Table 5.4. Analysis of variance of the effects of storage time on the survival of *S. aureus* (log cfu/g) in Boerewors batters, stored at 4 °C. Means with different superscripts in the same row differed significantly.

	Day 0	Day 3	Day 6	Sign. level (n = 8)
S. aureus **(log cfu/g)**	0.952^{a} ± 0.253	0.692^{a} ± 0.387	0.322^{b} ± 0.335	$p < 0.0001$

The treatment means differed significantly ($p < 0.0001$) in terms of the growth and survival of *S. aureus*, stored at 4 °C, over a 6-day shelf-life (Table 5.5 and Figure 5.4). The NC group had the highest count (1.084 ± 0.231 log cfu/g), whereas the two plant extract treatments, KD1 (0.65 ± 0.267 log cfu/g) and KD2 (0.592 ± 0.348 log cfu/g), were significantly lower ($p < 0.0001$) than NC (1.084 ± 0.231 log cfu/g) but significantly ($p < 0.0001$) higher than PC (0.295 ± 0.377 log cfu/g). This was an indication that SO_2 was the best inhibitor of growth of *S. aureus* in Boerewors during a storage time of 6 days, followed by both plant extracts mixtures.

When the effect of storage day on the growth and survival of *S. aureus* was evaluated, on day 0, NC had a significantly higher ($p < 0.0001$) count than the rest of the treatments, which was expected due to no preservative being present. Treatment groups KD1 and KD2 did not differ significantly from PC, which showed that adding any of the two natural preservatives, immediately inhibited the growth of *S. aureus*. According to Gonelimali, Lin, Miao, Xuan, Charles, Chen, & Hatab (2018), plant extracts have a remarkable effect on Gram-positive bacteria, such as *S. aureus*, by disrupting the potential in the membrane, which changes the cytoplasmic pH of the microorganism.

On day 3, the NC group continued to have higher ($p < 0.0001$) *S. aureus* counts (1.134 ± 0.086 log cfu/g), however, PC had significantly ($p < 0.0001$) lower counts than the KD1 (0.784

± 0.081 log cfu/g) and KD2 (0.720 ± 0.083 log cfu/g) treatments as well. On the last day, NC had a significantly higher ($p < 0.0001$) count (0.808 ± 0.071 log cfu/g) than the other treatments, and KD1 (0.330 ± 0.204 log cfu/g) had a significantly ($p < 0.0001$) higher *S. aureus* count than PC (0 ± 0 log cfu/g), but PC did not differ significantly from KD2 (0.151 ± 0.165 log cfu/g). These results were an indication that, over a 3-day storage time at 4 °C, SO_2 would still be the best preservative to control *S. aureus* growth and survival. However, over a 6-day storage time at 4 °C, SO_2 and KD2 would give similar preservative effect for *S. aureus* growth and survival. Several studies have shown that extracts from plants may be active against *S. aureus strains*, including methicillin-resistant *S. aureus* (MRSA) (Martin & Ernst, 2003; Yu, Liu, Liu, Qin, Jin, & Wang, 2019).

According to Del Campo, Amiot, & Nguyen-The (2000), rosemary extract had a greater inhibitory effect on Gram-positive bacteria, like *S. aureus*, than Gram-negative bacteria. Some studies suggested that the cell wall of Gram-negative bacteria, like *E. coli*, are more resistant to the effects of plant extracts than Gram-positive bacteria (Stojiljkovic, Trajchev, Nakov, & Petrovska, 2018). However, in this study, the plant extract treatments inhibited the growth of *E. coli* better than that of *S. aureus*. *Staphylococcus aureus* is a halophilic bacterium; it can grow at NaCl concentrations of 5% and synthesize compounds that can help it survive in salty environments, such as processed meat products (Nostro, Ninham, Nostro, Pesavento, Fratoni, & Baglioni, 2005).

No interactions between day and treatment, in Boerewors inoculated with *S. aureus*, were significant. The repetitions were also analysed, but were not significant at all, which shows that the repetitions were independent and had no random effect on the data.

Table 5.5. Analysis of variance of the effects of the preservative treatments, on the survival of *S. aureus* (log cfu/g) in Boerewors over a storage time of 6 days at 4 °C. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same row differed significantly.

	NC	KD1	KD2	PC	Sign. level (n = 8)
S. aureus **(log cfu/g)**	$1.084^{a} \pm 0.231$	$0.65^{b} \pm 0.267$	$0.592^{b} \pm 0.348$	$0.295^{c} \pm 0.377$	$p < 0.0001$

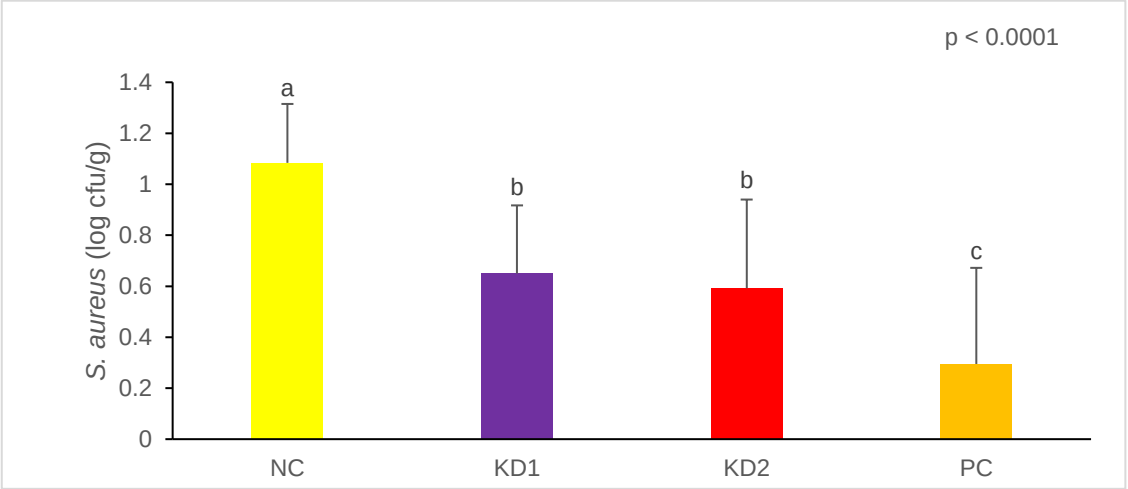

Figure 5.4. The effects of the preservative treatments, on the survival of *S. aureus* (log cfu/g) in Boerewors, over a storage time of 6 days at 4 °C (n = 8). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Treatment means with different superscripts differed significantly.

Table 5.6. Analysis of variance of the effects of the preservative treatments, on the survival of *S. aureus* (log cfu/g) in Boerewors, on days 0, 3, and 6 at 4 °C. NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Means with different superscripts in the same row differed significantly.

	NC	KD1	KD2	PC	Sign. Level (n = 8)
Day 0	$1.311^{a} \pm 0.114$	$0.836^{b} \pm 0.098$	$0.906^{b} \pm 0.082$	$0.756^{b} \pm 0.215$	$p < 0.0001$
Day 3	$1.134^{a} \pm 0.086$	$0.784^{b} \pm 0.081$	$0.720^{b} \pm 0.083$	$0.130^{c} \pm 0.208$	$p < 0.0001$
Day 6	$0.808^{a} \pm 0.071$	$0.330^{b} \pm 0.204$	$0.151^{bc} \pm 0.165$	$0^{c} \pm 0$	$p < 0.0001$

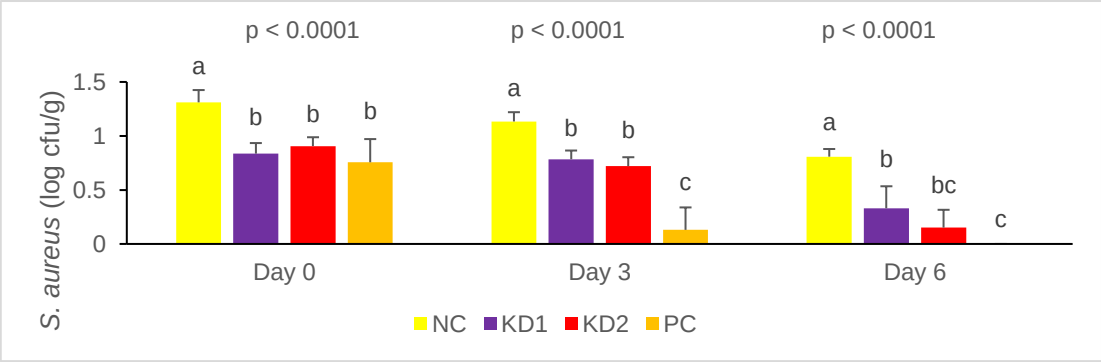

Figure 5.5. The effects of the preservative treatments, on the survival of *S. aureus* (log cfu/g) in Boerewors, on days 0, 3, and 6 of storage at 4 °C (n = 8). NC = negative control with no added preservative; PrC1 = treatment with protective culture 1 (0.025% BLC 20); PrC2 = treatment with protective culture 2 (0.0125% BLC 48); KD1 = treatment with Kalsec Duralox plant extract 1 (0.35% NR20); KD2 = treatment with Kalsec Duralox plant extract 2 (0.5% NV3 + 0.05% RD30); PC = positive control containing the SO_2 inclusion level recommended for Boerewors (0.0682% $Na_2S_2O_5$; DoH of South Africa, 2020). Treatment means with different superscripts differed significantly.

5.4 Conclusions

The impact of SO_2 replacement on the growth and survival of *E. coli* and *S. aureus* reference strains in Boerewors, were investigated. In the Boerewors inoculated with *E. coli*, the two plant extracts evaluated in this study, KD1 and KD2 had an equal preservative effect on *E. coli* than the PC containing SO_2, throughout the entire shelf-life.

However, in the Boerewors inoculated with *S. aureus*, the PC with SO_2 better controlled the growth and survival of *S. aureus* during a 6-day storage at 4 °C than the plant extract treatments (KD1 and KD2).

The storage temperature of 4 °C contributed to the inhibition of both pathogens, while the NaCl content in the Boerewors formulations only affected the growth of *E. coli*.

Considering the above-mentioned conclusions, with the fact that the *S. aureus* counts were higher than the *E. coli* counts throughout the 6-day shelf-life, the Gram-positive bacteria proved to be more opportunistic in SO_2 replaced Boerewors. KD1 and KD2 could possibly replace SO_2 in Boerewors, as a food safety preservative, if only *E. coli*, and not *S. aureus*, was a concern.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Consumers demanding healthier and natural ingredients in the food they eat, have been the driving force behind the vast number of commercially available natural preservatives. These natural preservatives, however, first need to be evaluated in the product where a chemical preservative (SO_2) needs to be replaced. This will ensure that these products with the natural preservatives will still be in demand after replacement because no compromise should be made when it comes to food safety, food waste by spoilage and consumer acceptability.

Chapter 2 is a review on the literature that was available on the topic of SO_2 and natural preservatives. The problems of SO_2 were discussed with regard to the health effects and the consumer demands for the exclusion of chemical preservatives. The functions of SO_2 in meat products were discussed, as well as identifying the crucial areas, where challenges might come to light, due to the replacement of SO_2. The information on natural preservatives, and the effects thereof on food products and consumer acceptability, have been obtained from previous research on the topic. It was established that there is a shortage of information on the replacement of SO_2, and the use of natural preservatives in Boerewors, and it needed to be investigated.

Two protective cultures and two plant rosemary extract blends were selected as the natural preservatives in this study. The protective cultures, BLC-20 (PrC1) and BLC-48 (PrC2) were from the Chr. Hansen company that uses sustainable microbial solutions for better food and healthier living (Chr. Hansen, 2023). The rosemary extract blends, KD1 and KD2, were from Kalsec's Duralox range, which offers a wide range of natural food and beverage ingredients that provide protection (Kalsec, 2023).

In this study, Boerewors with six different preservative treatments, NC, PrC1, PrC2, KD1, KD2, and PC, were formulated and manufactured. The six types of Boerewors were stored at 4 °C, and sampled on days 0, 3 and 6, in terms of physical and microbiological parameters. Samples of the six Boerewors treatments were frozen at -18 °C, and sampled on day 0, 6 and 90, for lipid oxidative stability analysis. Microbial stability and lipid oxidative stability analysis results were encouraging. The natural preservatives had an effect on the pH, as KD2 had the highest pH values at the beginning and the end of the 6-day shelf-life. On the first day, the treatments differed in terms of the a_w, with PrC1 having highest a_w, while KD1 had the lowest a_w. No effects on moisture content were observed, in part due to all formulation types containing the

same amount of meat. The plant extract groups, KD1 and KD2, had a positive effect on the lipid oxidative stability of the Boerewors, by having the lowest SLOP levels throughout the storage period, compared to the rest of the treatment groups. KD1 and KD2 had lower SLOP levels on day 90 than that of SO_2 preserved Boerewors on day 0. Even with one group of preservatives having these outstanding results, the other natural preservative Boerewors samples also had TBARS values below the primary detection threshold of rancidity, from day 0 to day 90 of frozen storage. In terms of the microbial quality, it was evident that TBC and LAB counts would not be suitable for quality analysis in cases where protective cultures are added to products. The plant extract preservatives did maintain the microbial quality, with values closest to that of the PC, and were significantly lower than that of the other treatment groups. The protective cultures could not inhibit the growth of yeasts; PrC1 and PrC2 had significantly higher yeast counts than the PC on the third day.

The Boerewors treatments had an influence on the colour parameters. KD1 was the only treatment that maintained the lightness as well as the PC, but the NC had the lowest lightness, which proved that adding any preservative is better than not adding anything at all. The redness, which is probably the most important parameter, decreased from day 0 to 6, but PC had the highest redness values throughout the 6-day shelf-life. No treatment could compare to the SO_2 in terms of redness. Yellowness was higher when KD1 was used in the formulation, but for the most part, there were no significant differences in the yellowness of the Boerewors. The yellowness could probably be due to the physical colour of the acerola extract in the treatment solutions. In terms of brightness, the PC treatment maintained it the best, followed by KD2. Even though all the Boerewors treatments had great red colour purity values, the PC consistently had the values closest to the red side of the spectrum than the other treatments. The colour results proved how difficult it would be to replace SO_2 as a colour preservative in Boerewors, but from the natural preservatives, the plant extracts would be the better option. However, to maintain the same redness in the Boerewors when replacing it with KD1 or KD2, it is suggested that future research focus on natural colours as additives to improve the redness value.

Another part of the sensory analysis was the consumer sensory evaluation. The panel consisted of 100 members, 70% female and 30% male. The formulation type did not have a significant effect on the sensory attributes, but PrC2 had the highest scores in most attributes. Insignificant results were a good outcome because it showed that the natural preservatives did not impart a bad taste and the consumers did not have a preference to SO_2 specifically. The women gave higher attribute scores than the men. The null hypothesis of the first aim stated that replacing SO_2 with natural preservatives resulted in Boerewors with unacceptable

changes in chemical, microbial and/or sensory quality. When all of the results of Chapter 3 are considered, the plant derived natural preservatives, KD1 and KD2, did maintain the quality and stability of Boerewors, and the null hypothesis was, therefore, rejected.

In Chapter 4, the same six Boerewors treatments were used to investigate the microbial communities by using Biolog™ EcoPlates™. These results were obtained by physiologically profiling the microbial communities in the Boerewors, which were incubated in the Omnilog machine on day 0, at 25 °C, for 72.5 hours. The raw data was statistically analysed by using Shannon indices and heatmaps to compare the treatments, and in the second part of this chapter, the Gompertz function was used to analyse the colour development and growth parameters of the microbial communities in each Boerewors treatment. The microbial communities oxidised the carbon compounds at different rates; however, in all the treatment groups, 2-hydroxybenzoic acid was metabolized the least, and L–asparagine was metabolized at the highest rate.

In terms of treatments, the NC had the highest growth rate, diversity, and richness, which indicated that adding no preservative to Boerewors would increase the functional diversity of the bacterial communities. KD1 had the lowest growth rate, diversity, and richness in the raw data analysis. Grouping the substrates into guilds did not show meaningful results, because the growth in the different treatments only showed that polymers were oxidised more than the other guilds, but it was not treatment specific.

The results of the first part of Chapter 4 indicated that the natural preservatives did restrict microbial growth and diversity, especially KD1 with the lowest Shannon values and growth rate. The results based on the Gompertz curve parameters did not differ significantly. Nevertheless, subtle differences were observed in terms of the position and explosiveness of growth of the microbial communities; the first treatment to show some growth was KD2 with a midpoint of 12.1 h, and PrC1 had irregular growth positions and started growing at a later stage, with a midpoint growth at 24.4 h. Even though KD2 was the first to grow, this treatment had the least explosive growth, with an average of 4.8 The treatment with the most variety of explosiveness was KD1, with an average of 6.5. Considering the results of the second part of this chapter, KD2 would be the best option as a SO_2 replacer in Boerewors, because it had the least explosive growth. The null hypothesis of the second aim stated that replacing SO_2 with natural preservatives would result in adverse changes in the microbial communities in Boerewors. Both plant extracts, KD1 and KD2, showed promising results in different aspects of analysing the microbial communities in Boerewors and the null hypothesis was, therefore, rejected. For future research, the experiment should include multiple repetitions to establish

variability between repetitions and also determine if there could possibly be variations between the suppliers and sources.

The last chapter of this study was based on the food safety of Boerewors treated with different preservatives. Only four of the original six formulation types were included: NC, KD1, KD2, and PC. These samples were stored at 4 °C for 6 days, and analysed on day 0, 3, and 6. The protective culture treated Boerewors were excluded from this part of the study based on the result of the previous chapters. In the Boerewors inoculated with *E. coli*, the NC had a higher count than the other treatments. Initially, the formulation type did not have an immediate effect on the *E. coli* count, however, the plant extract treatments and the PC had significantly lower counts than the NC on the third day. The counts on day 6 were too low to show any significant differences between treatments. In terms of *S. aureus*, the formulation type had an immediate effect on the counts. The NC had the highest counts, and the natural preservatives did not differ significantly from the PC. On the last day, the NC continued to have the highest counts and even though the PC had the lowest count, KD2 did not differ significantly from it. The null hypothesis of the last aim stated that replacing SO_2 with plant derived, natural preservatives would allow for the survival and growth of either one of the inoculated bacterial strains. The plant extracts groups did not have significantly higher *E. coli* counts than the SO_2 treated Boerewors, however, the KD1 and KD2 did not consistently have similar *S. aureus* counts than the PC, but at the end of the shelf-life, KD2 did not differ significantly from the PC. The null hypothesis was, therefore, rejected. In future research, more pathogens that are commonly found in meat products, should be evaluated to get clearer results on the effectiveness of the natural preservatives on maintaining food safety of Boerewors. For future research, more pathogens, like Listeria monocytogenes, could be included in the experiment, to see the effects of all the natural preservatives used in this study, on the food safety of Boerewors.

CHAPTER 7

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CHAPTER 8

SUMMARY

Boerewors is a traditional fresh South African sausage, made from beef and pork, with a variety of herbs and spices added for flavour. Currently, SO_2 is still the main preservative used in Boerewors, with a maximum inclusion level of 450 mg/kg. However, it has come to light that SO_2 causes adverse reactions in some consumers; it is severely irritating to the mucous membranes and respiratory tract. As consumers have become more aware of what they eat, there has been an increased demand for minimally processed food products that contain natural ingredients.

In this study, Boerewors was used to evaluate the chemical, microbial and sensory efficacy of natural preservatives that could potentially replace SO_2. The six Boerewors treatments were formulated according to the recommended inclusion levels of each preservative. To replicate retail conditions, the Boerewors was stored at 4 °C for 6 days for most of the analyses and stored at -18 °C for lipid oxidation stability analysis. The effects of no preservatives, microbial preservatives, plant extract preservatives, and SO_2 as preservative, were evaluated. Water activity, pH and moisture content were inconsistently influenced. However, the lipid oxidative stability results were encouraging, as the effects of the plant extract preservatives surpassed that of SO_2. The microbial results were inconclusive, due to the protective culture treatments containing viable microorganisms. The colour of the Boerewors remained quite stable, but SO_2 maintained the red colour of Boerewors the best. In the consumer sensory evaluation, it was established that there was not a preference for a specific treatment.

The Boerewors was sampled on the first day and evaluated by profiling the microorganisms on a community level. The results showed that the treatment with no preservatives added had the largest and most diverse microbial community, while the microbial community in KD1 was the smallest and least diverse and was functionally more similar to the SO_2 treatment than the other treatments. The Gompertz based results showed that the microbial community in PrC1 started to grow at a much later stage than the other treatments, and KD2 had the least explosive growth of the treatments.

The protective cultures were excluded from the spiking experiment and the efficacy of four treatments, on the microbial safety of the Boerewors, inoculated with either *E. coli* or *S. aureus*, were monitored. The treatments affected the growth and survival of *E. coli*, as the results showed that plant extracts maintained the product safety similar to that of the SO_2

Boerewors treatment. The SO_2 Boerewors inhibited the growth of *S. aureus* the most but adding the plant extracts proved to be better than adding no preservative. Survival rates of both inoculated strains decreased from day 0 to 6, while it was stored at 4 °C.

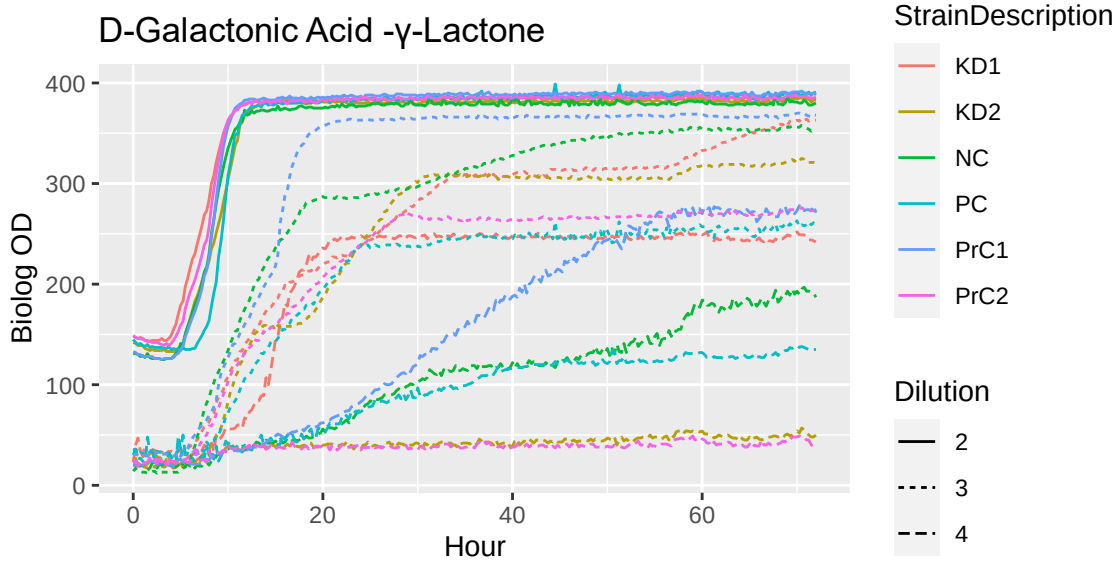
Overall, the results of this study showed that replacing SO_2 with natural preservatives, specifically plant extract preservatives, KD1 and KD2, maintained the chemical, microbial, and sensory quality, and product safety as efficient as SO_2. Thus, it is recommended that the meat industry consider including natural plant preservatives when reformulating Boerewors.

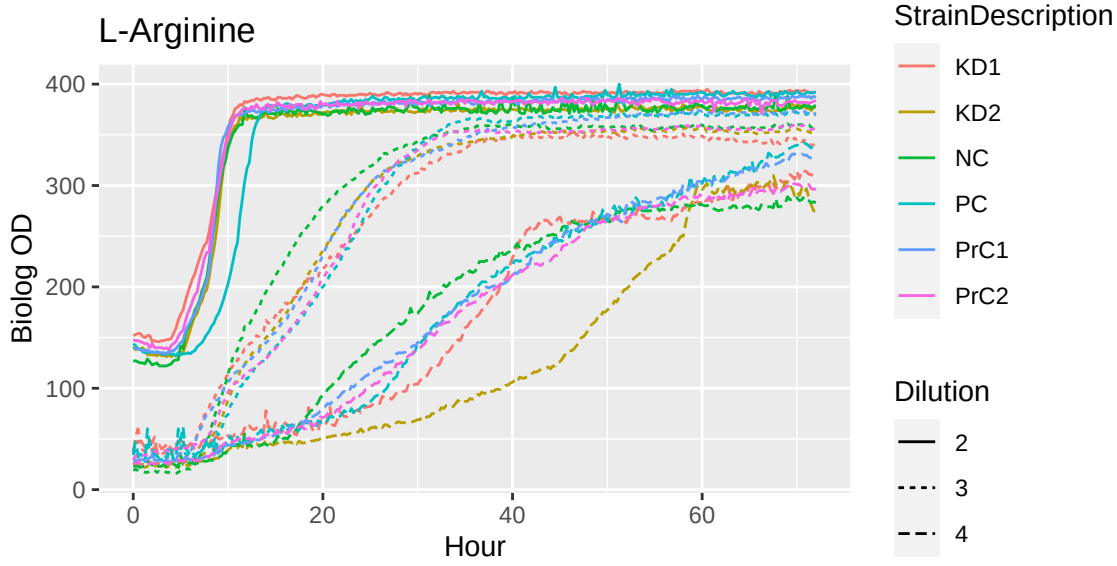
The preliminary cost estimates for inclusion of the natural plant extracts as replacers of SO_2 in Boerewors indicated that it will be financially viable.

Keywords: sulphur dioxide replacement, protective cultures, plant extracts, product safety, microbial quality, chemical stability, sensory quality, community level physiological profiling, Boerewors.

