In situ biofortification of folate in sorghum with *Lactobacillus plantarum* and its microbiome analysis using 16s rRNA gene sequencing

by

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Submitted in fulfilment of the requirements for the degree

MSc Food Science

In the

Department of Consumer and Food Sciences
Faculty of Natural and Agricultural Sciences
University of Pretoria

August 2020

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Declaration

I, Adriana Salomina du Plessis, declare that the dissertation, which I hereby submit for the degree MSc Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Adriana Salomina du Plessis

17 August 2020

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Acknowledgements

I hereby wish to express my extreme gratitude to my supervisor, Prof Elna Buys, for her patience, support, and valuable contribution to this study. To Prof Gyebi Duodu, thank you for your professional guidance.

I would like to thank the Quadram Institute for sponsoring my attendance to the 'Analysis of the Microbiomes of Naturally Fermented Foods Training Course' in Ghana, it is an experience I will forever be grateful for.

I sincerely would like to thank my family for their patience and continuous support throughout this journey. I would like to thank my father, Barend, for his excellent advice and contribution to my education, without him I would not have been able to achieve what I did. Thank you to my mother, Martjie, for always believing in me and her unconditional love and motivation, I would be genuinely lost without you. To my brother, Barrie, and his fiancé, Jackie, thank you for all your love and support, I truly appreciate everything you do for me.

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Dedication

This work is dedicated to my parents, Barend and Martjie du Plessis.

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Abstract

In situ biofortification of folate in sorghum with *Lactobacillus plantarum* and its microbiome analysis using 16s rRNA gene sequencing

By Adriana Salomina du Plessis

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Folate deficiency is an unfortunate reality in South Africa. Folate (vitamin B9) is important for many cellular pathways in the body, one of which is the prevention of neural tube defects (NTD's) during pregnancy. We cannot synthesise folate ourselves and therefore we rely on exogenous sources such as our diets to obtain folate. Folate is found in a variety of sources such as green leafy vegetables, legumes, fortified foods and even dietary supplements. Even though supplements can prevent folate deficiencies, the high folate content in the blood plasma caused by synthetic folate can mask a vitamin B12 deficiency. The objective of this study was to ferment sorghum with folate producing *Lactobacillus plantarum* with the aim of increasing the folate content through *in situ* biofortification. The application of four *L. plantarum* strains (FS2, B411, S7 and S49) as starter cultures in fermented sorghum were successfully used to biofortify these fermentations with folate. The use of *L. plantarum* B411 and FS2 generated a folate increase of 50% or more from the spontaneous fermentation. Furthermore, it was found that the addition of 10% sorghum malt had a significant effect on the folate content of the sorghum fermentations. The sorghum malt contained enzymes, such as α-amylase, that are capable of promoting growth of

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microorganisms, such as folate producing *L. plantarum*. Sorghum fermented with *L. plantarum* B411 and sorghum malt had a folate content of 31.82 µg per 100ml fermentation. This is a 118% increase from the spontaneous fermentation. Sorghum fermented with *L. plantarum* FS2 and sorghum malt had a folate content of 30.39 µg per 100ml fermentation. This is a 109% increase from the spontaneous fermentation. These two *L. plantarum* strains will be ideal to use as starter cultures in sorghum fermentations