ANNEXURE 1

BIOLOG OD RESULTS SHOWING ALL DILUTIONS




L-Arginine
Biolog OD
400
300
200
100
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60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
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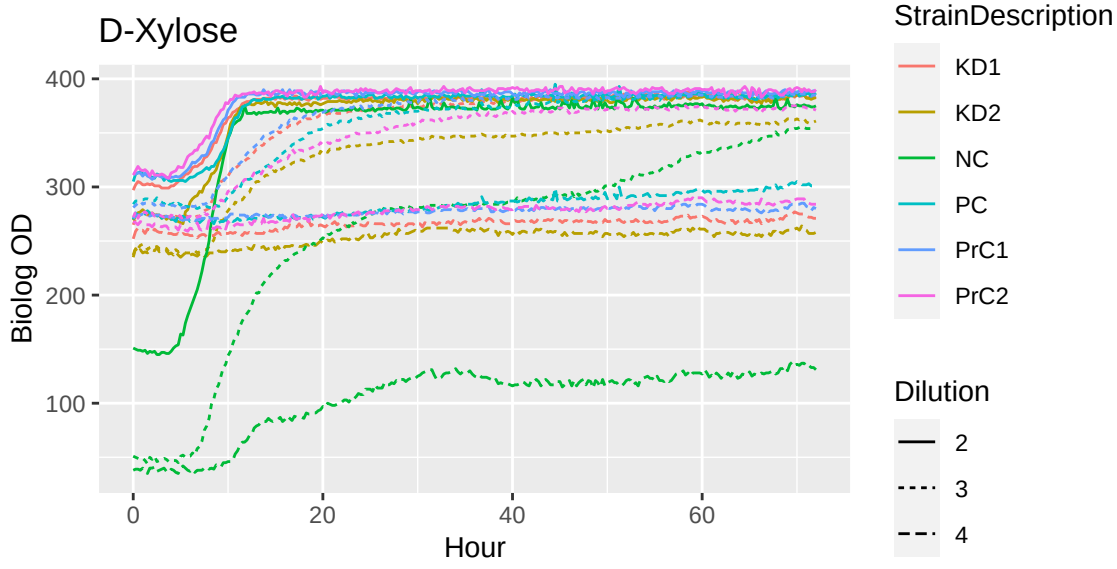
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Biolog OD
400
300
200
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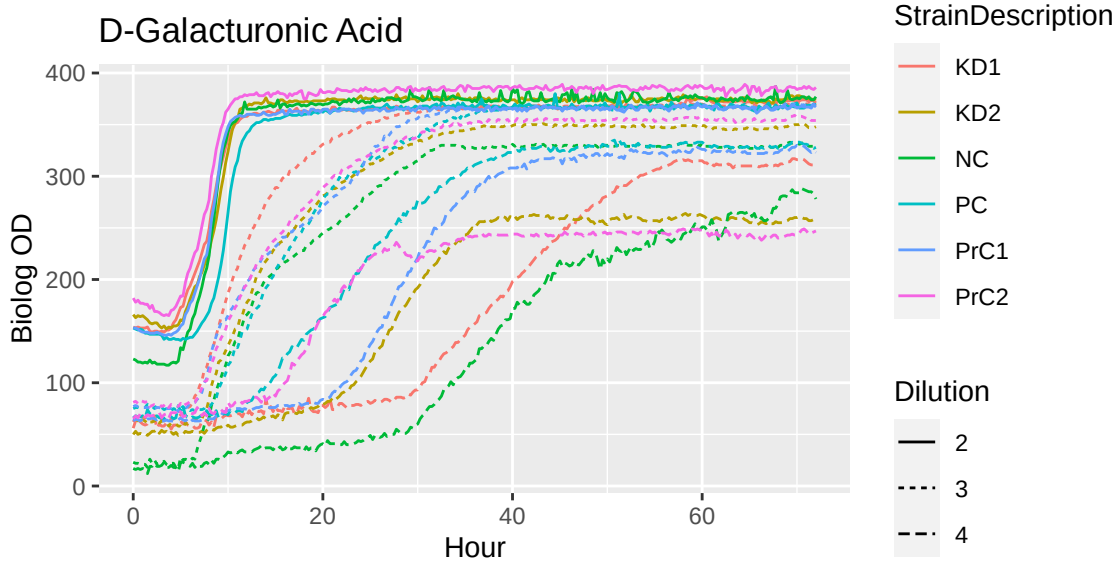
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4


D-Galacturonic Acid
Biolog OD
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Hour
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PrC1
PrC2
Dilution
2
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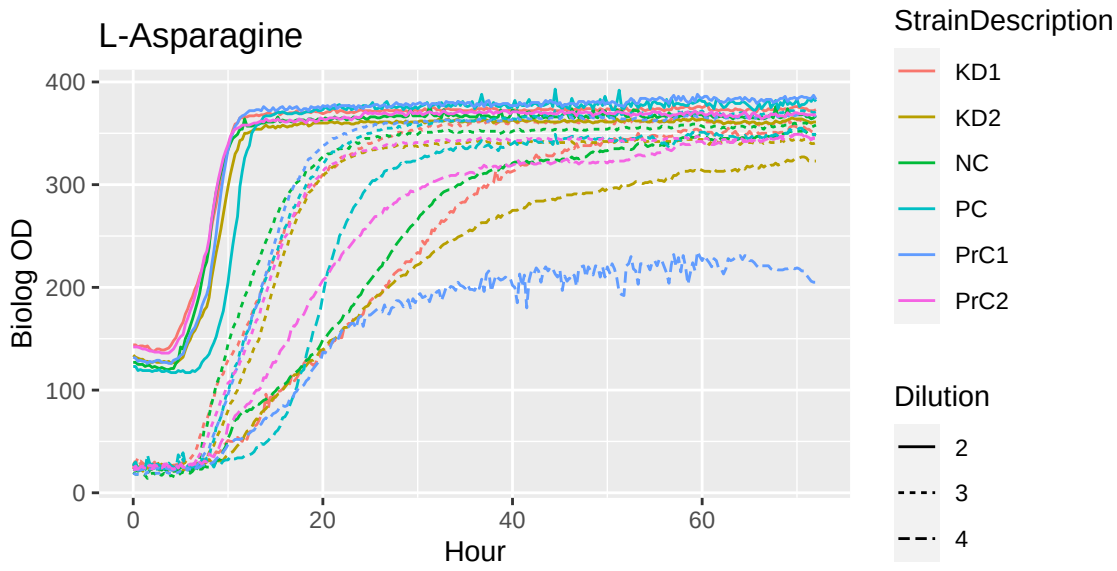
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Biolog OD
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100
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2
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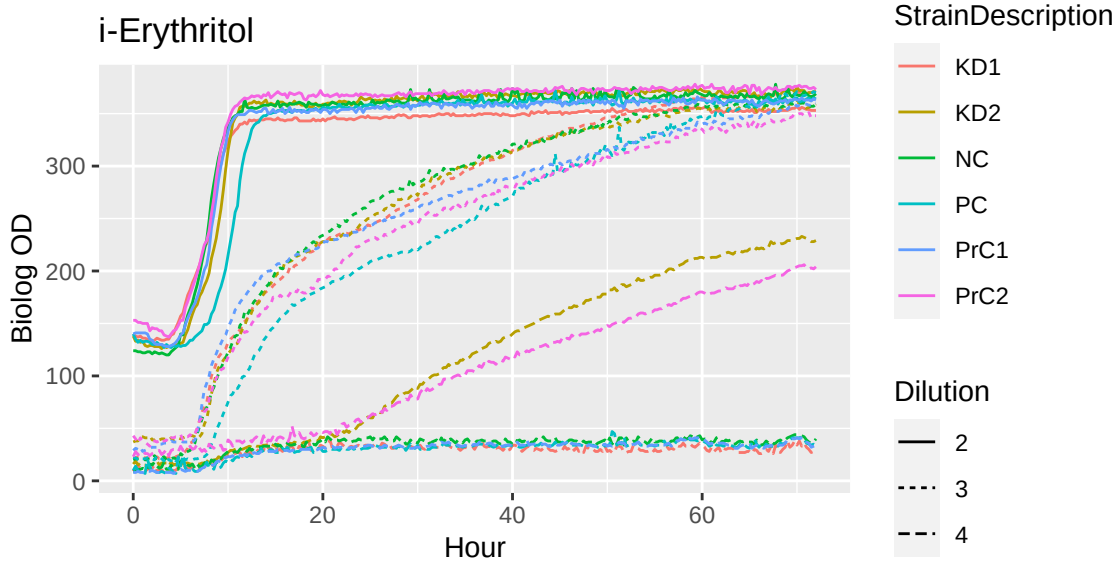
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PC
PrC1
PrC2
Dilution
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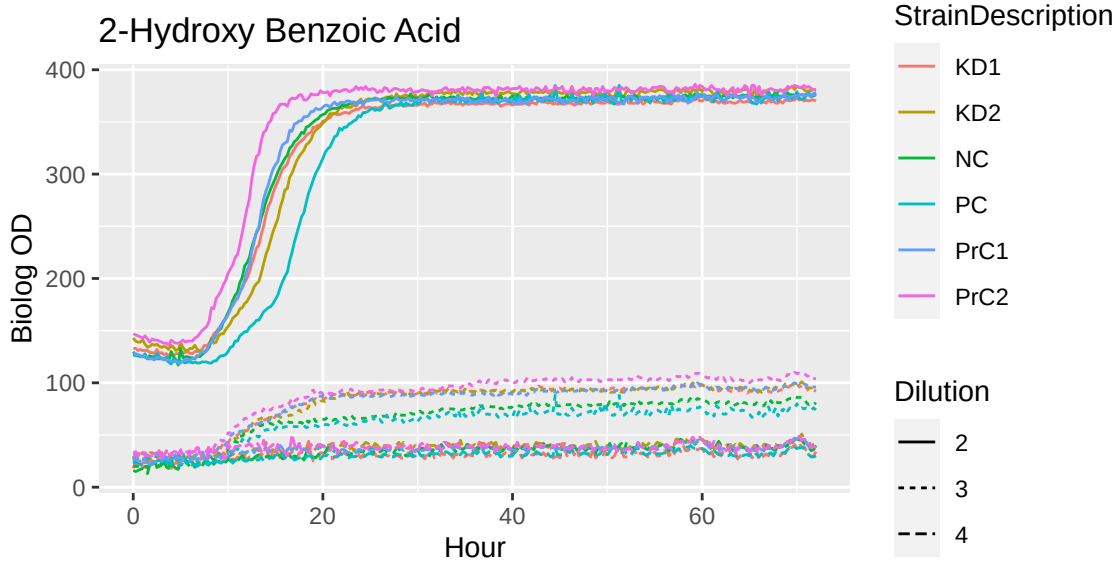
2-Hydroxy Benzoic Acid
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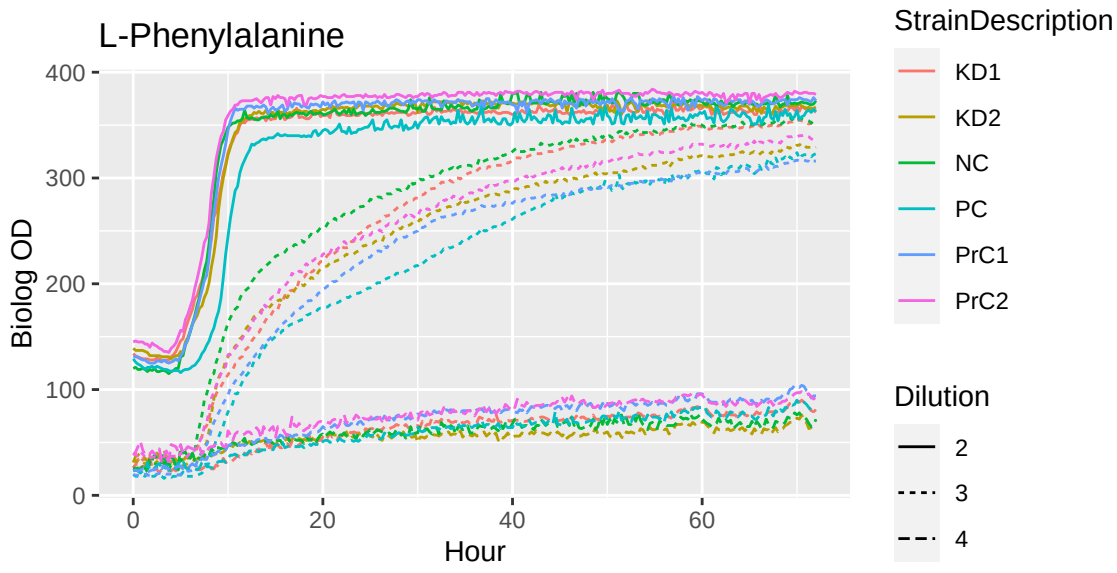
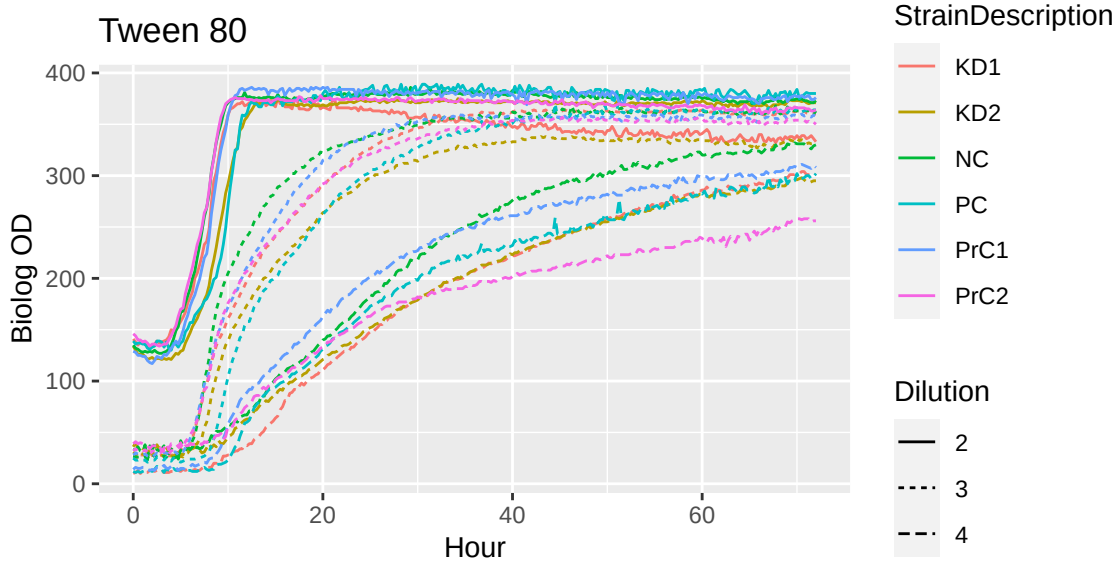
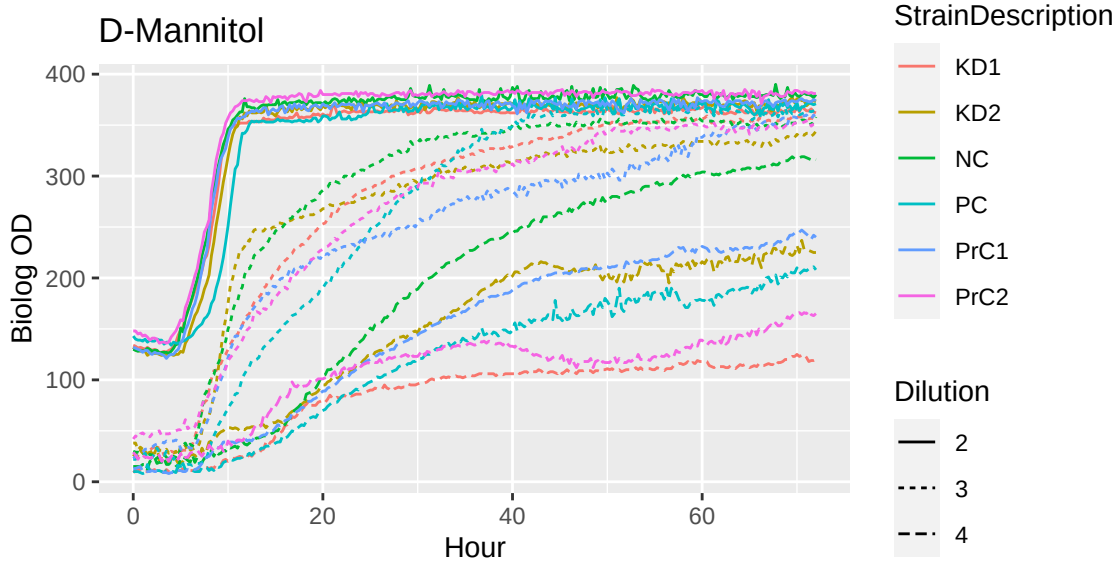
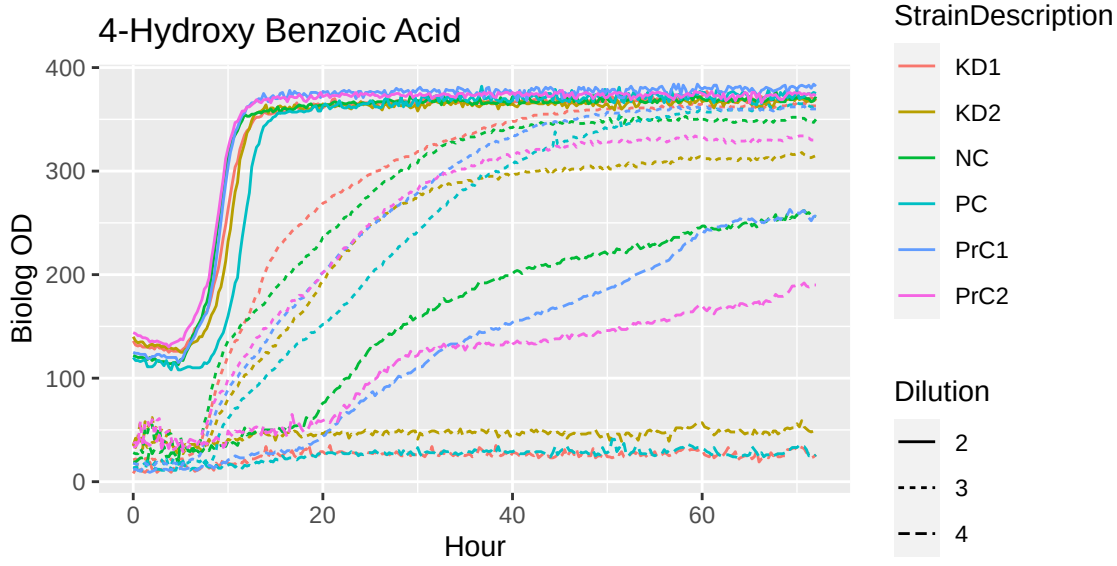
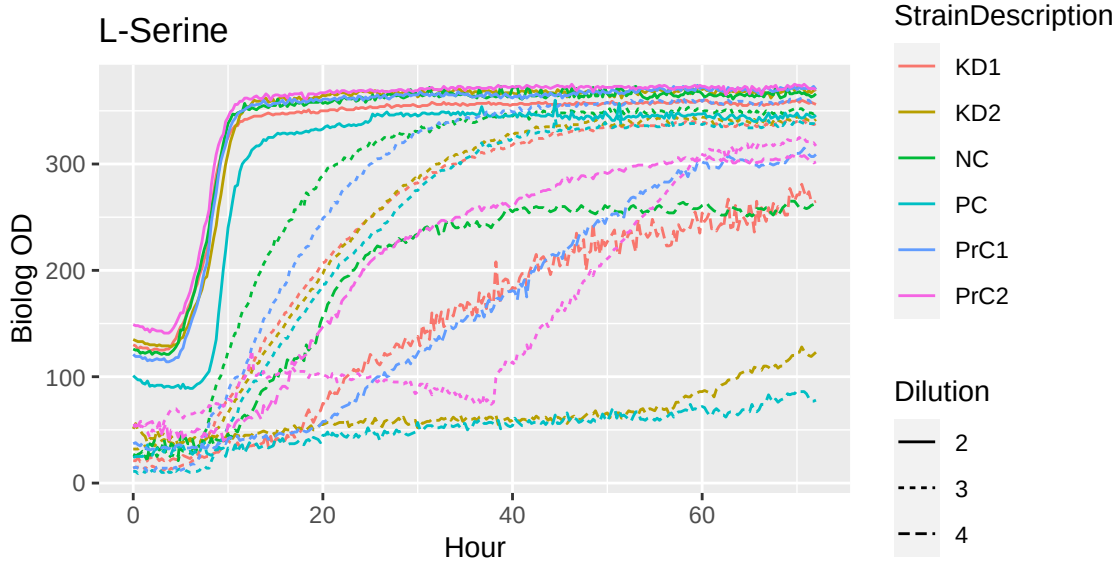
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

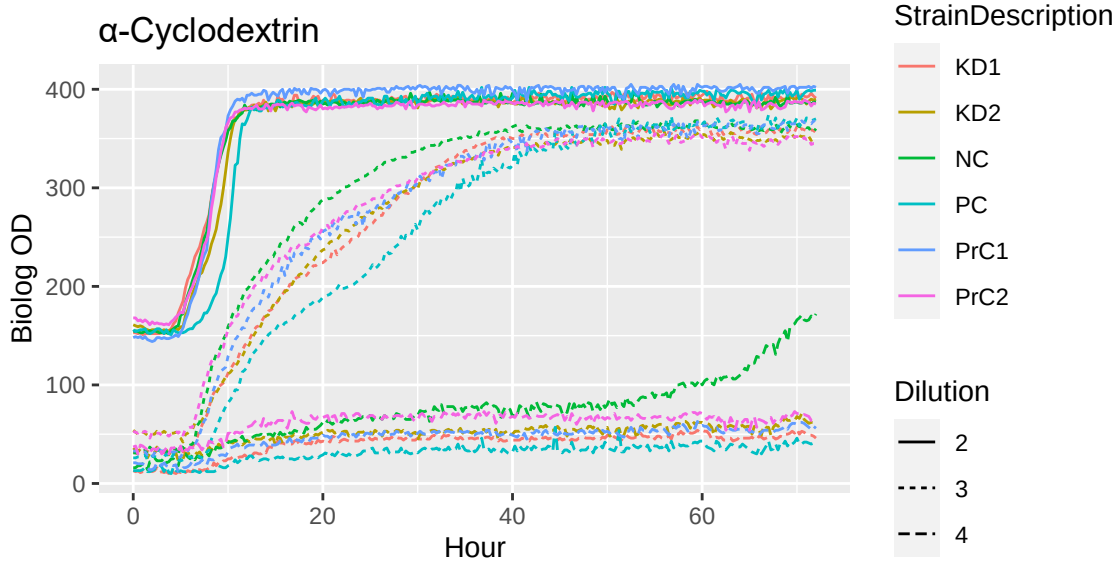

L-Phenylalanine
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

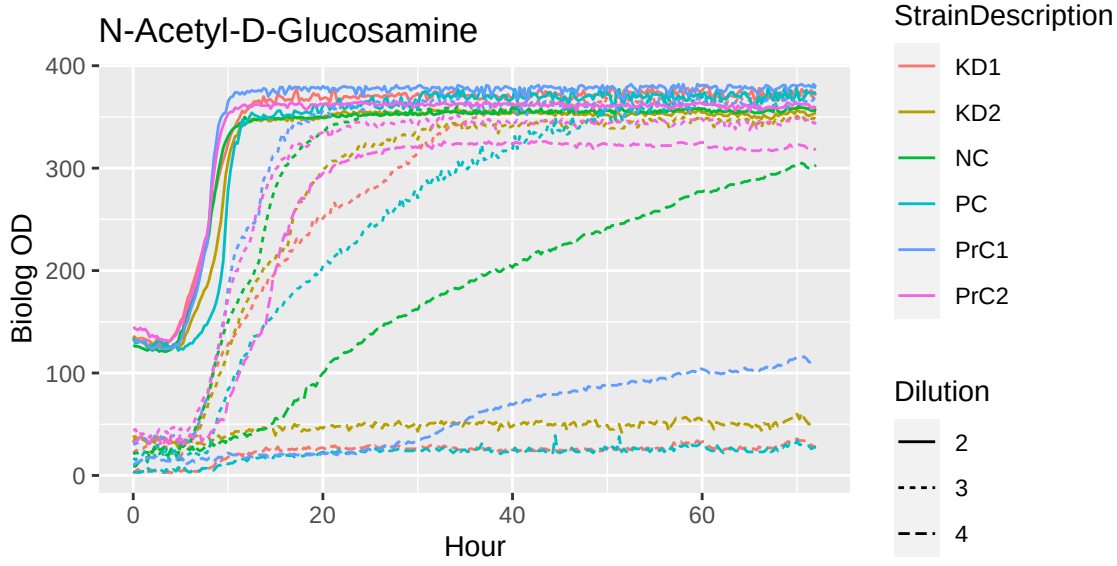

Tween 80
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

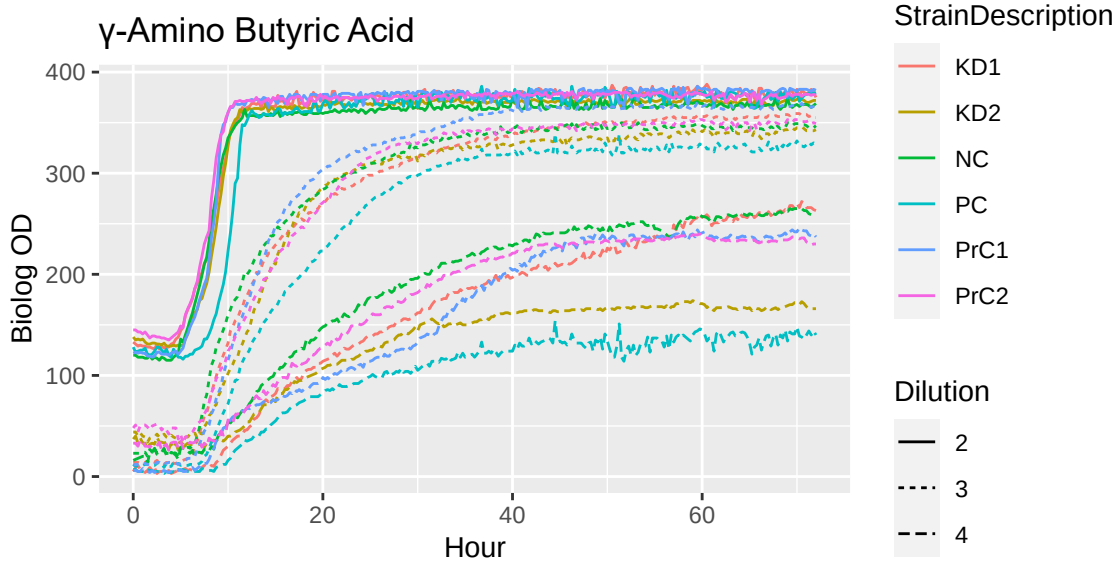

D-Mannitol
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

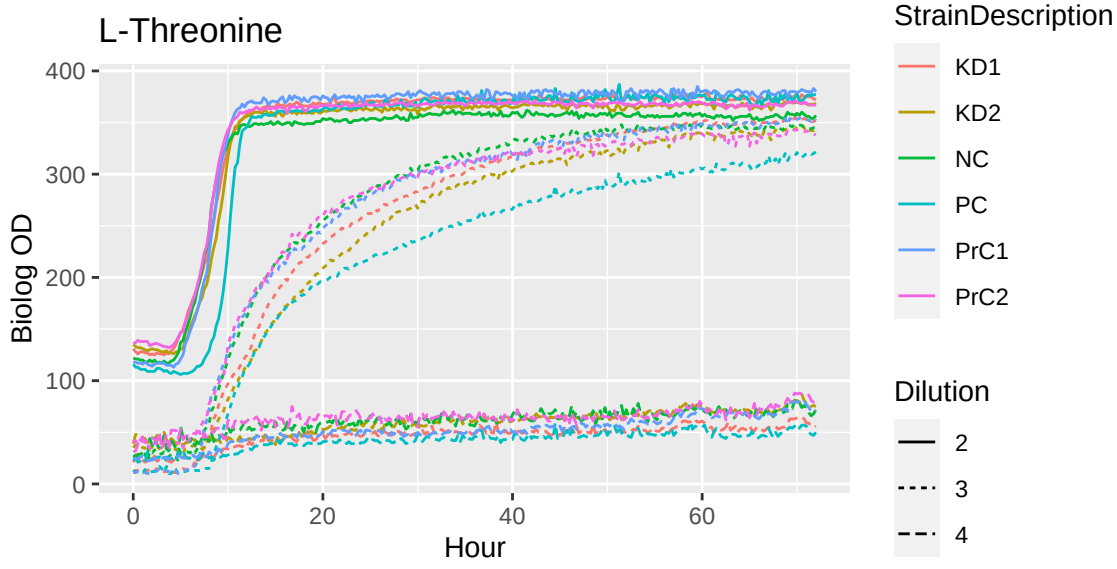

4-Hydroxy Benzoic Acid
Biolog OD
Hour
0
100
200
300
400
0
20
40
60
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

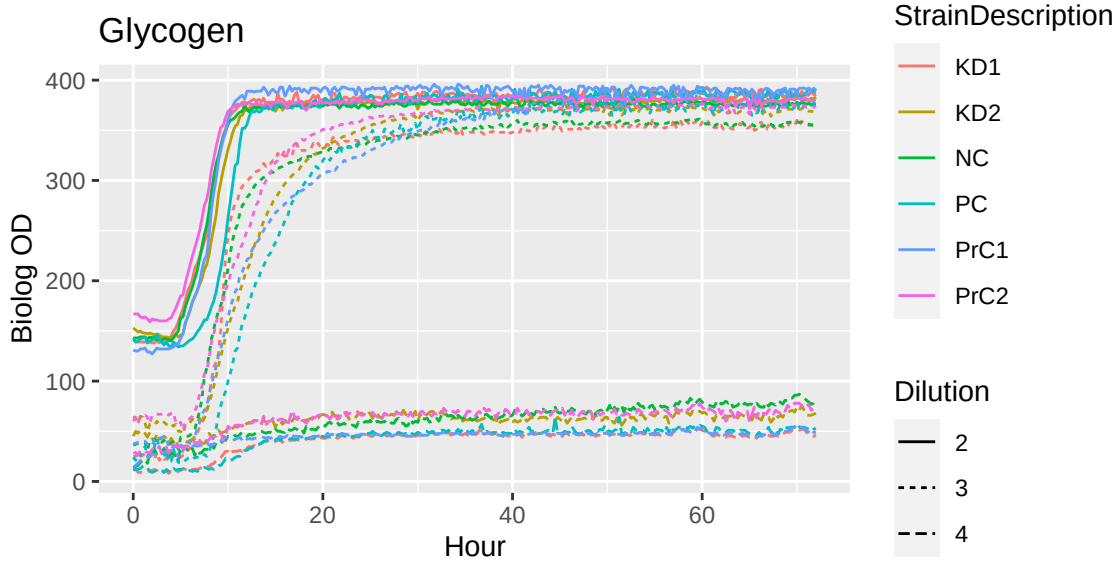

L-Serine
Biolog OD
Hour
0
100
200
300
0
20
40
60
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

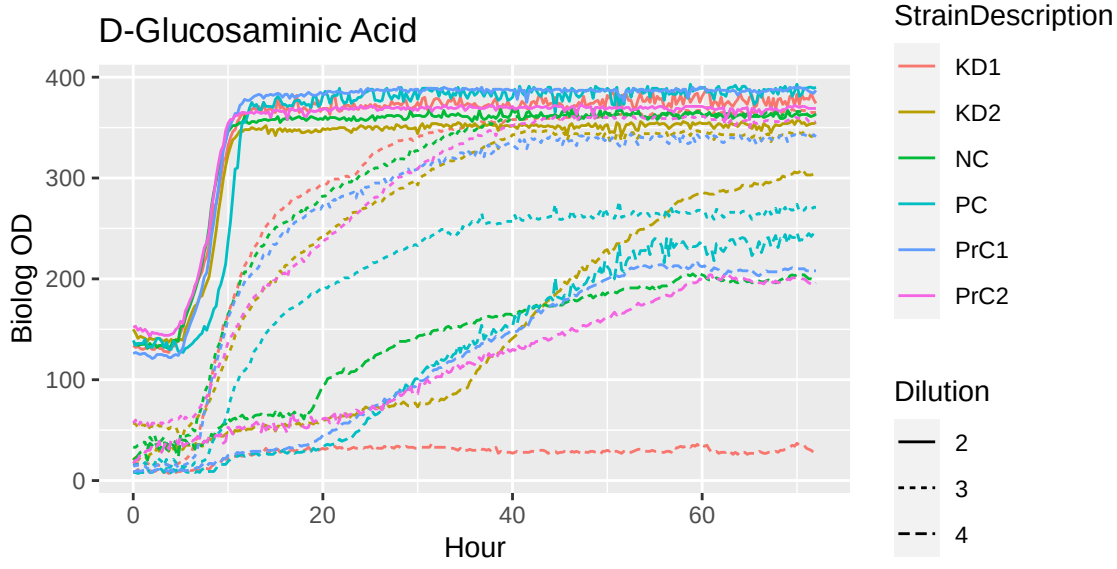

α-Cyclodextrin
Biolog OD
Hour
0
100
200
300
400
0
20
40
60
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

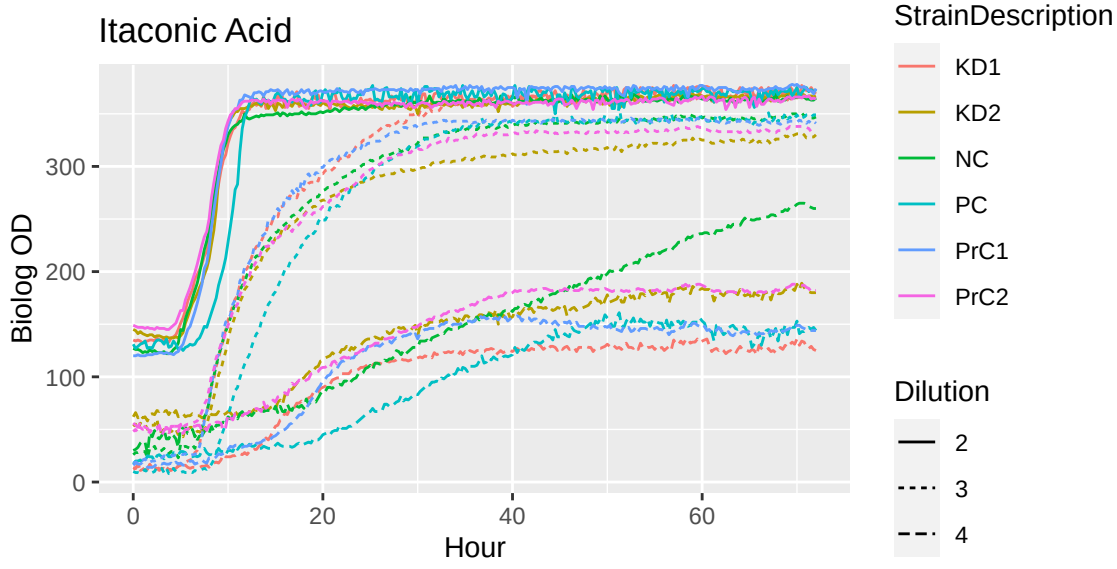

N-Acetyl-D-Glucosamine
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

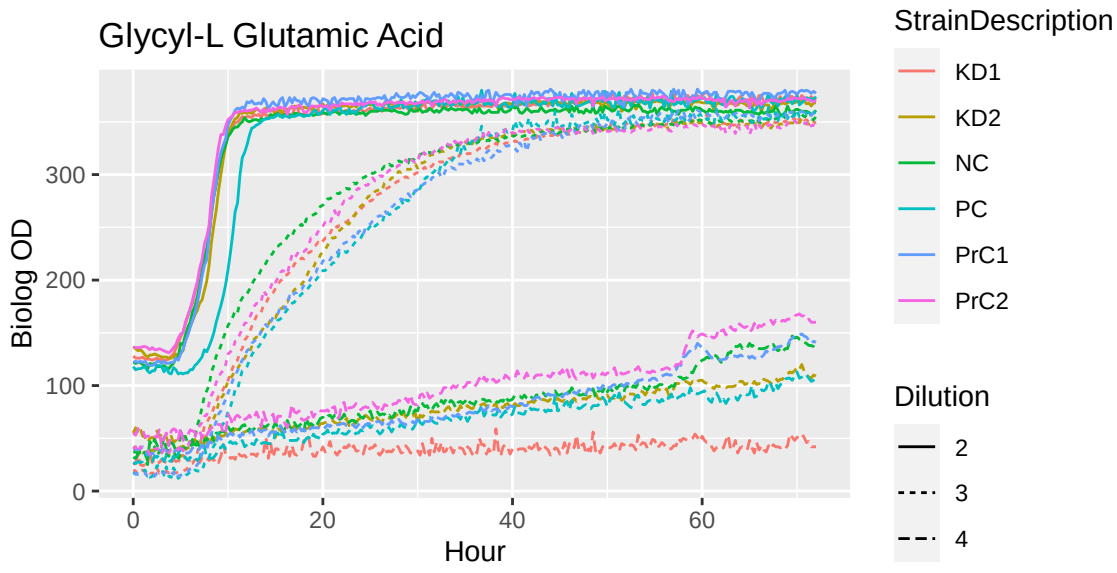

γ-Amino Butyric Acid
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

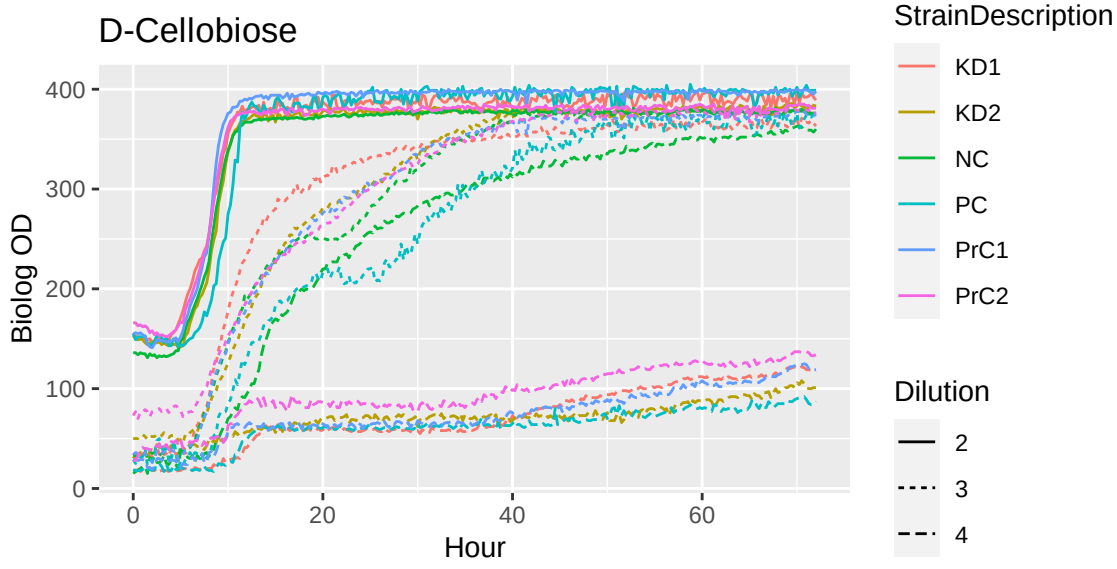

L-Threonine
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

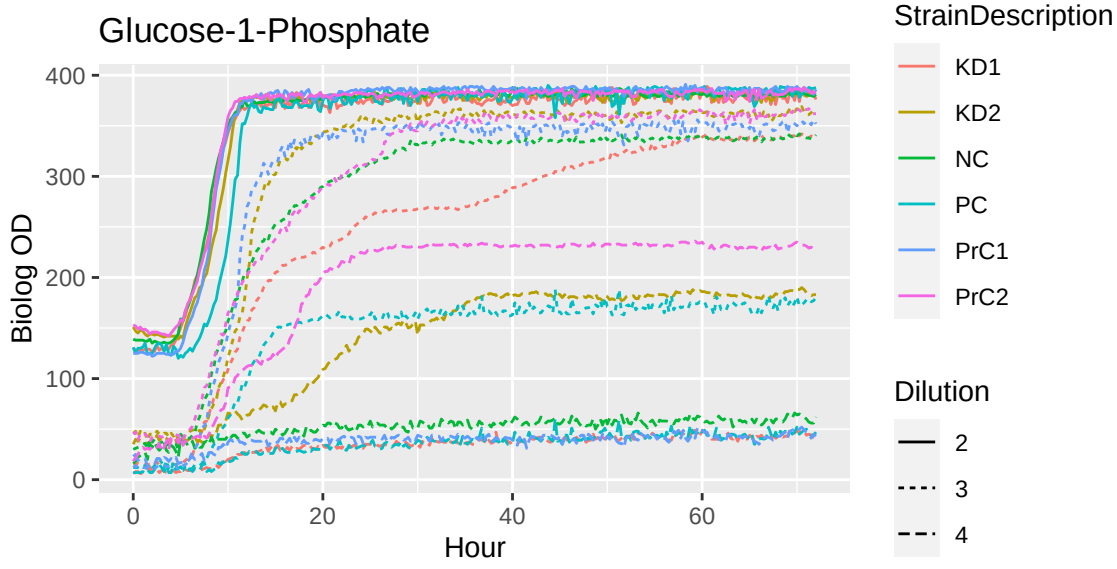

Glycogen
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

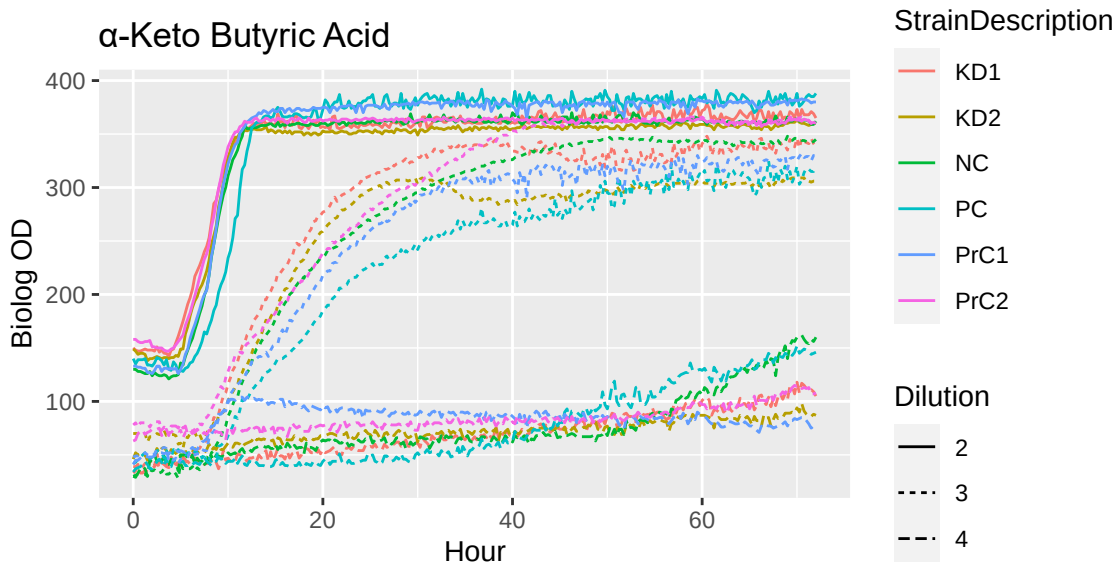

D-Glucosaminic Acid
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

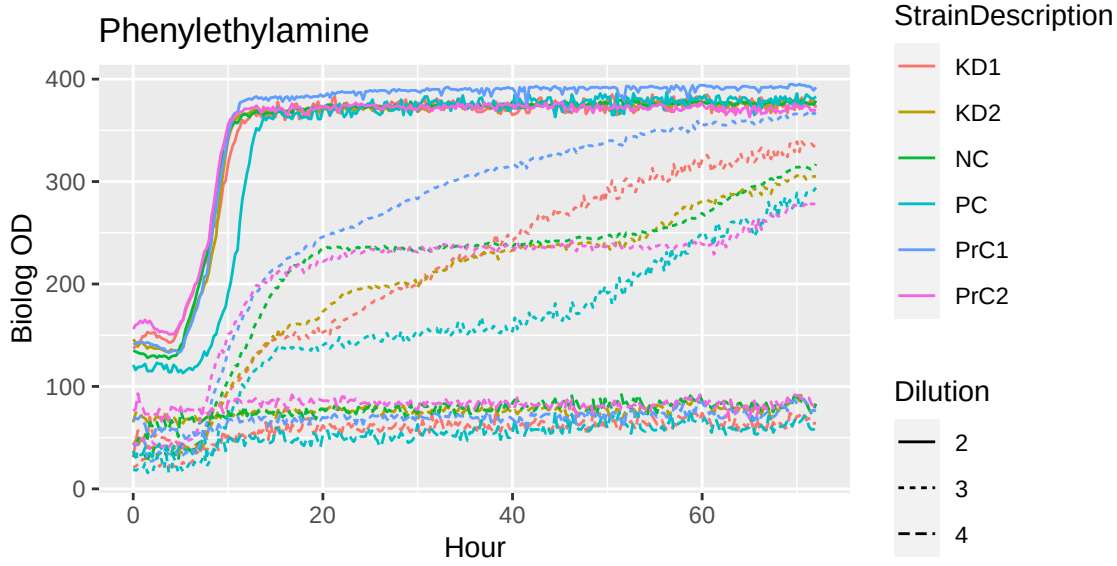

Itaconic Acid
Biolog OD
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

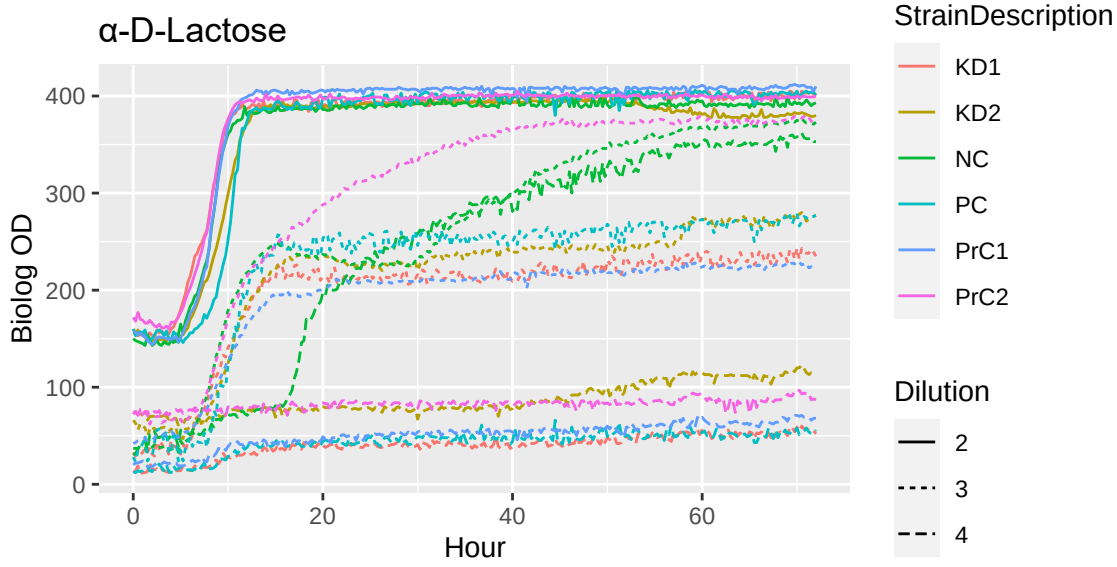

Glycyl-L Glutamic Acid
Biolog OD
0
100
200
300
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

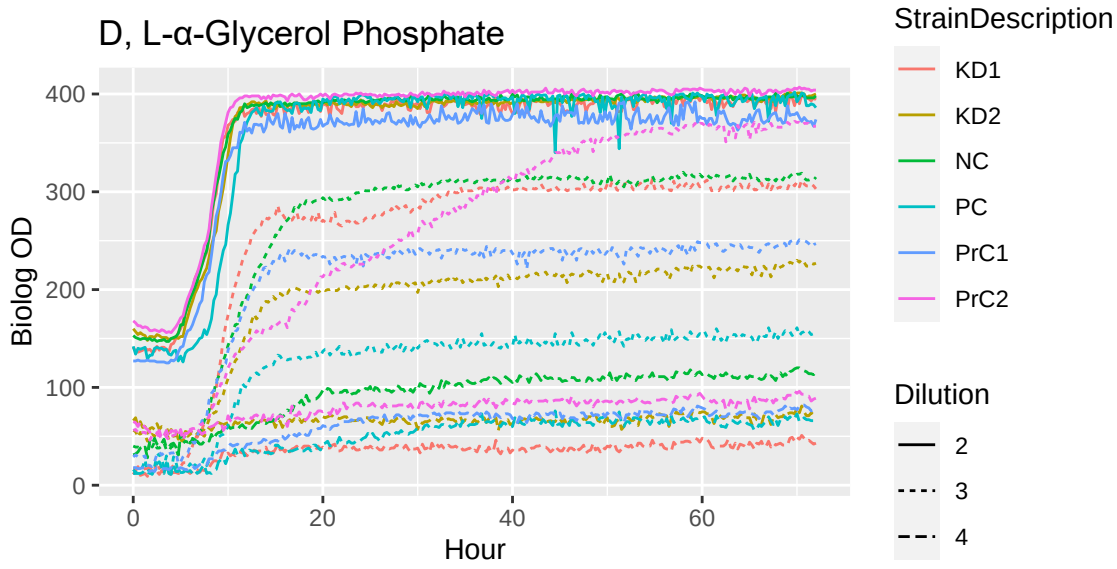

D-Cellobiose
Biolog OD
0
100
200
300
400
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

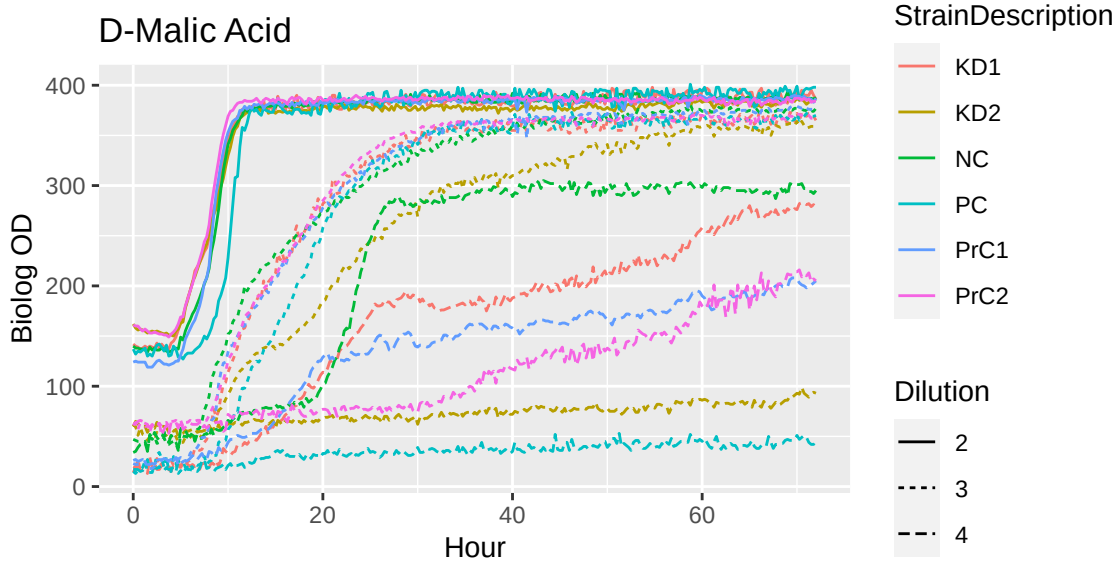

Glucose-1-Phosphate
Biolog OD
0
100
200
300
400
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

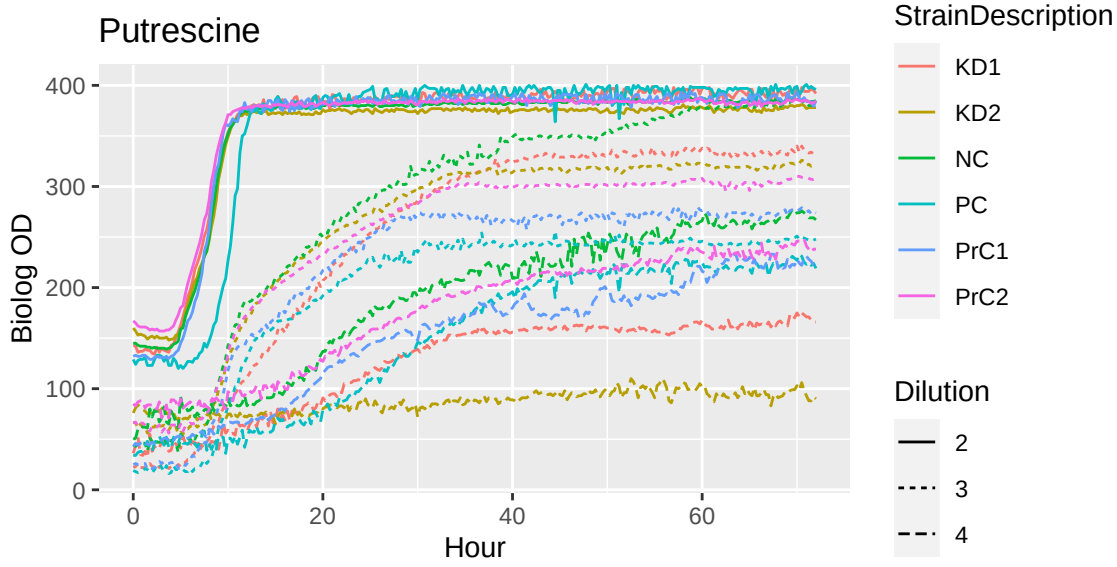

α-Keto Butyric Acid
Biolog OD
400
300
200
100
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4


Phenylethylamine
Biolog OD
400
300
200
100
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4


α-D-Lactose
Biolog OD
400
300
200
100
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

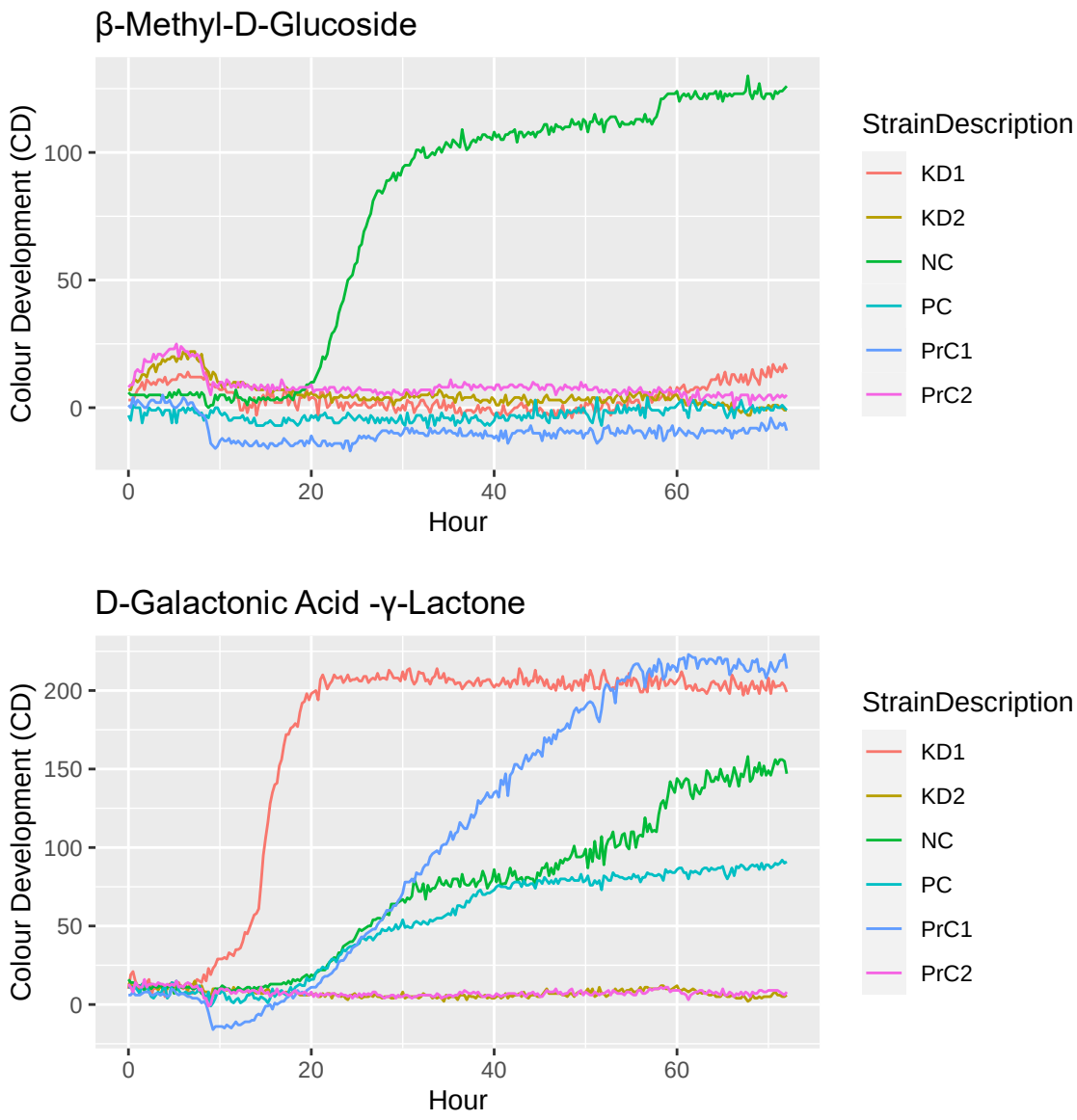

D, L-α-Glycerol Phosphate
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

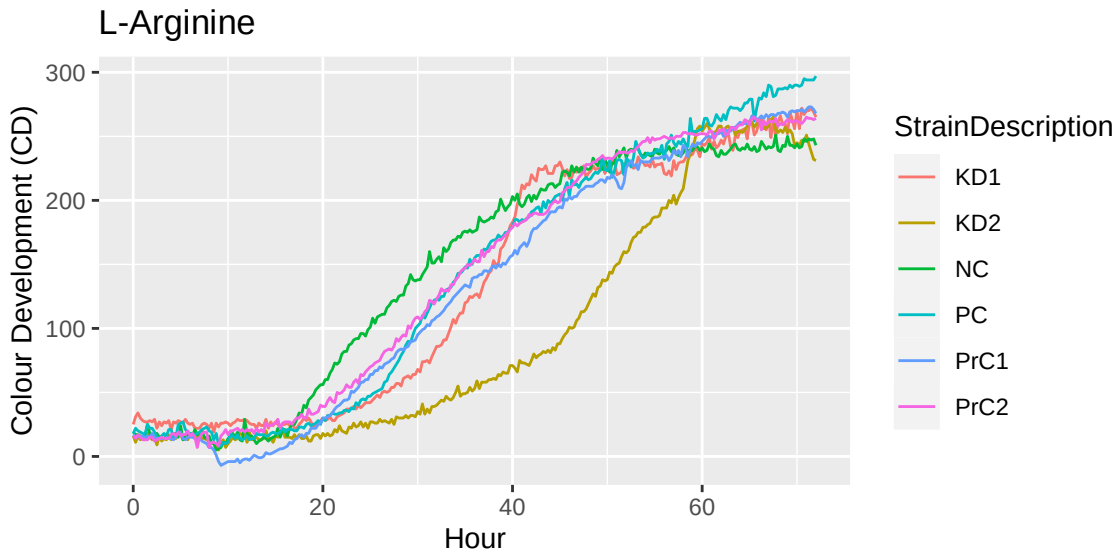

D-Malic Acid
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

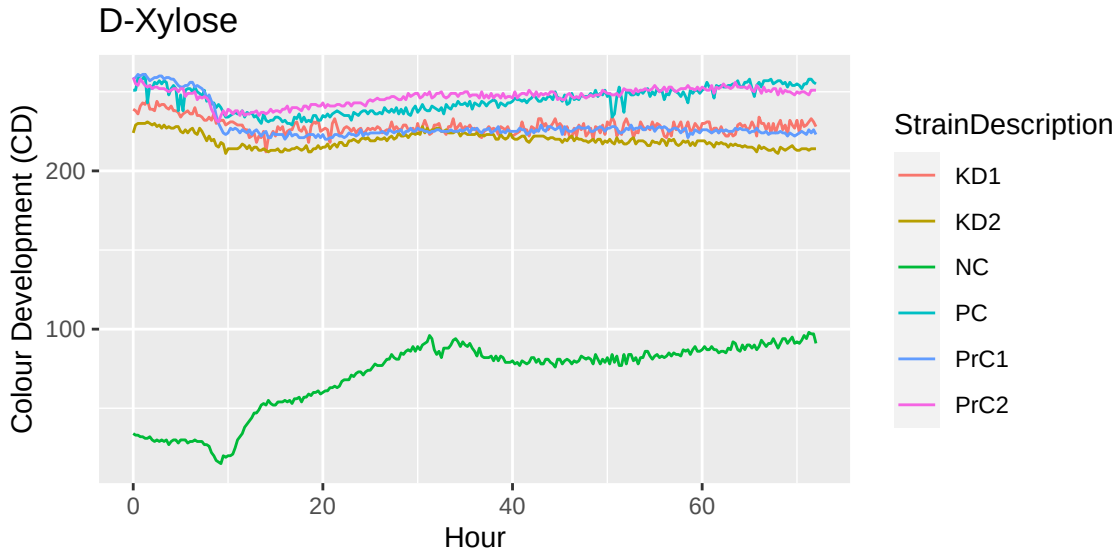

Putrescine
Biolog OD
400
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2
Dilution
2
3
4

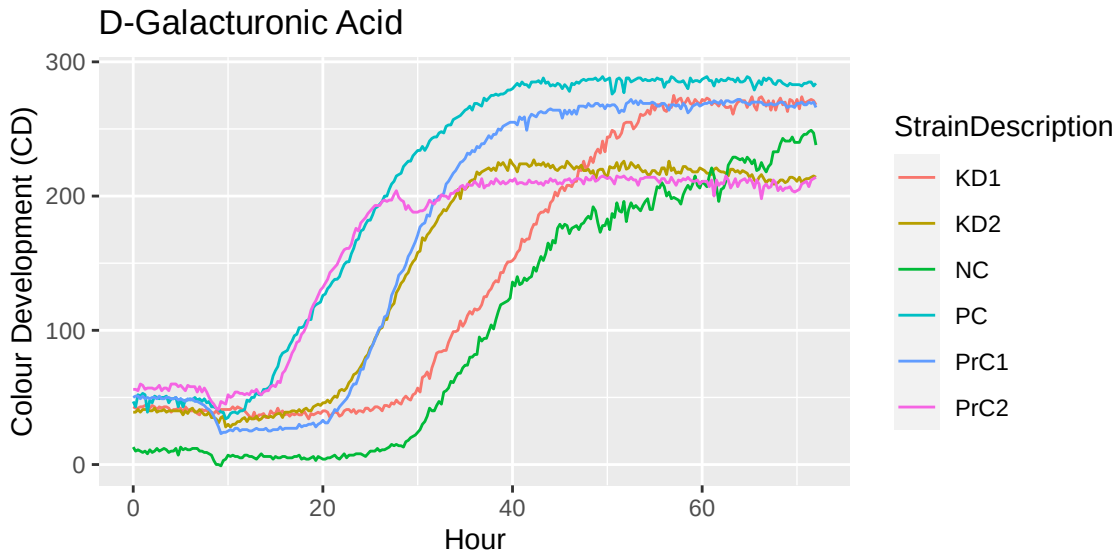
ANNEXURE 2

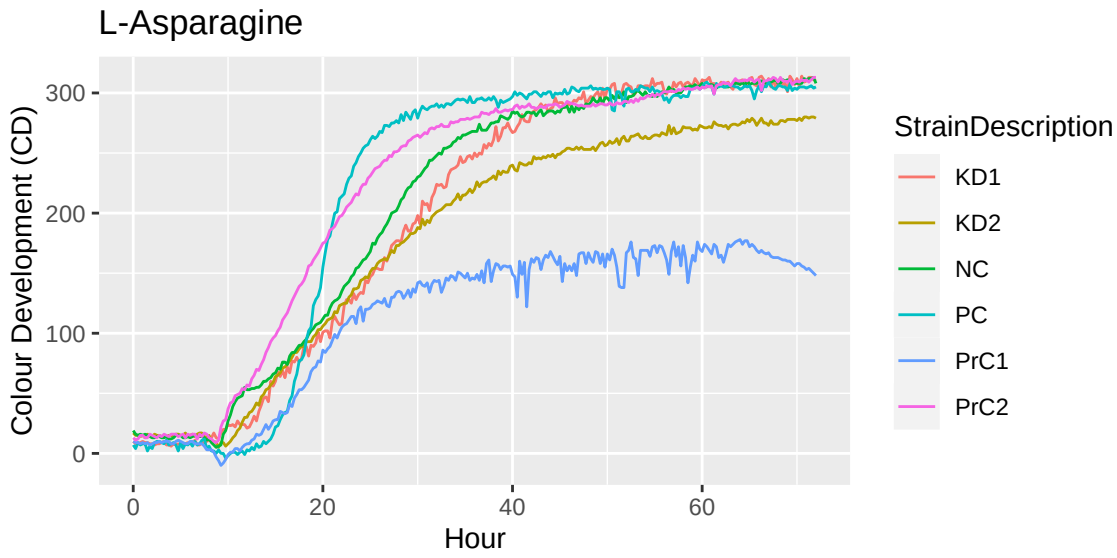
COLOUR DEVELOPMENT OF THE 10^{-4} DILUTION

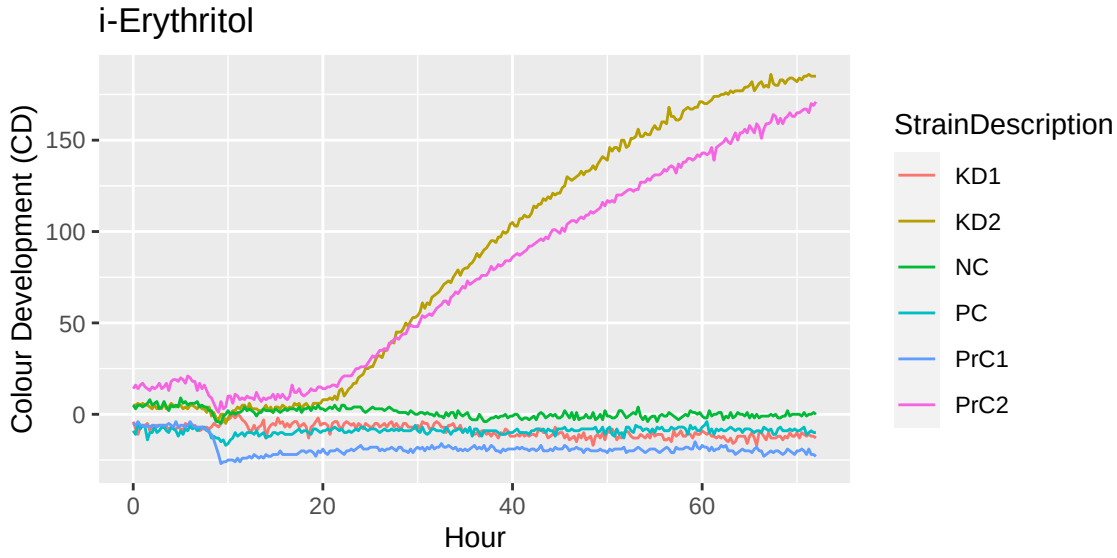


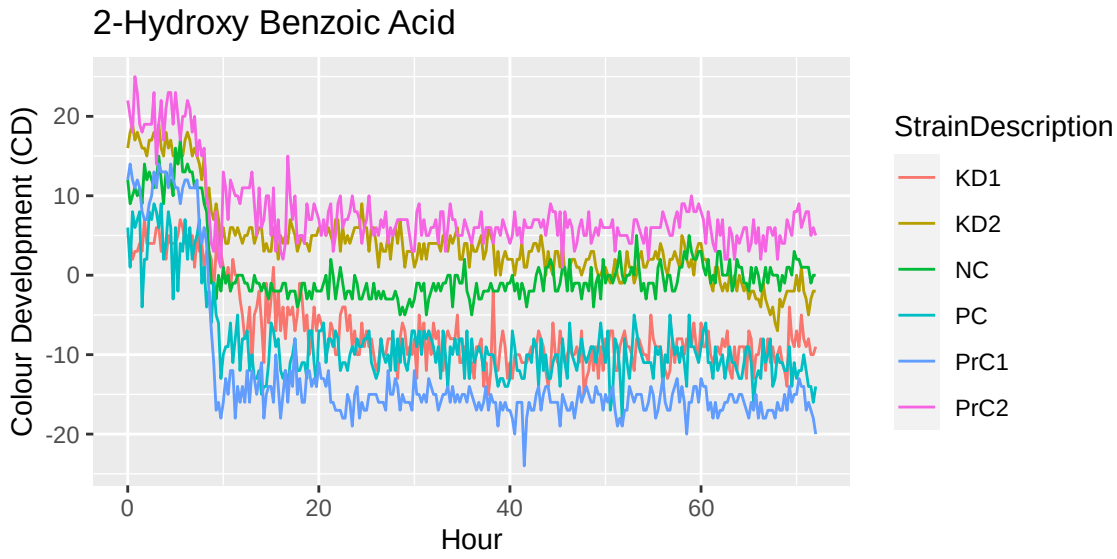

L-Arginine
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

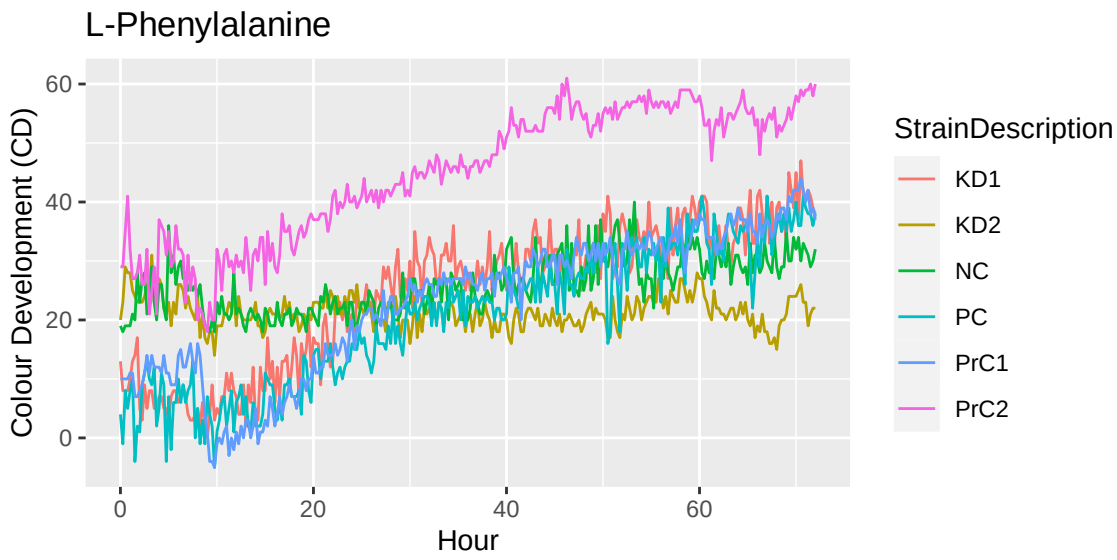

D-Xylose
Colour Development (CD)
200
100
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

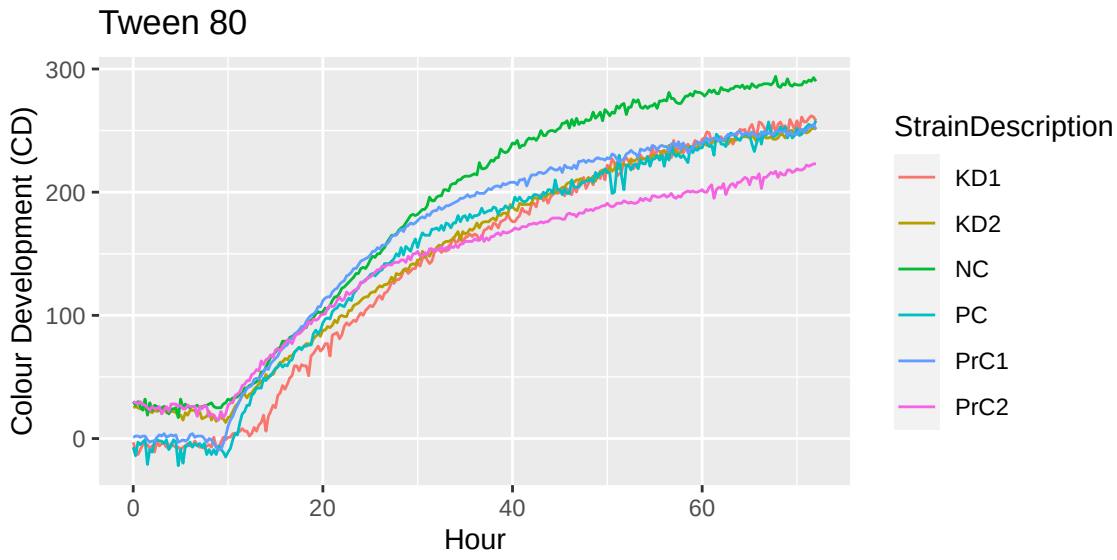

D-Galacturonic Acid
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

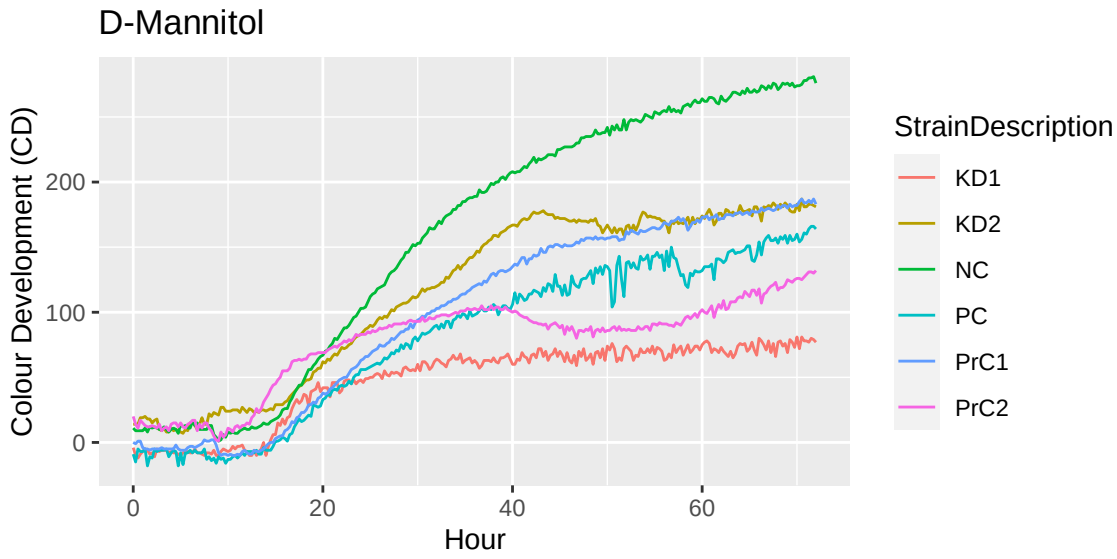

L-Asparagine
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

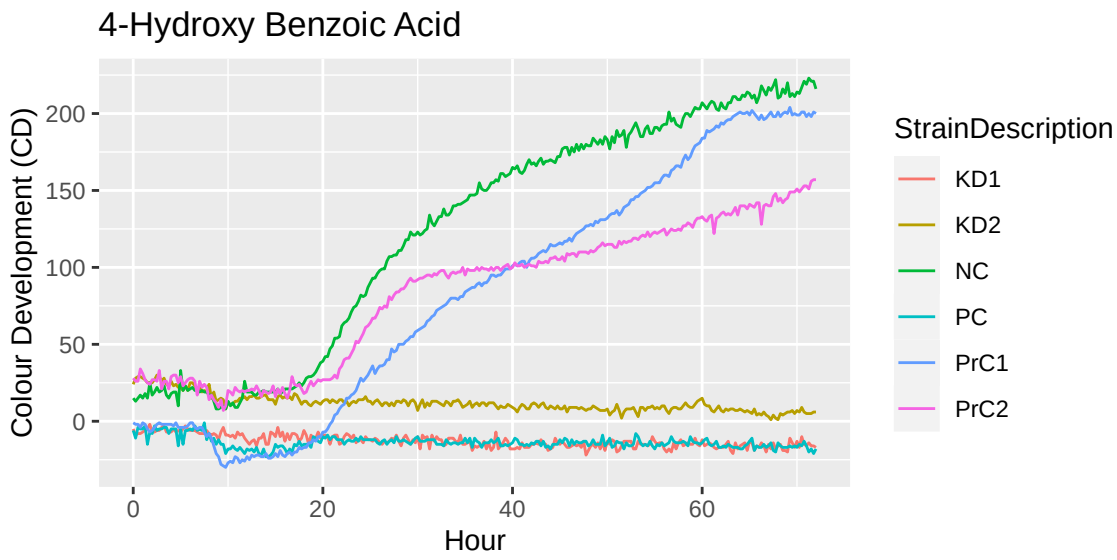

i-Erythritol
Colour Development (CD)
150
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

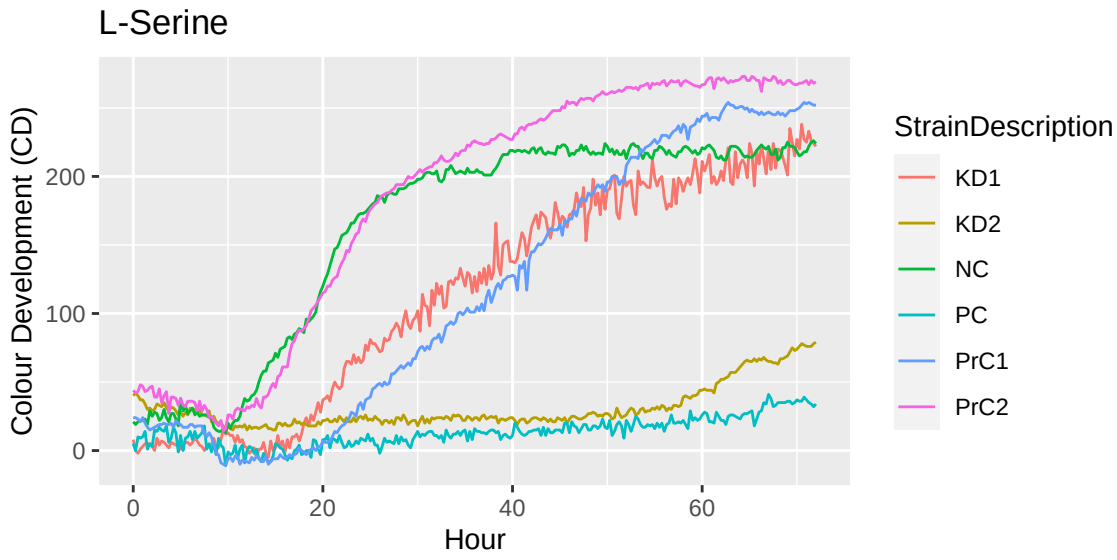

2-Hydroxy Benzoic Acid
Colour Development (CD)
20
10
0
-10
-20
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

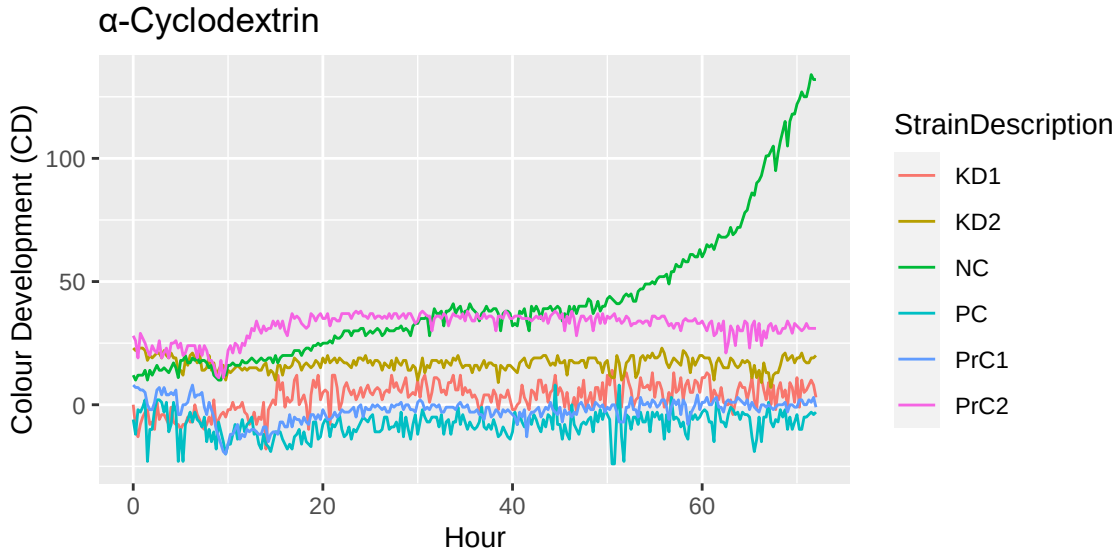

L-Phenylalanine
Colour Development (CD)
60
40
20
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

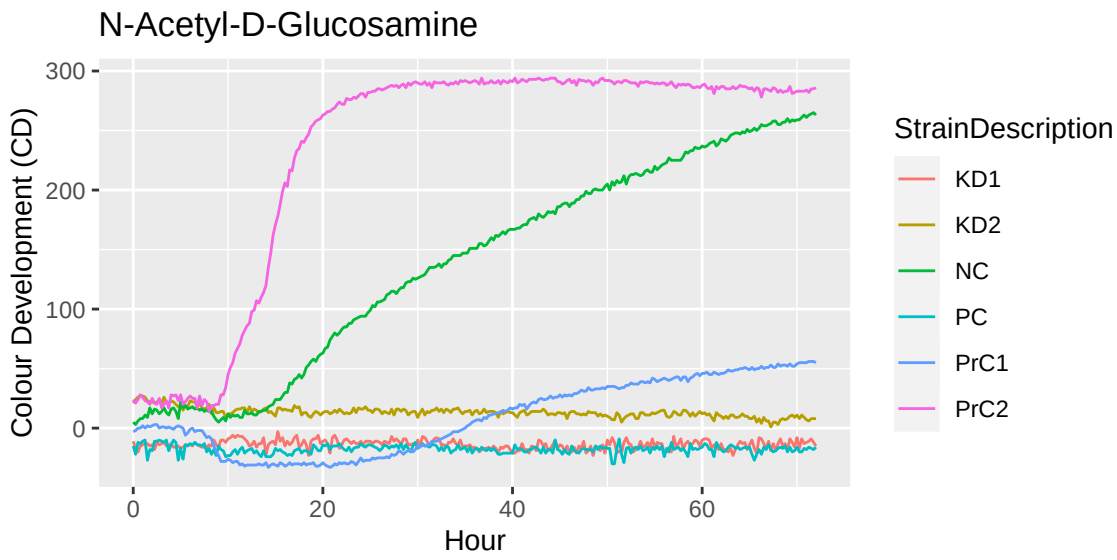

Tween 80
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

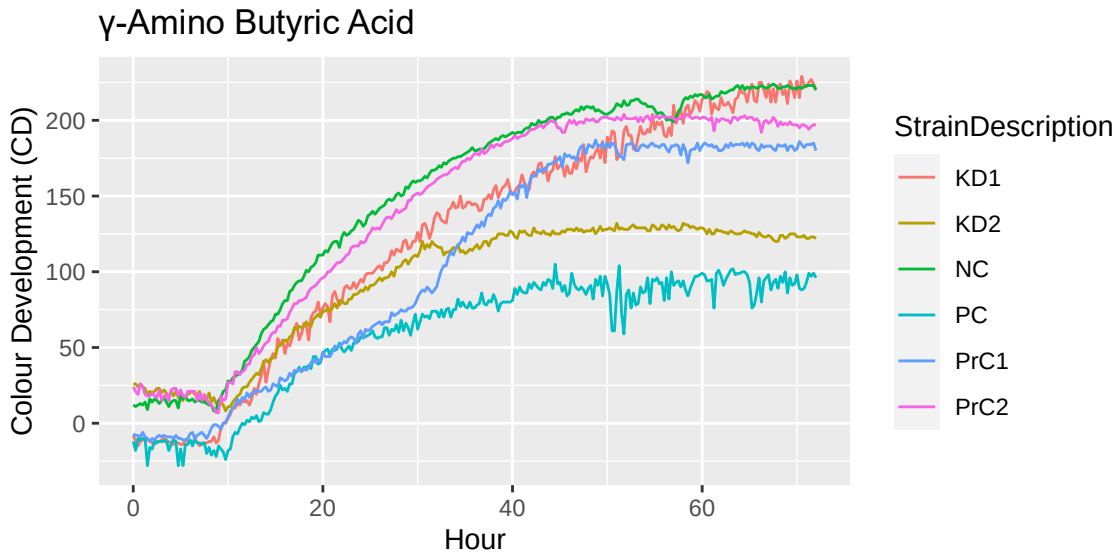

D-Mannitol
Colour Development (CD)
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

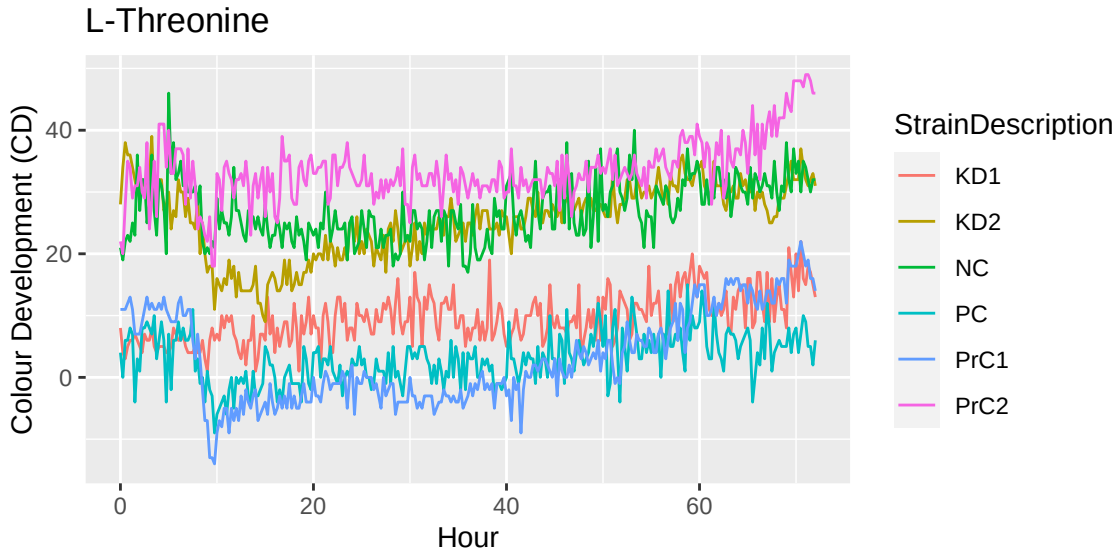

4-Hydroxy Benzoic Acid
Colour Development (CD)
200
150
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

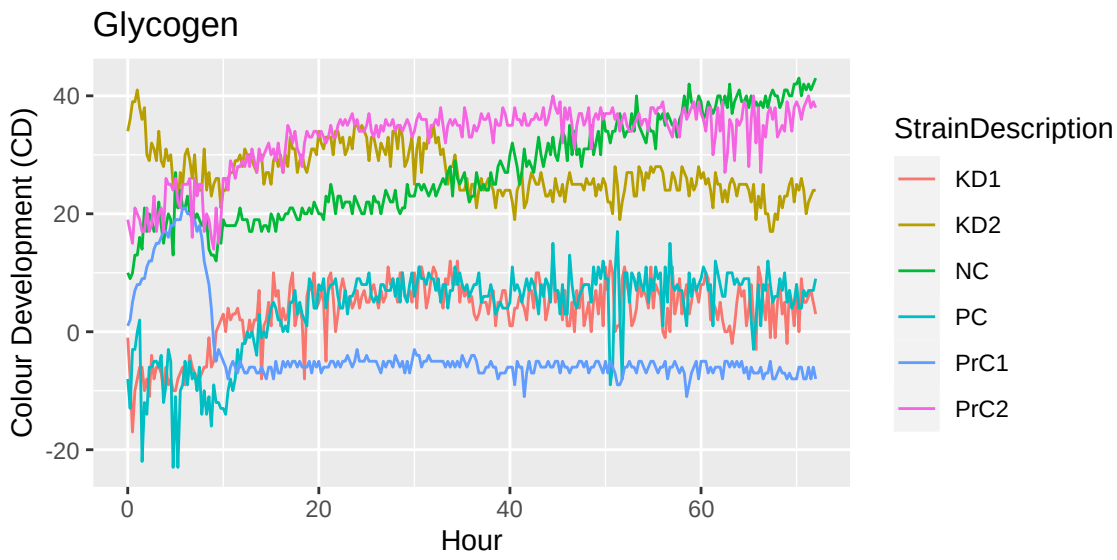

L-Serine
Colour Development (CD)
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

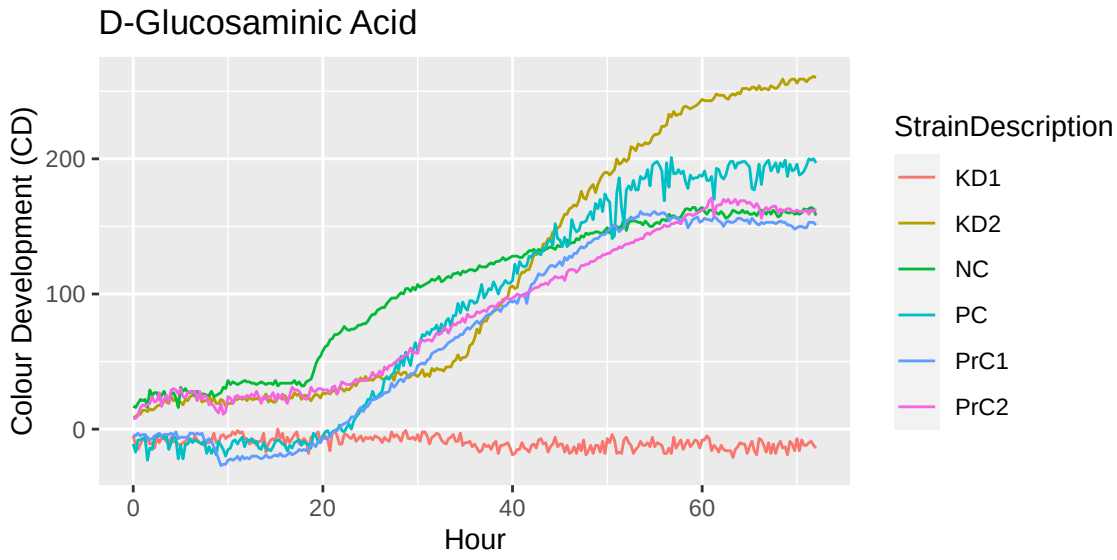

α-Cyclodextrin
Colour Development (CD)
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

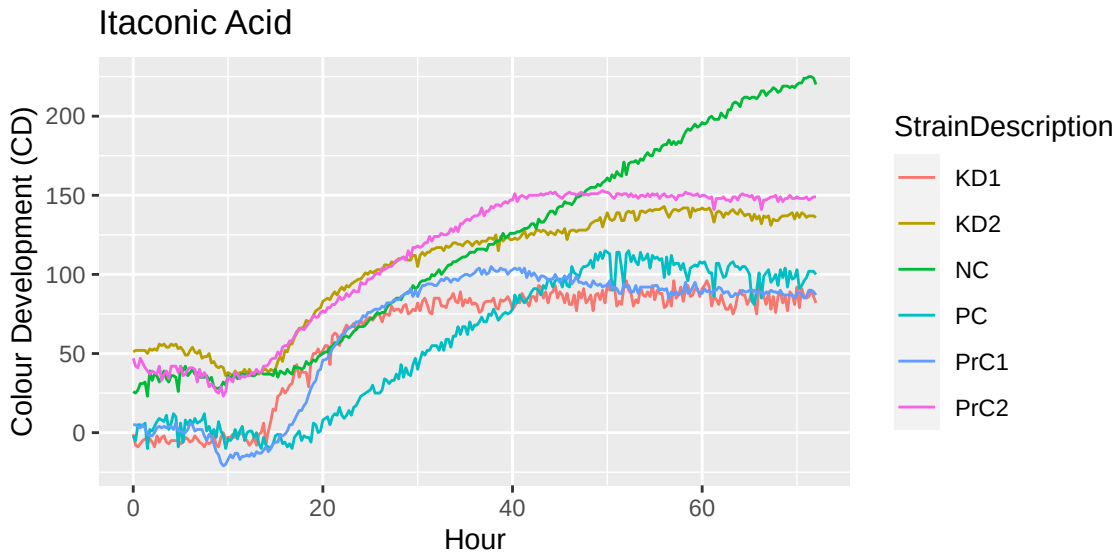

N-Acetyl-D-Glucosamine
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

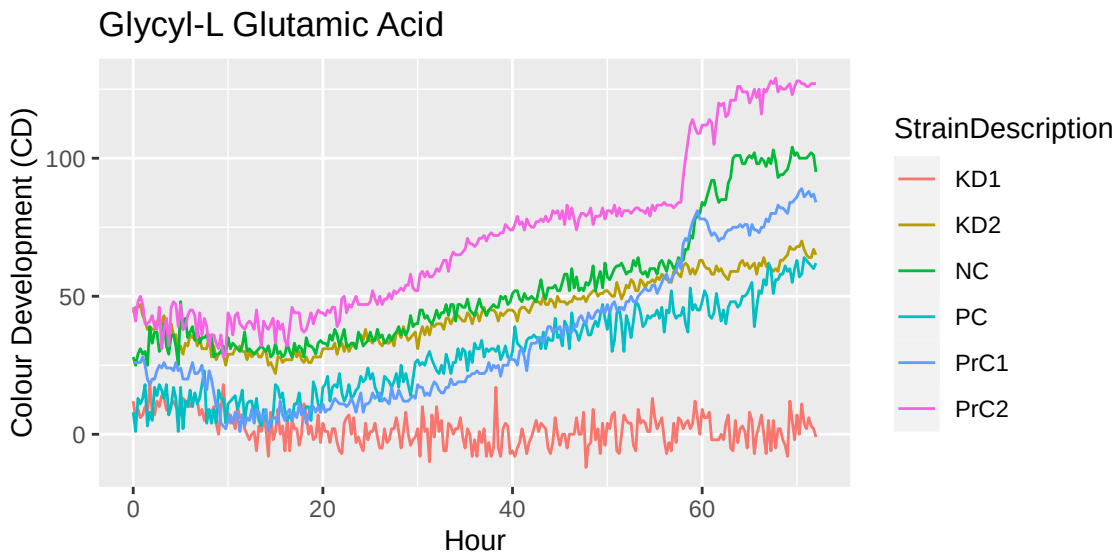

γ-Amino Butyric Acid
Colour Development (CD)
200
150
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

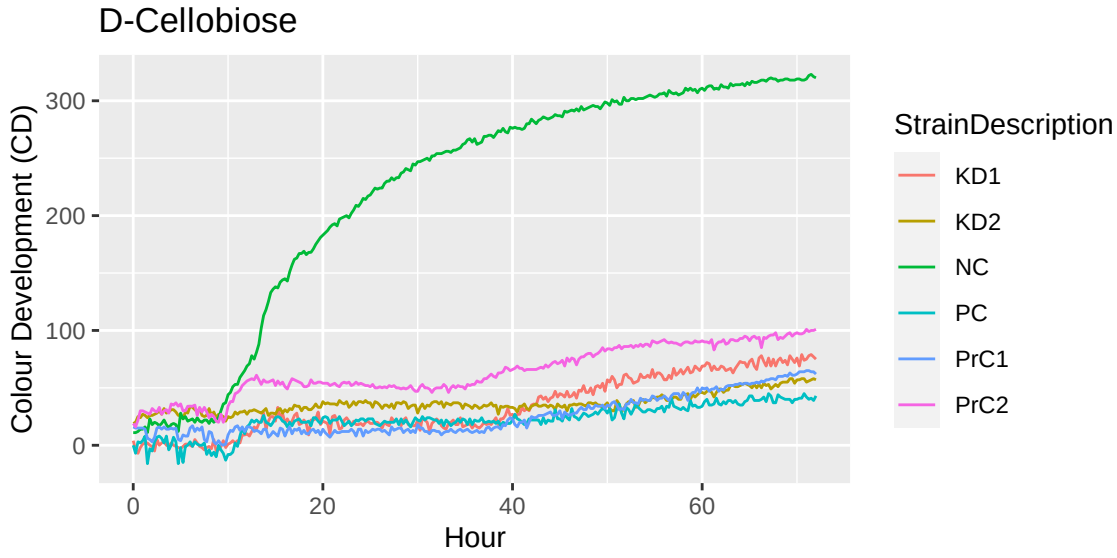

L-Threonine
Colour Development (CD)
40
20
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

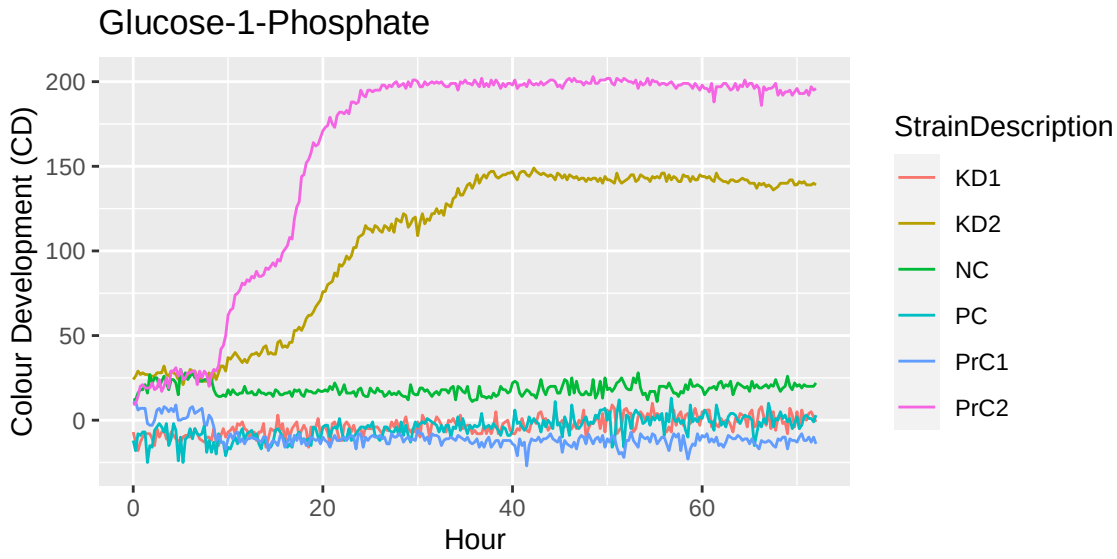

Glycogen
Colour Development (CD)
40
20
0
-20
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

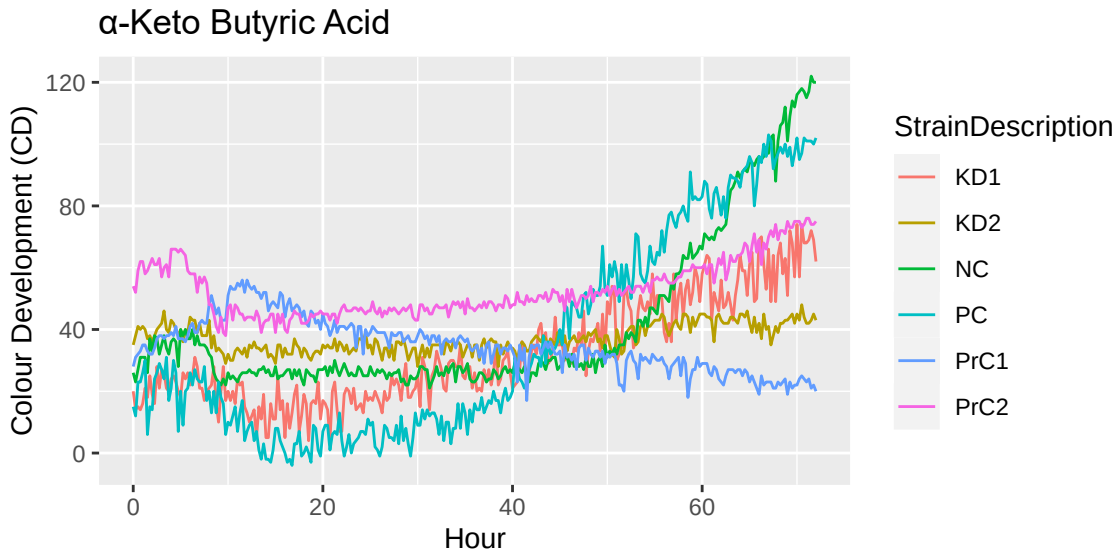

D-Glucosaminic Acid
Colour Development (CD)
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

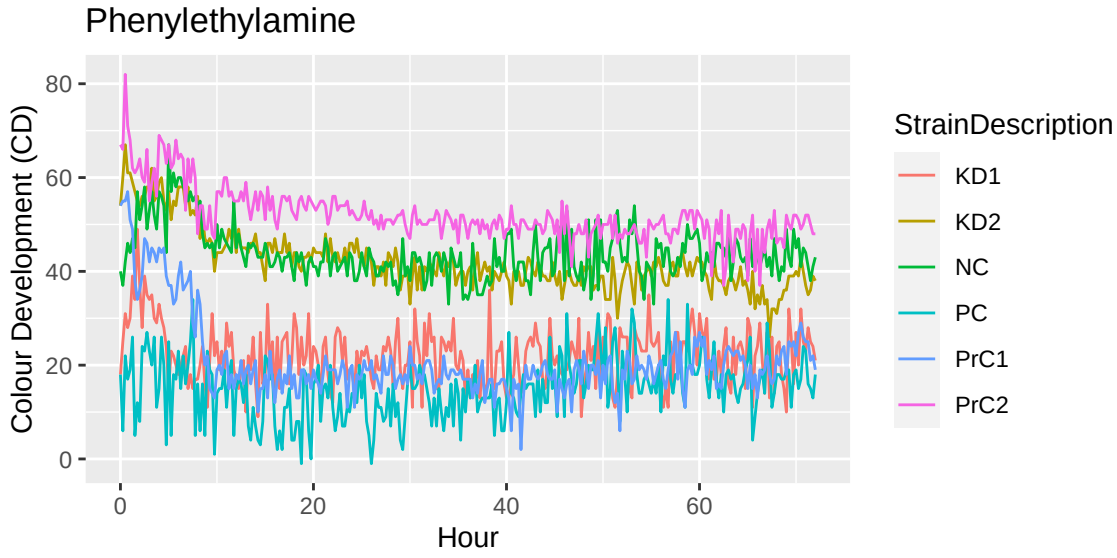

Itaconic Acid
Colour Development (CD)
200
150
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

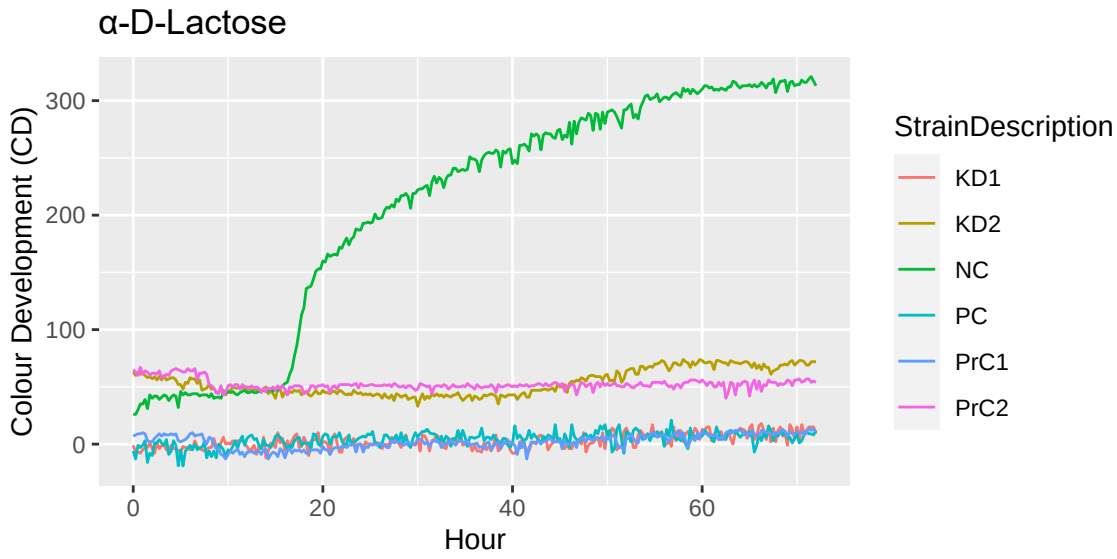

Glycyl-L Glutamic Acid
Colour Development (CD)
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

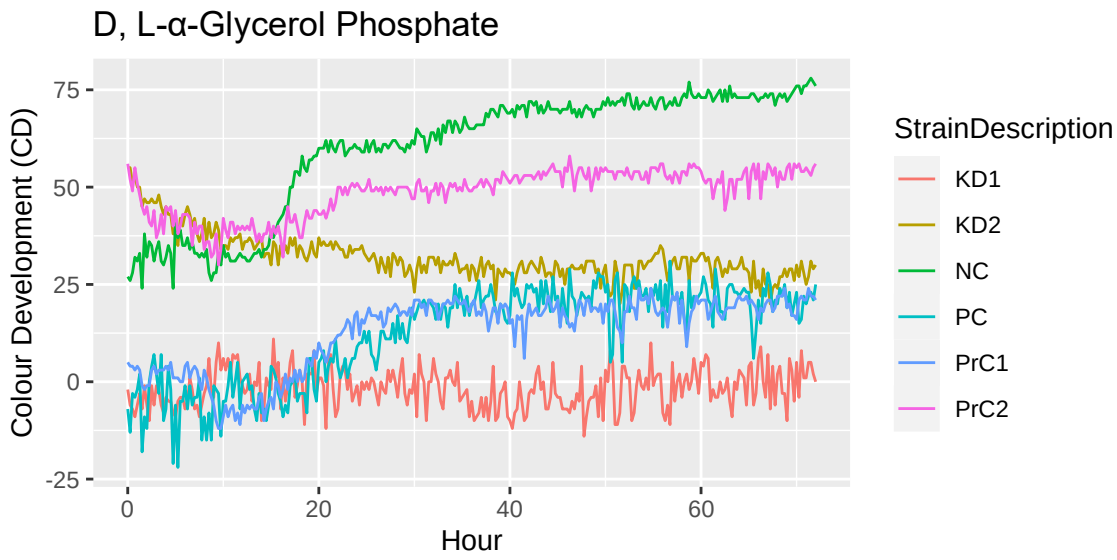

D-Cellobiose
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

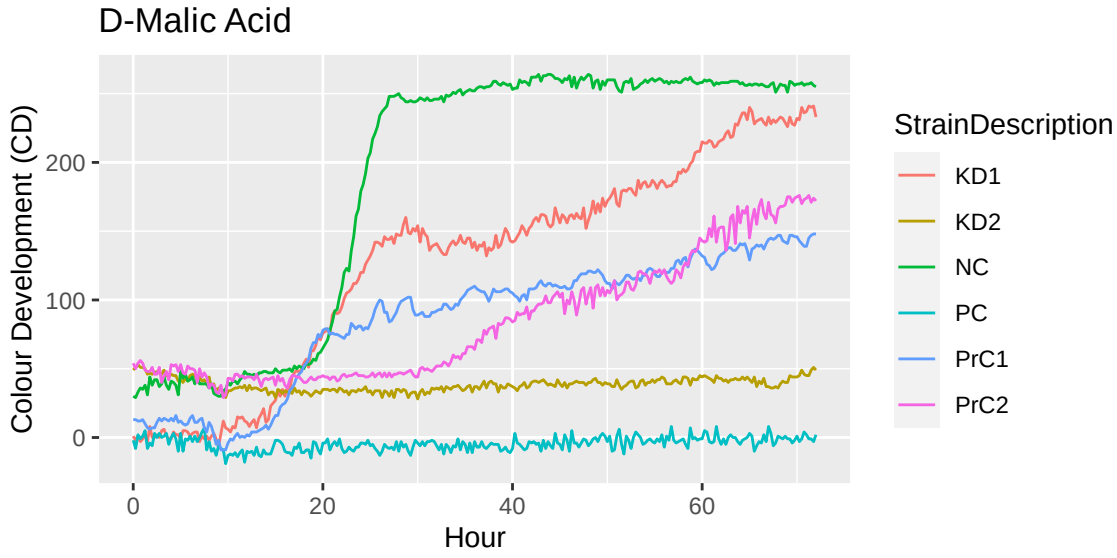

Glucose-1-Phosphate
Colour Development (CD)
200
150
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

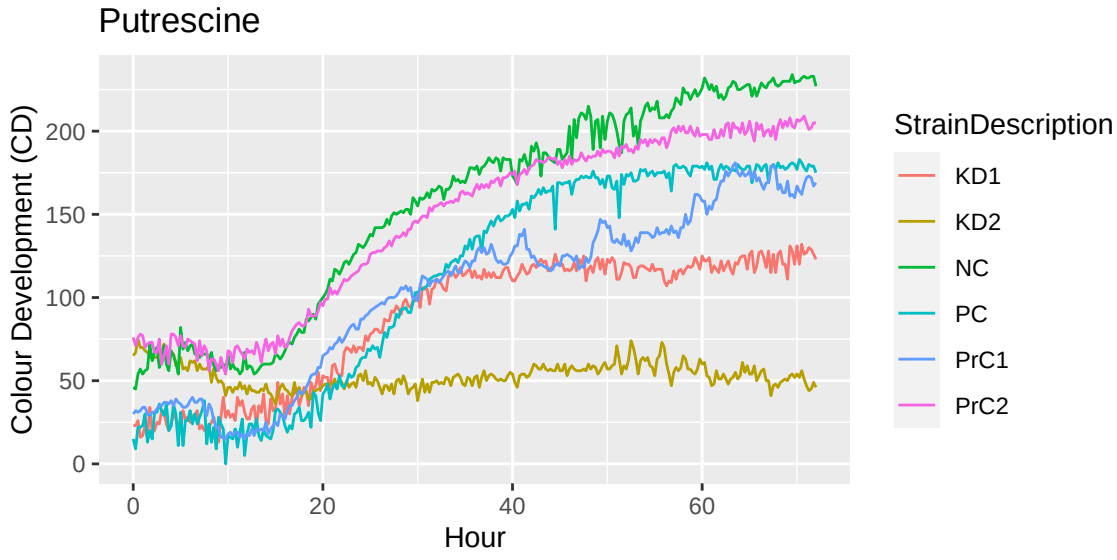

α-Keto Butyric Acid
Colour Development (CD)
120
80
40
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2


Phenylethylamine
Colour Development (CD)
80
60
40
20
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2


α-D-Lactose
Colour Development (CD)
300
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

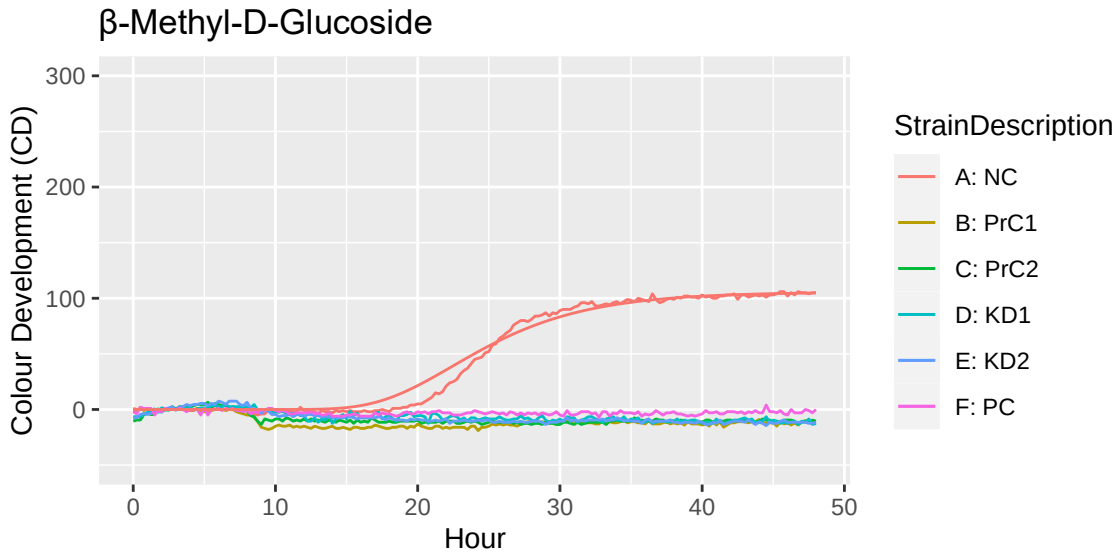

D, L-α-Glycerol Phosphate
Colour Development (CD)
75
50
25
0
-25
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

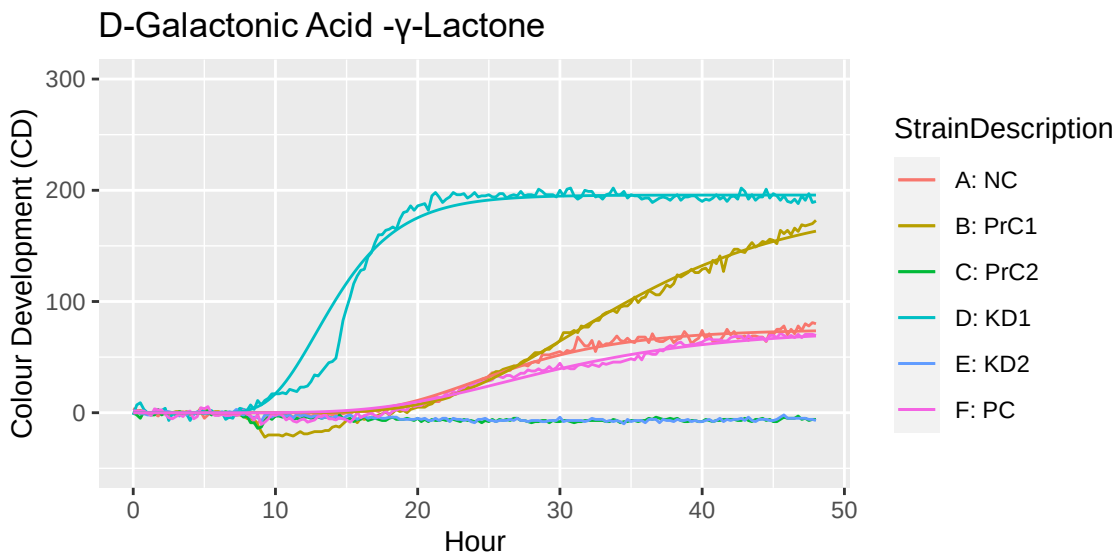

D-Malic Acid
Colour Development (CD)
200
100
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

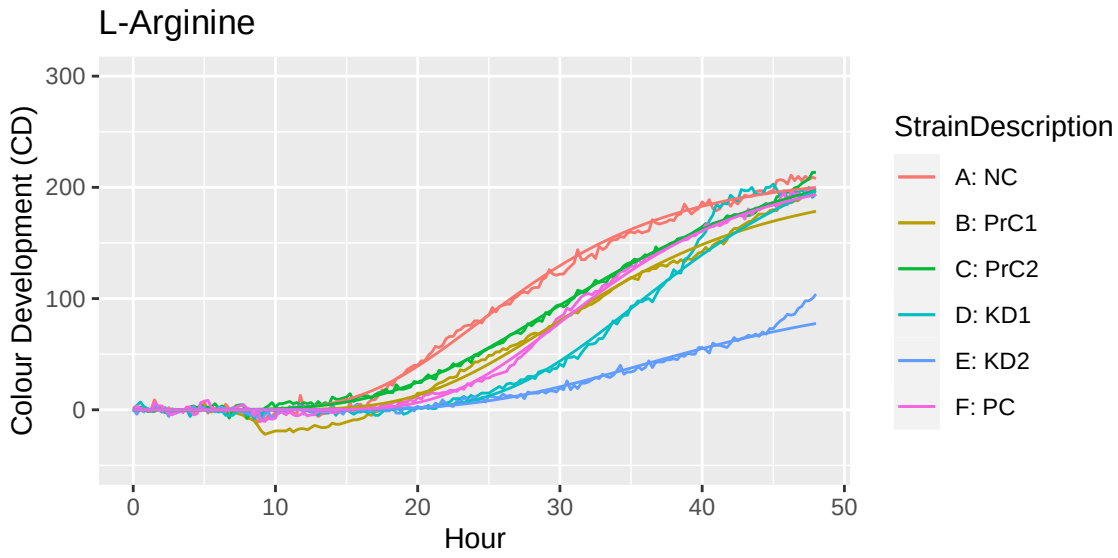

Putrescine
Colour Development (CD)
200
150
100
50
0
0
20
40
60
Hour
StrainDescription
KD1
KD2
NC
PC
PrC1
PrC2

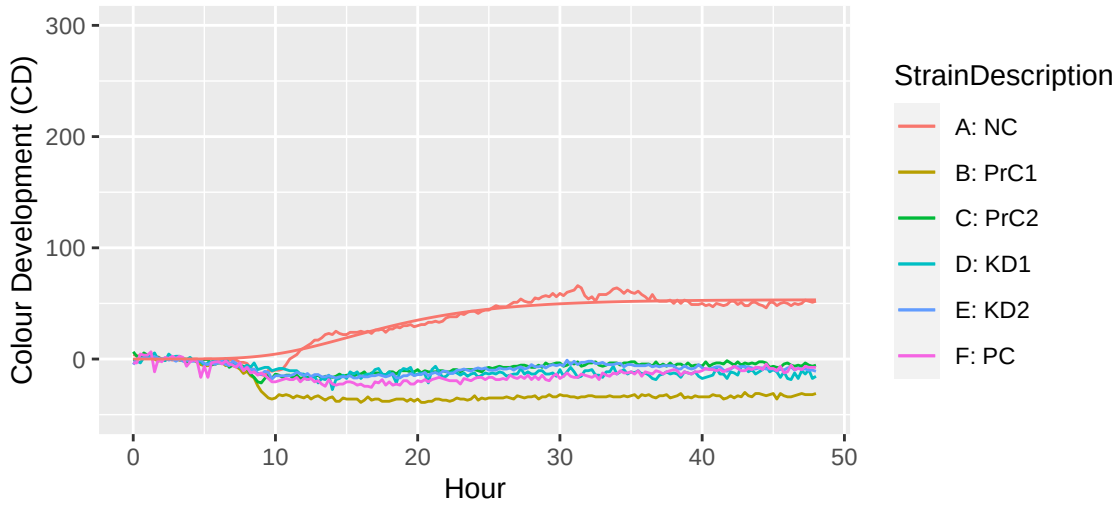
ANNEXURE 3

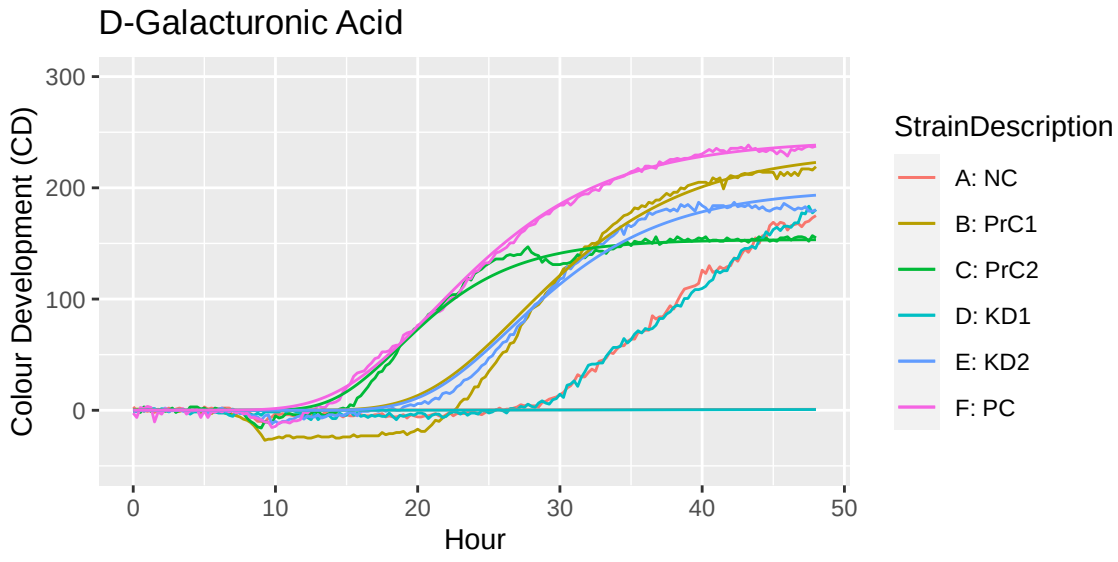
COLOUR DEVELOPMENT BASED ON THE GOMPERTZ FUNCTION



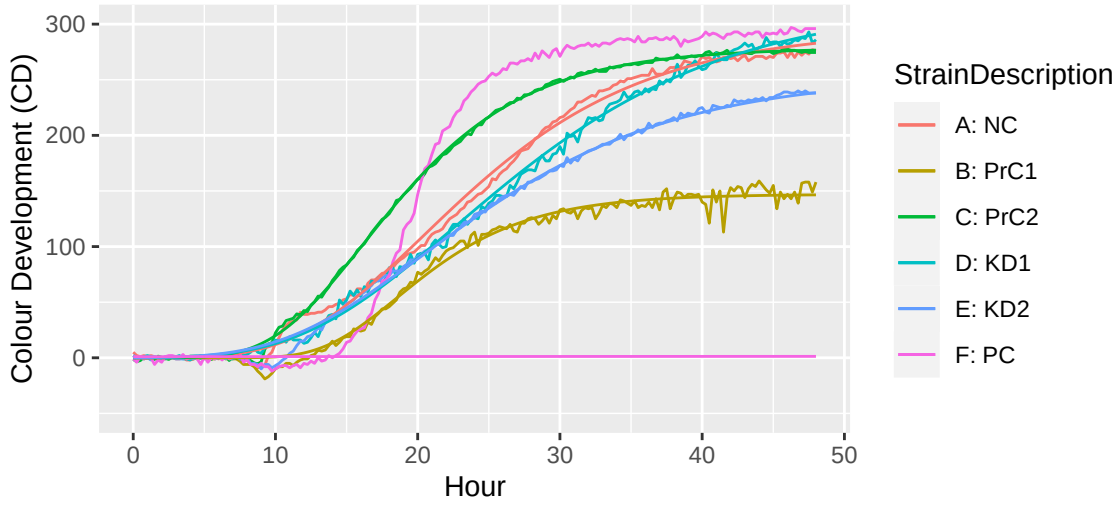



L-Arginine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

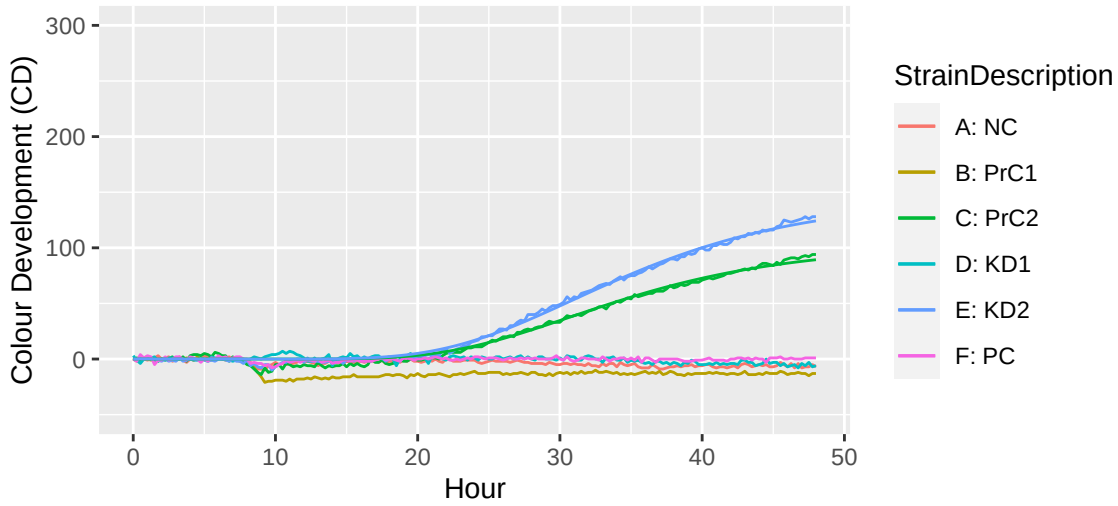

D-Xylose
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC


D-Galacturonic Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

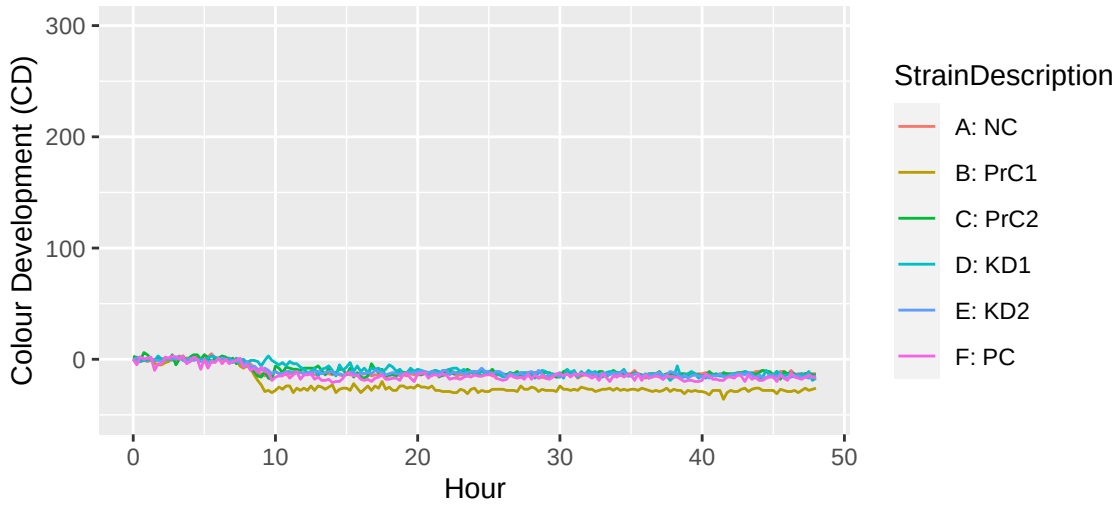
L-Asparagine

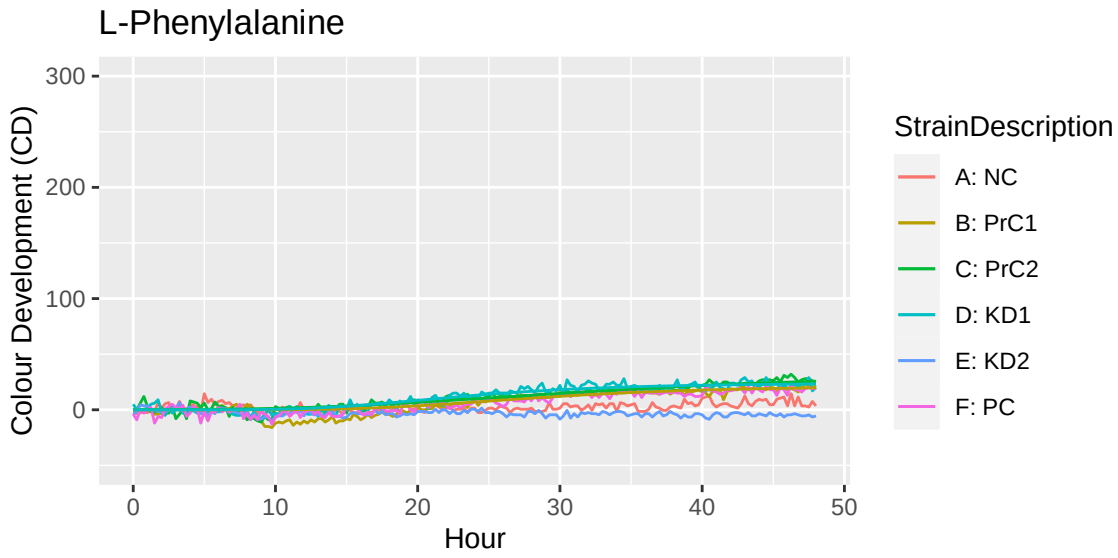


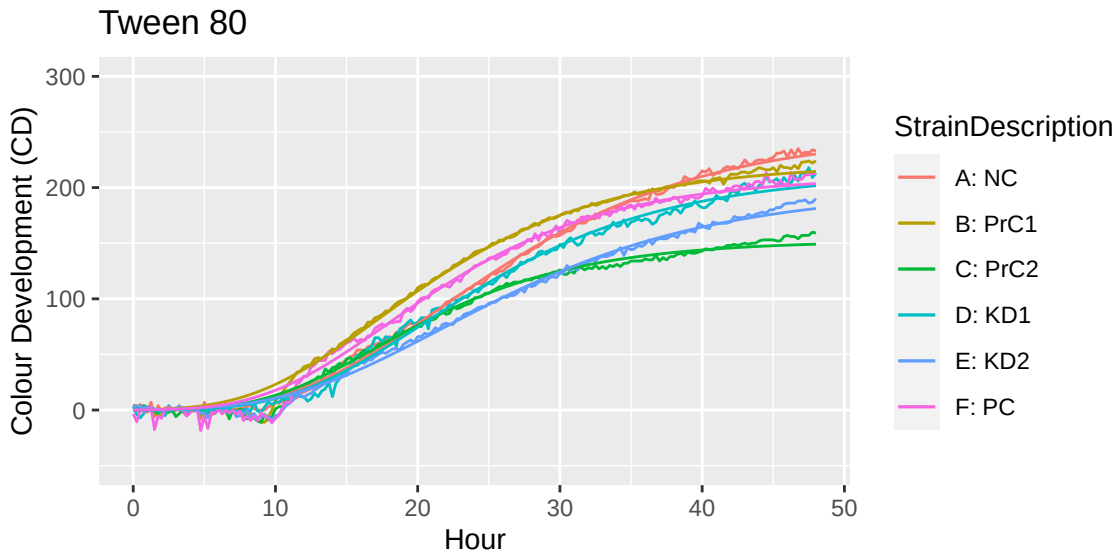
i-Erythritol

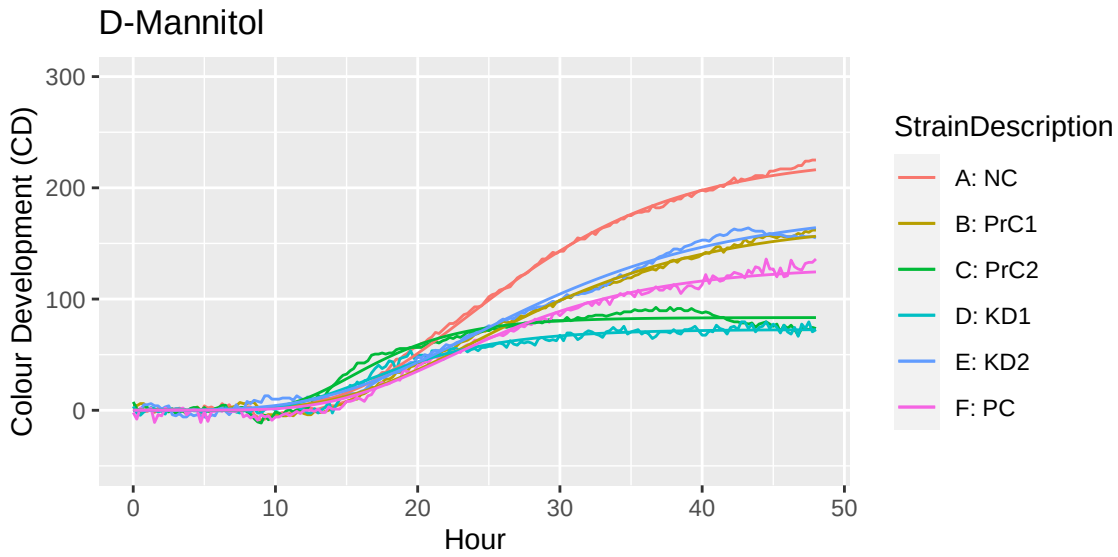


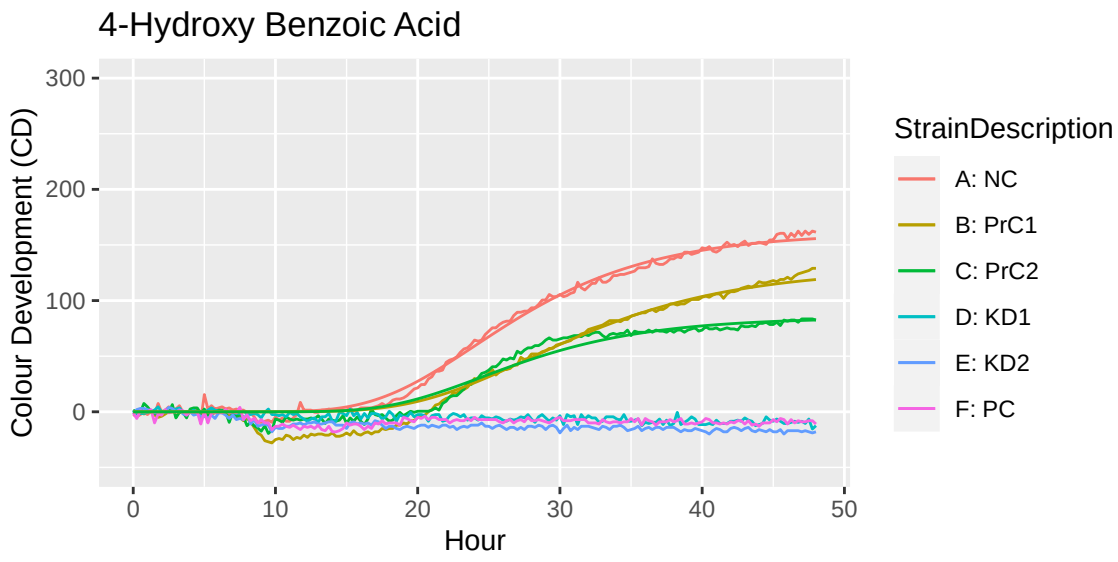
2-Hydroxy Benzoic Acid

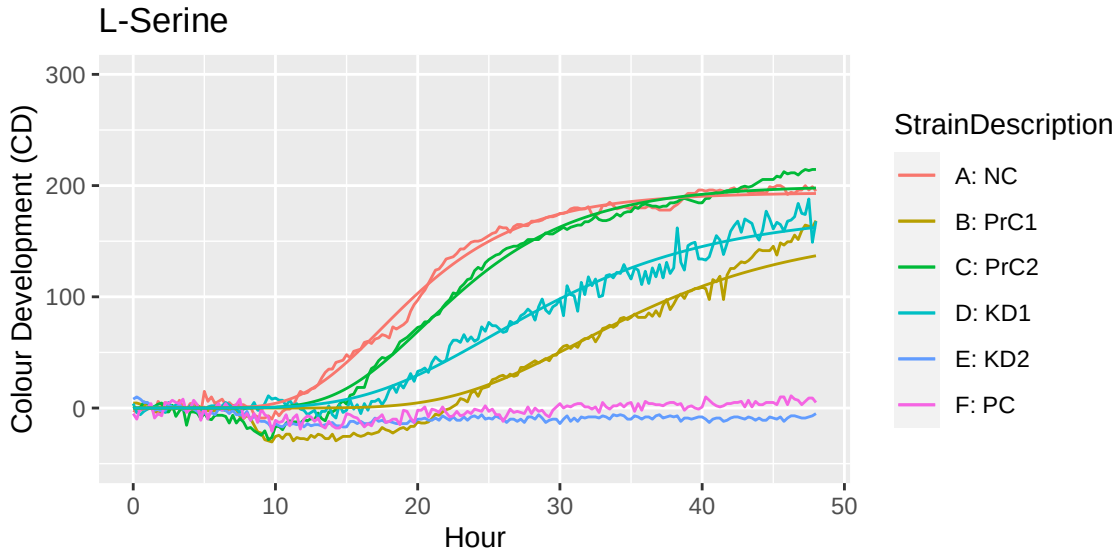


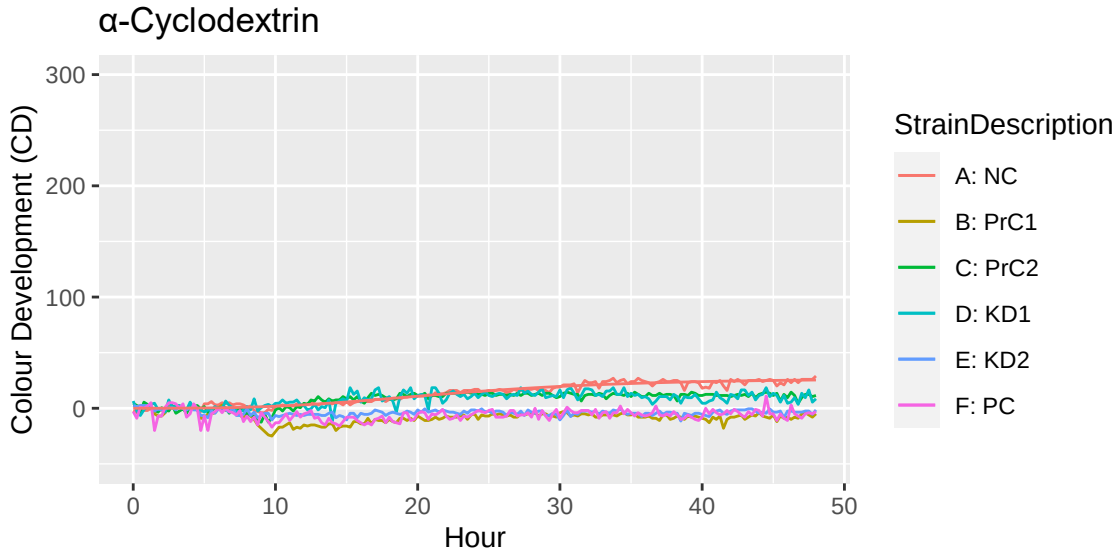

L-Phenylalanine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

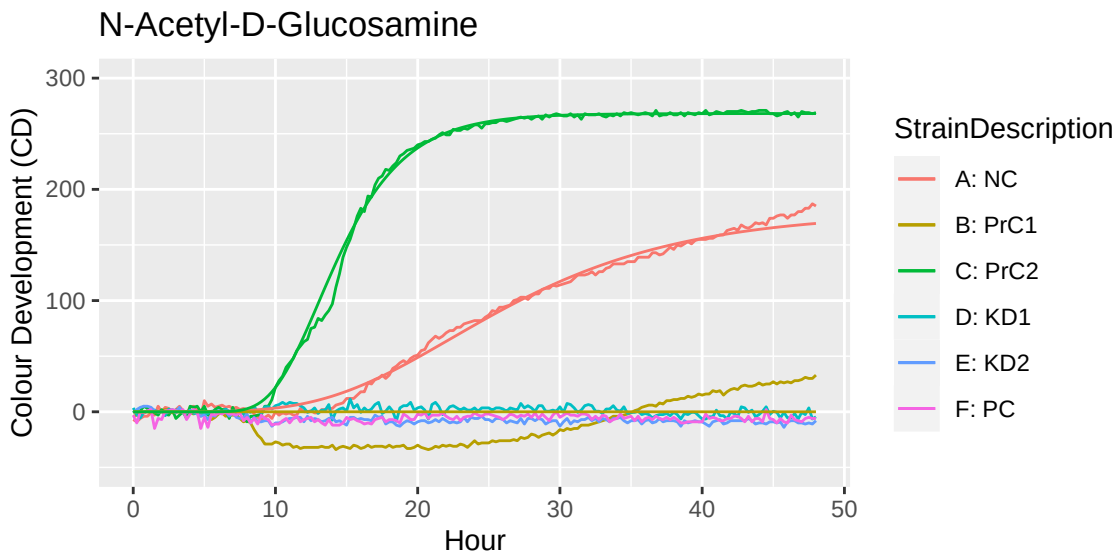

Tween 80
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

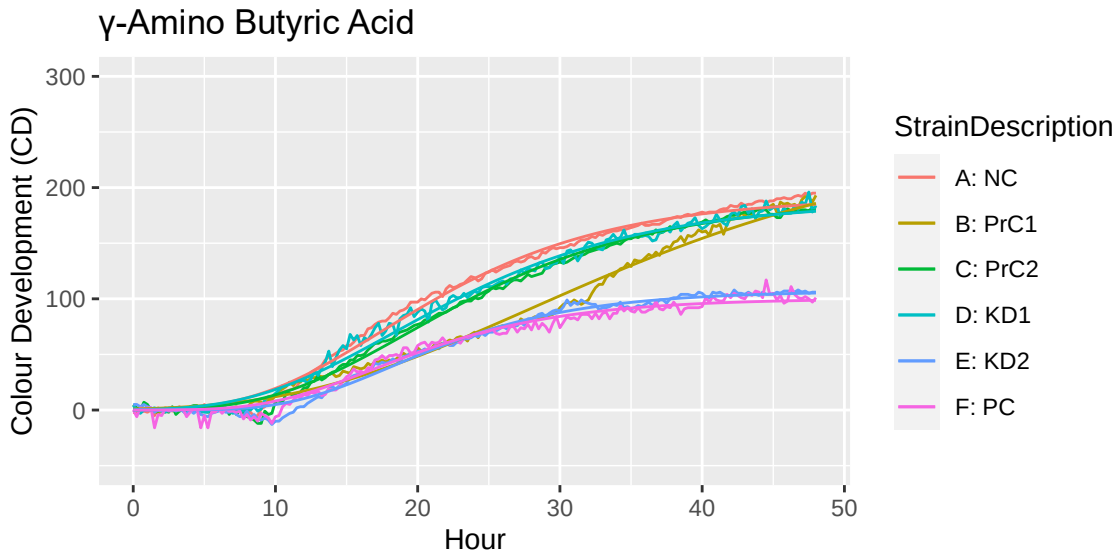

D-Mannitol
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

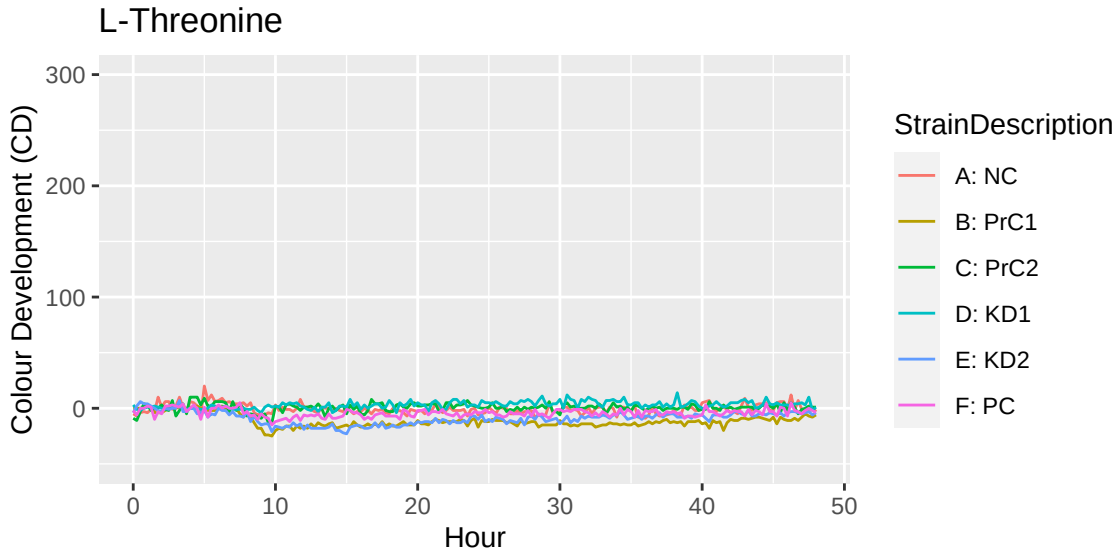

4-Hydroxy Benzoic Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

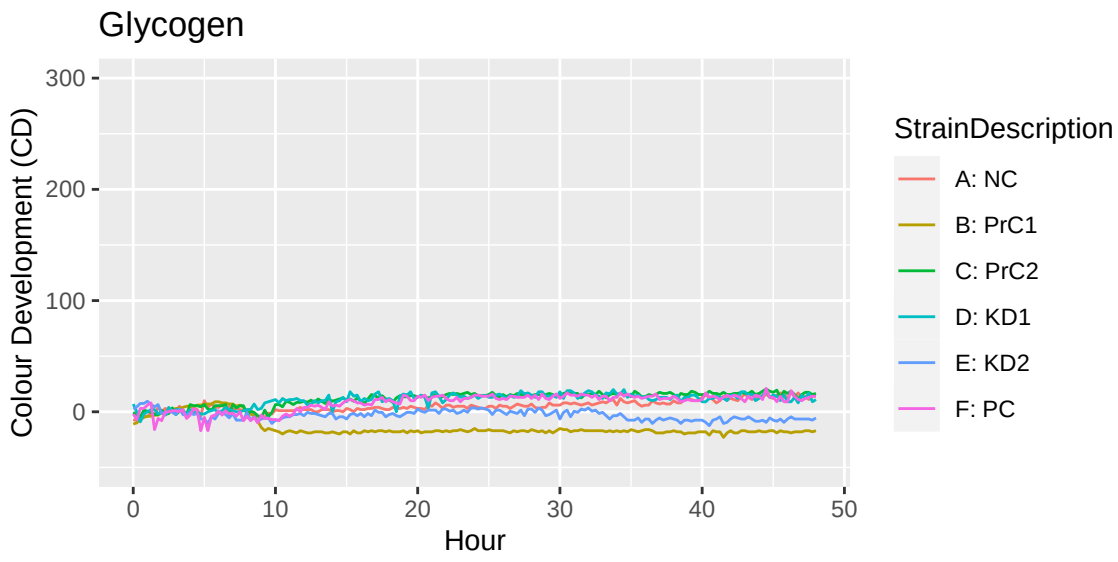

L-Serine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

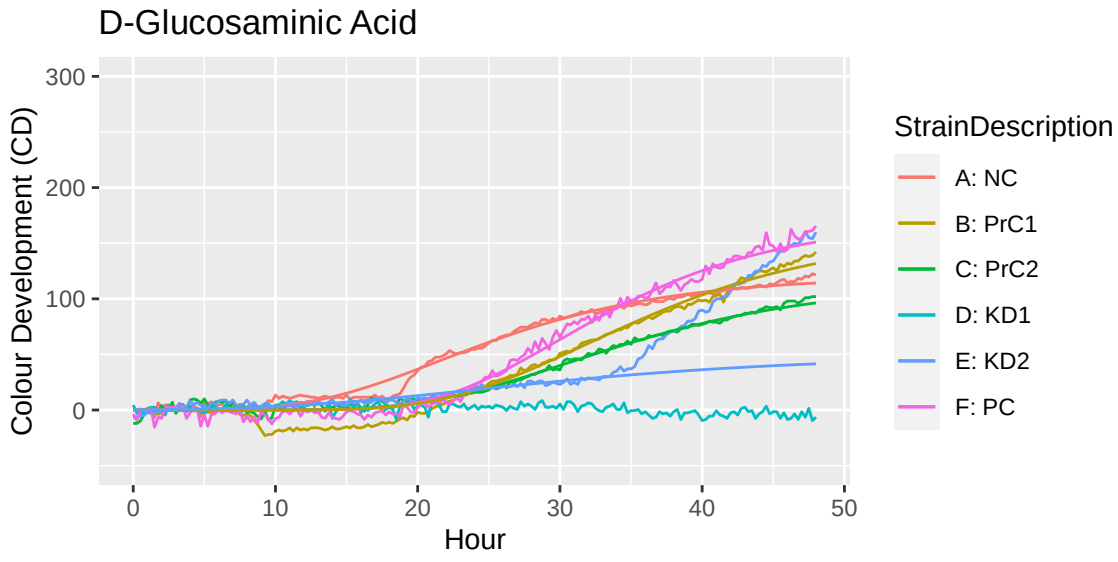

α-Cyclodextrin
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

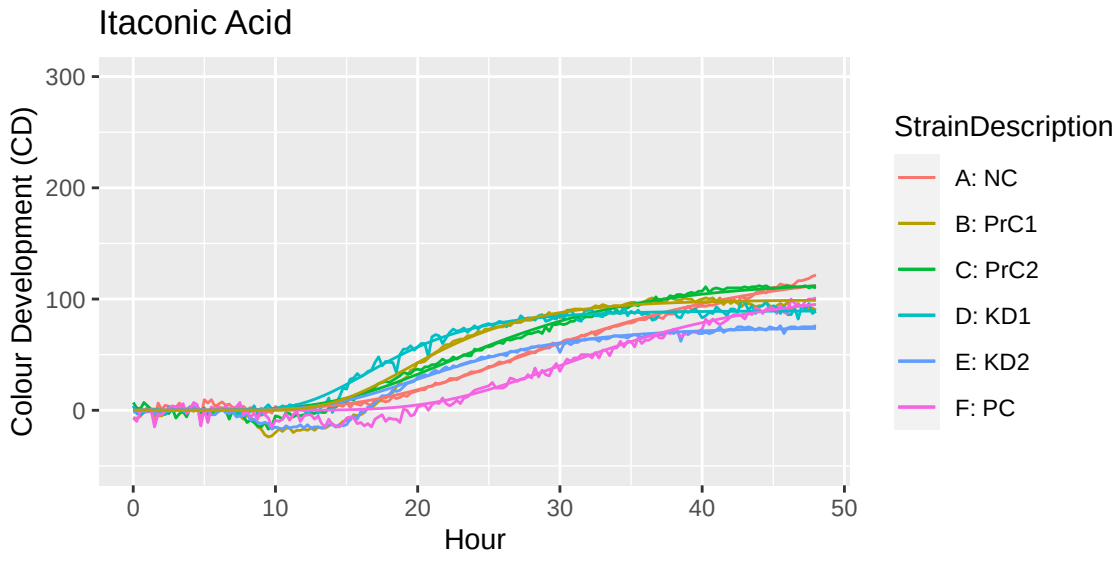

N-Acetyl-D-Glucosamine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

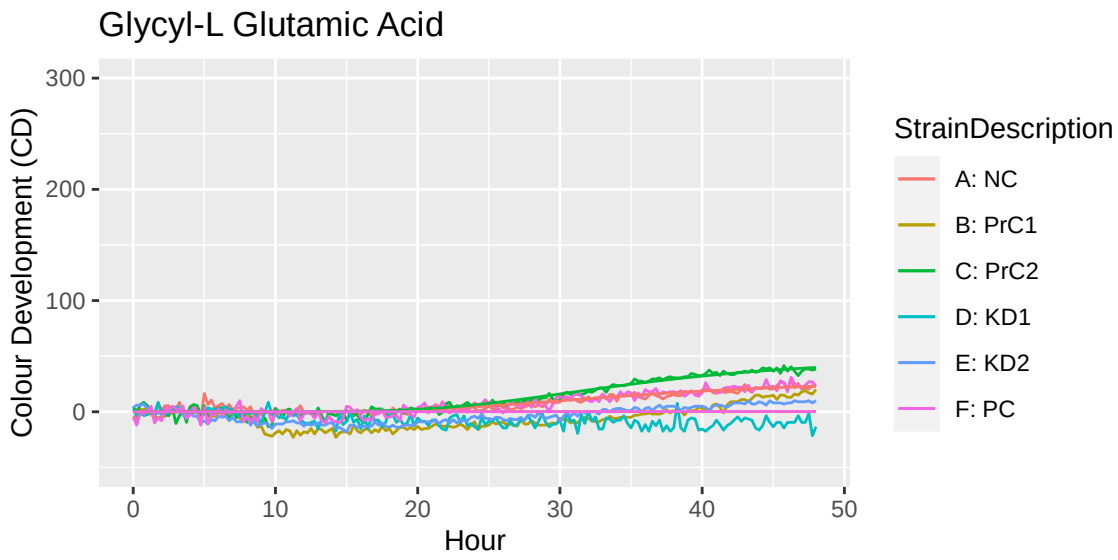

γ-Amino Butyric Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

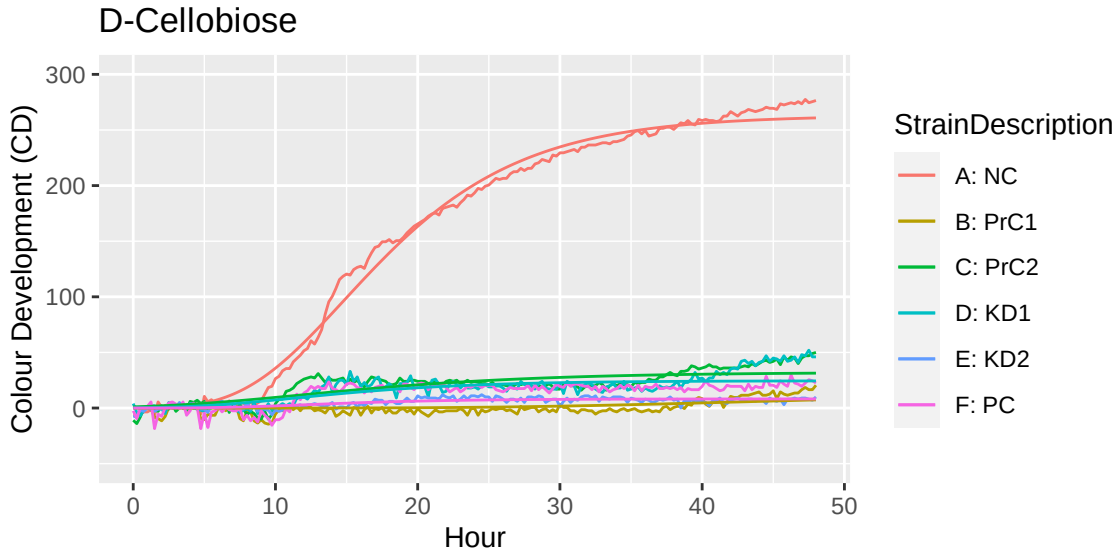

L-Threonine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

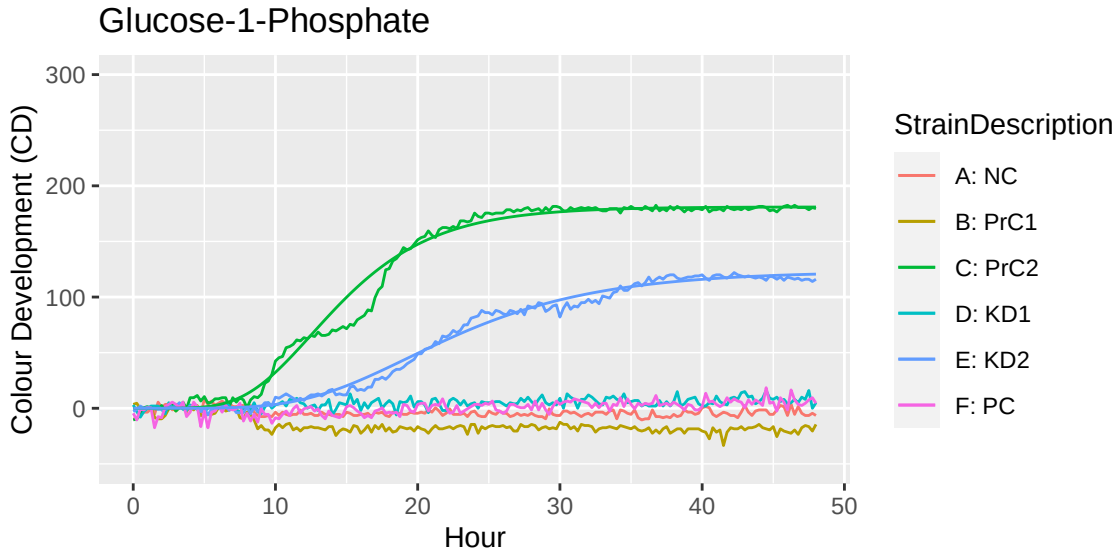

Glycogen
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

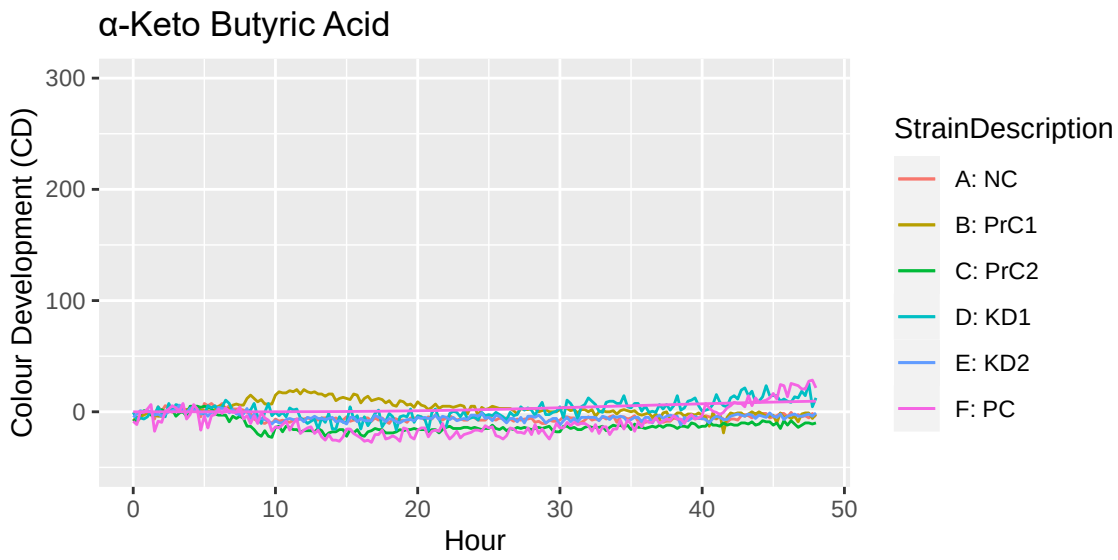

D-Glucosaminic Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

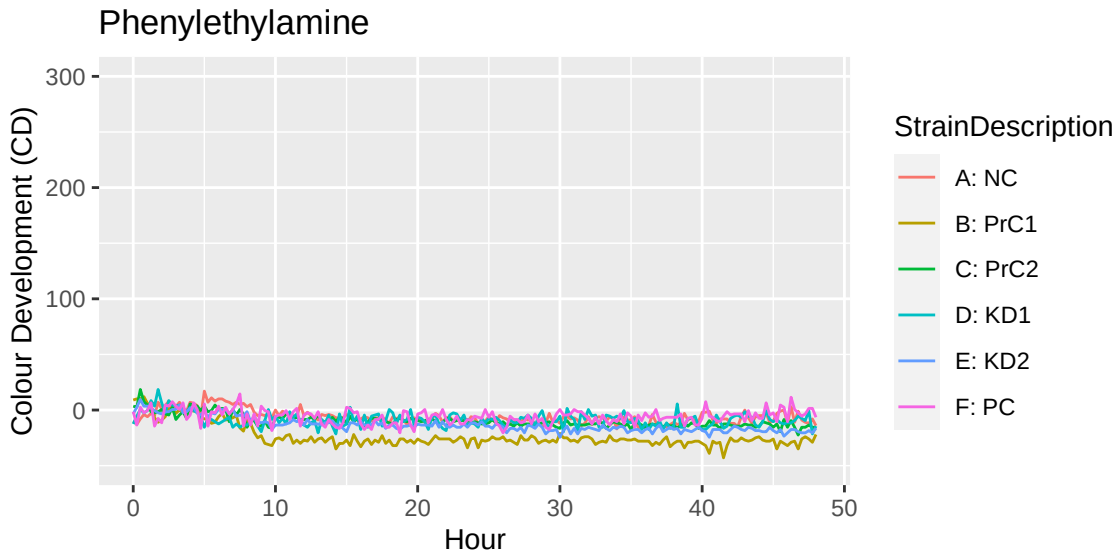

Itaconic Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

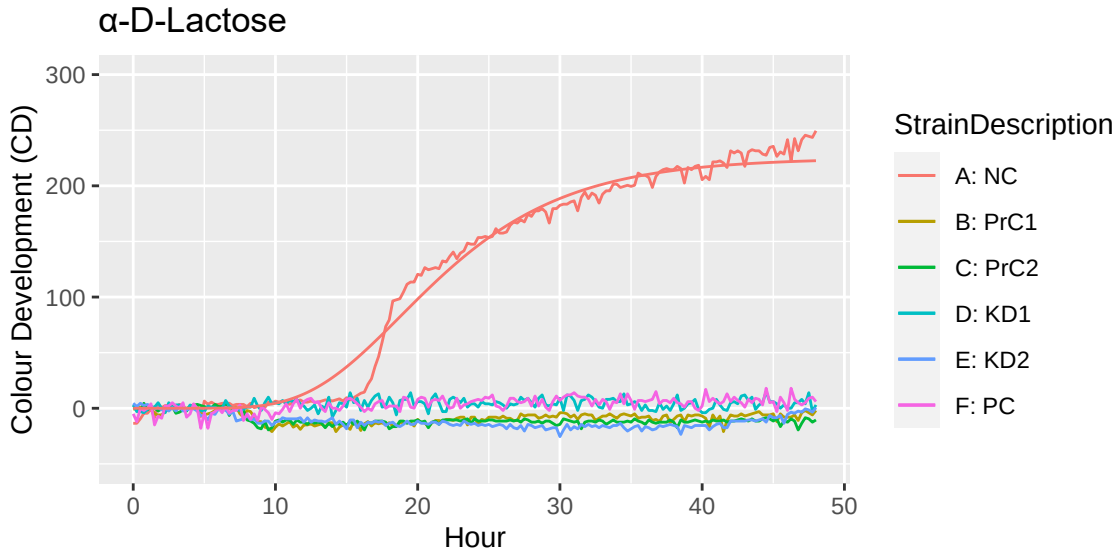

Glycyl-L Glutamic Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

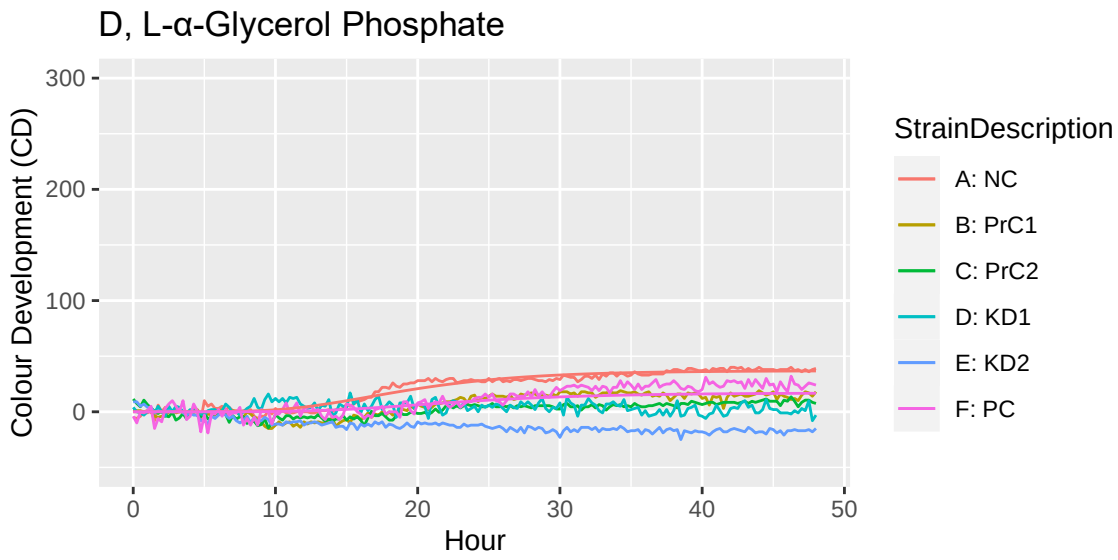

D-Cellobiose
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

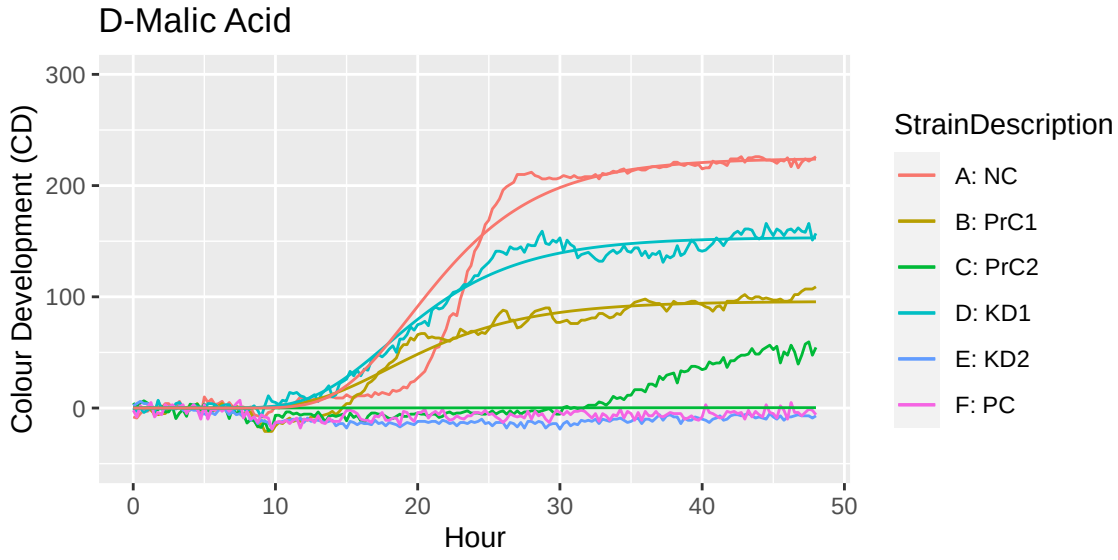

Glucose-1-Phosphate
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

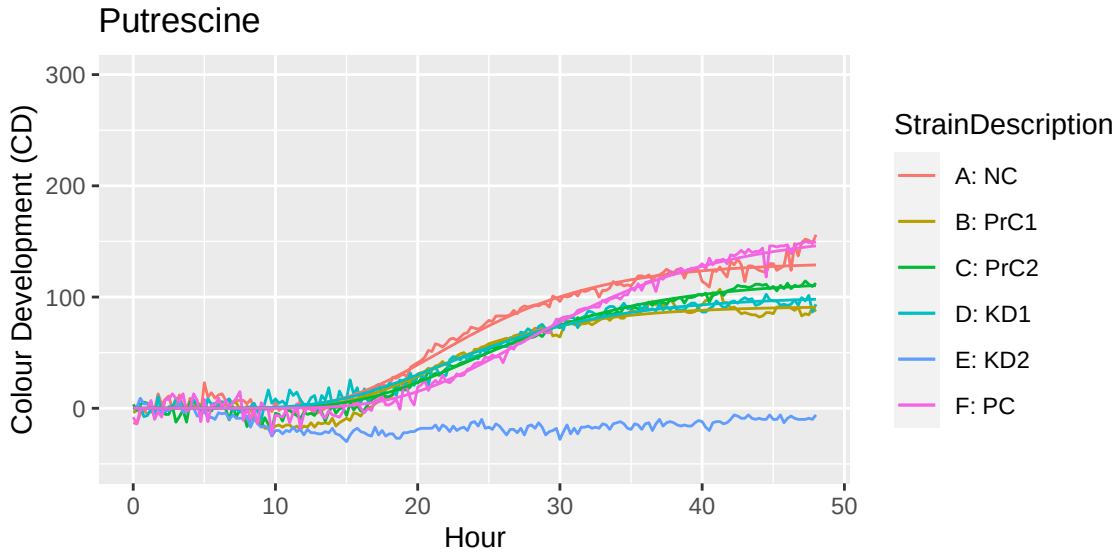

α-Keto Butyric Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC


Phenylethylamine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC


α-D-Lactose
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC


D, L-α-Glycerol Phosphate
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC


D-Malic Acid
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC


Putrescine
Colour Development (CD)
300
200
100
0
0
10
20
30
40
50
Hour
StrainDescription
A: NC
B: PrC1
C: PrC2
D: KD1
E: KD2
F: PC

ANNEXURE 4

GOMPERTZ CURVE PARAMETERS

β-Methyl-D-Glucoside

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	105.6	71.7	9.1	2.4	1.2	104.8	87.6	125.5
B	0.0	0.0	0.0	5.4	100.0	0.0	-18.0	0.0
C	0.0	0.0	0.0	4.8	100.0	0.0	-13.5	2.5
D	0.0	0.0	0.0	4.5	100.0	0.0	-12.5	2.5
E	0.0	0.0	0.0	5.4	100.0	0.0	-14.5	4.5
F	0.0	0.0	0.0	2.0	100.0	0.0	-6.0	1.0

D-Galactonic Acid-γ-Lactone

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	75.0	51.4	7.9	3.1	11.0	73.7	66.5	80.8
B	189.7	29.0	5.3	3.6	1.7	163.5	146.8	182.2
C	0.0	0.0	0.0	2.7	100.0	0.0	-9.0	0.0
D	195.7	53.8	14.9	4.3	1.7	195.7	171.6	220.2
E	0.0	0.0	0.0	2.7	100.0	0.0	-8.0	1.0
F	74.6	20.7	5.6	4.0	31.5	69.2	60.7	77.8

L-Arginine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	210.6	20.1	6.0	5.7	20.4	199.9	188.3	212.0
B	204.2	23.7	5.2	7.8	6.7	178.2	159.6	196.9
C	237.6	13.4	4.3	3.5	5.0	197.9	189.3	206.6
D	253.3	44.6	5.2	3.2	1.3	195.3	173.3	221.7
E	107.6	24.4	4.3	2.4	2.3	76.6	67.9	86.4
F	215.5	42.1	6.0	4.3	14.3	194.0	184.6	203.5

D-Xylose

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	53.4	14.6	8.5	4.6	4.9	53.2	42.0	65.9
B	0.0	0.0	0.0	12.2	100.0	0.0	-38.0	0.0
C	0.0	0.0	0.0	5.2	100.0	0.0	-17.5	0.5
D	0.0	0.0	0.0	5.6	100.0	0.0	-18.5	2.5
E	0.0	0.0	0.0	4.8	100.0	0.0	-17.0	0.0
F	0.0	0.0	0.0	6.8	100.0	0.0	-24.5	0.5

D-Galacturonic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	1.9	4.0	1.6	5.3	0.6	0.9	-368.6	479.3
B	233.3	54.9	7.1	16.8	44.4	220.3	184.9	254.6
C	153.6	58.6	10.5	6.2	7.9	153.7	139.9	168.5
D	1.2	7.7	2.5	4.3	0.5	0.9	-412.1	403.4
E	199.2	75.6	7.8	7.9	14.5	193.1	176.6	211.3
F	243.1	21.7	7.0	5.3	11.5	238.5	226.8	249.8

L-Asparagine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	295.5	10.0	5.4	6.3	7.2	282.2	267.2	297.9
B	147.0	35.3	9.2	5.0	6.0	146.4	134.0	158.8
C	278.5	12.6	7.5	1.8	3.0	276.5	270.4	282.6
D	319.9	8.1	4.5	4.9	26.1	290.9	280.1	301.2
E	253.9	7.8	4.8	2.4	1.7	238.6	227.0	249.8
F	1.6	0.5	0.8	206.3	89.4	0.8	-394.7	440.2

i-Erythritol

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0.0	0.0	0.0	2.7	100.0	0.0	-9.0	1.0
B	0.0	0.0	0.0	5.6	100.0	0.0	-20.0	0.0
C	102.3	34.4	5.5	4.2	88.7	89.3	80.7	98.2
D	0.0	0.0	0.0	2.9	100.0	0.0	-7.0	4.0
E	144.5	30.5	5.3	2.5	12.8	124.1	118.4	129.8
F	0.0	0.0	0.0	1.8	100.0	0.0	-5.0	2.0

2-Hydroxy Benzoic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0	0	0	5.4	100	0	-17	1
B	0	0	0	10.1	100	0	-31	0
C	0	0	0	5.4	100	0	-18	2
D	0	0	0	5.7	100	0	-18	1
E	0	0	0	5.0	100	0	-17	0
F	0	0	0	6.0	100	0	-20	1

L-Phenylalanine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0.0	0.0	0.0	3.9	100.0	0.0	-3.5	9.5
B	21.1	18.0	5.6	2.3	1.6	19.6	7.6	32.1
C	31.9	6.5	3.4	2.3	3.2	26.0	18.8	33.1
D	23.7	14.8	6.4	3.4	34.0	23.3	16.3	30.7
E	0.0	0.0	0.0	2.5	100.0	0.0	-7.5	1.5
F	0.0	0.0	0.0	8.8	100.0	0.0	-9.0	20.0

Tween 80

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	249.3	7.9	4.6	4.1	22.2	230.1	221.4	238.6
B	221.1	7.0	5.4	2.5	1.8	214.7	201.5	226.8
C	152.2	8.6	6.0	3.5	3.3	149.2	139.0	160.4
D	212.8	8.8	5.1	5.6	16.3	201.9	189.4	213.1
E	198.1	7.3	4.4	4.2	4.5	181.2	170.1	192.3
F	210.0	7.8	5.5	4.3	2.2	203.7	185.9	219.8

D-Mannitol

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	229.2	15.3	5.6	4.2	12.5	216.1	207.0	225.4
B	170.1	12.4	5.0	4.4	29.0	156.7	147.3	165.5
C	83.4	31.4	10.8	5.0	19.8	83.3	72.3	93.8
D	72.6	19.8	8.8	3.3	4.3	72.3	63.5	81.3
E	181.0	9.8	4.6	5.2	27.4	162.9	152.4	174.0
F	129.4	15.8	6.0	4.6	13.2	124.7	114.1	134.0

4-Hydroxy Benzoic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	160.9	30.4	6.8	4.3	33.6	155.8	147.3	164.9
B	128.9	31.6	6.0	2.2	0.8	119.1	68.8	173.2
C	84.8	42.2	7.3	6.7	47.1	82.3	68.9	96.1
D	0.0	0.0	0.0	4.0	100.0	0.0	-12.5	1.5
E	0.0	0.0	0.0	5.4	100.0	0.0	-20.0	0.0
F	0.0	0.0	0.0	4.2	100.0	0.0	-17.0	0.0

L-Serine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	193.7	23.1	8.6	5.8	22.9	193.2	180.8	205.1
B	159.9	33.0	5.4	14.1	17.8	137.5	107.0	168.6
C	199.9	28.7	7.9	8.6	13.3	198.4	180.4	218.4
D	175.5	16.8	5.4	7.7	7.5	162.0	142.7	179.2
E	0.0	0.0	0.0	4.8	100.0	0.0	-18.0	0.0
F	0.0	0.0	0.0	6.9	100.0	0.0	-18.0	8.0

α-Cyclodextrin

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	26.5	7.9	5.2	1.9	4.9	25.4	20.5	30.1
B	0.0	0.0	0.0	5.1	100.0	0.0	-18.0	2.0
C	0.0	0.0	0.0	6.3	100.0	0.0	-4.5	14.5
D	0.0	0.0	0.0	6.1	100.0	0.0	-1.5	18.5
E	0.0	0.0	0.0	2.5	100.0	0.0	-8.5	1.5
F	0.0	0.0	0.0	4.9	100.0	0.0	-15.0	4.0

N-Acetyl-D-Glucosamine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	179.3	12.1	5.4	6.7	22.9	169.6	155.3	183.8
B	0.0	1.5	0.1	23.3	92.2	0.1	-46.4	44.7
C	268.2	51.3	14.5	2.0	1.6	268.2	257.9	279.5
D	0.0	0.0	0.0	3.7	100.0	0.0	-6.5	7.5
E	0.0	0.0	0.0	3.7	100.0	0.0	-13.0	1.0
F	0.0	0.0	0.0	3.4	100.0	0.0	-12.0	1.0

γ-Amino Butyric Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	191.2	7.0	5.4	5.5	13.0	185.0	173.3	197.5
B	249.0	5.6	3.0	7.0	38.8	187.3	173.1	201.9
C	188.7	7.4	5.0	4.0	5.6	179.3	168.8	190.0
D	187.6	6.4	4.9	6.1	13.6	178.0	165.4	191.1
E	107.0	12.1	6.6	4.2	6.0	105.3	95.6	115.7
F	100.2	9.6	6.4	4.7	4.6	98.3	86.1	111.6

L-Threonine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0	0	0	4.5	100	0	-8	7
B	0	0	0	5.7	100	0	-18	2
C	0	0	0	3.8	100	0	-9	7
D	0	0	0	3.2	100	0	-4	8
E	0	0	0	5.7	100	0	-20	0
F	0	0	0	3.6	100	0	-10	3

Glycogen

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0	0	0	5.0	100	0	-5.0	15.0
B	0	0	0	8.0	100	0	-20.0	7.0
C	0	0	0	6.3	100	0	-2.5	18.5
D	0	0	0	5.9	100	0	-2.0	18.0
E	0	0	0	4.1	100	0	-10.5	3.5
F	0	0	0	7.6	100	0	-8.0	16.0

D-Glucosaminic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	120.4	11.0	5.3	4.0	5.3	113.8	104.2	124.3
B	159.9	25.1	4.9	4.1	1.6	131.7	107.2	155.3
C	114.6	21.2	4.8	4.0	24.0	96.5	88.5	104.9
D	0.0	0.0	0.0	4.2	100.0	0.0	-8.5	7.5
E	49.3	5.8	3.5	2.8	0.6	41.4	-86.4	210.0
F	170.3	33.0	5.6	5.7	20.5	151.6	139.7	164.2

Itaconic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	129.1	12.7	4.5	3.0	12.3	111.9	105.3	118.7
B	99.2	39.4	9.2	2.8	1.1	98.9	71.2	124.8
C	116.5	14.9	5.9	3.8	6.3	112.0	102.5	121.1
D	89.3	35.5	10.5	2.8	4.1	89.5	81.5	98.6
E	74.5	22.1	7.5	2.1	1.2	73.6	55.3	92.6
F	108.1	30.6	5.5	5.5	15.3	94.4	82.1	106.5

Glycyl-L-Glutamic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	27.8	18.0	4.6	2.6	6.1	22.4	16.0	28.6
B	0.2	1.1	0.7	11.4	28.7	0.1	-24.1	23.3
C	47.0	24.5	5.0	2.7	3.2	40.0	32.2	47.8
D	0.0	0.0	0.0	6.0	100.0	0.0	-17.5	2.5
E	0.0	0.0	0.0	7.3	100.0	0.0	-14.0	9.0
F	0.4	2.6	1.6	7.7	132.8	0.2	-16.0	16.1

D-Cellobiose

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	263.1	8.3	6.9	8.8	15.8	260.9	242.5	279.8
B	15.5	13.4	2.9	4.9	19.1	7.9	-3.0	18.1
C	32.1	3.4	5.0	7.7	26.9	31.8	15.4	47.7
D	24.5	5.0	6.8	5.5	3.3	23.7	5.2	43.2
E	0.0	0.0	0.0	4.2	100.0	0.0	-3.0	11.0
F	8.2	7.6	8.0	2.4	11.8	8.1	2.9	13.6

Glucose-1-Phosphate

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0.0	0.0	0.0	3.6	100.0	0.0	-9.5	3.5
B	0.0	0.0	0.0	6.8	100.0	0.0	-23.5	0.5
C	181.0	14.4	10.2	4.1	1.6	181.0	160.0	202.3
D	0.0	0.0	0.0	4.4	100.0	0.0	-6.0	12.0
E	123.1	13.9	6.5	4.7	7.8	120.1	109.1	131.1
F	0.0	0.0	0.0	5.7	100.0	0.0	-11.5	9.5

α-Keto Butyric Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0.0	0.0	0.0	4.0	100.0	0.0	-10.5	4.5
B	0.0	0.0	0.0	6.8	100.0	0.0	-7.0	18.0
C	0.0	0.0	0.0	5.9	100.0	0.0	-23.0	1.0
D	0.0	0.0	0.0	8.0	100.0	0.0	-15.5	14.5
E	0.0	0.0	0.0	3.2	100.0	0.0	-12.0	1.0
F	13.8	11.4	3.4	5.9	11.8	9.6	-3.1	22.7

Phenylethylamine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	0	0	0	6.1	100	0	-16.0	7.0
B	0	0	0	10.1	100	0	-35.0	1.0
C	0	0	0	5.7	100	0	-16.5	3.5
D	0	0	0	6.3	100	0	-21.5	2.5
E	0	0	0	6.6	100	0	-21.5	1.5
F	0	0	0	6.5	100	0	-17.5	6.5

α-D-Lactose

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	225	18.4	7.4	8.8	3.6	222.4	195.6	247.5
B	0	0.0	0.0	5.2	100.0	0.0	-17.0	1.0
C	0	0.0	0.0	5.2	100.0	0.0	-19.5	1.5
D	0	0.0	0.0	4.6	100.0	0.0	-5.0	12.0
E	0	0.0	0.0	6.0	100.0	0.0	-20.5	0.5
F	0	0.0	0.0	6.1	100.0	0.0	-11.0	13.0

D,L-α-Glycerol Phosphate

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	36.9	15.6	7.9	3.5	57.2	36.6	29.7	44.2
B	0.0	0.0	0.0	9.9	100.0	0.0	-12.0	18.0
C	0.0	0.0	0.0	6.1	100.0	0.0	-9.5	10.5
D	0.0	0.0	0.0	4.9	100.0	0.0	-4.0	13.0
E	0.0	0.0	0.0	6.1	100.0	0.0	-21.0	1.0
F	16.9	17.8	6.9	3.8	3.6	16.6	5.1	27.8

D-Malic Acid

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	224.6	46.4	9.5	3.5	0.8	223.8	149.3	294.5
B	95.9	26.5	8.8	8.1	14.4	95.3	78.7	113.5
C	0.7	3.0	1.4	8.4	1.3	0.4	-55.6	61.0
D	153.4	30.3	9.2	7.5	16.0	152.9	137.1	169.6
E	0.0	0.0	0.0	4.9	100.0	0.0	-18.0	0.0
F	0.0	0.0	0.0	4.6	100.0	0.0	-13.0	3.0

Putrescine

	a_hat	b_hat	c_hat	sigma_hat	nu_hat	f_hat	f_low	f_high
A	131.2	24.5	7.2	4.6	3.1	128.5	114.3	142.3
B	91.7	33.2	8.1	4.7	2.1	91.2	70.9	110.5
C	115.1	21.6	6.2	3.3	2.5	110.3	99.8	121.7
D	100.7	18.4	6.5	4.4	9.6	98.0	87.5	108.3
E	0.0	0.0	0.0	7.4	100.0	0.0	-25.0	2.0
F	158.9	25.5	5.7	5.3	7.7	146.2	133.0	158.7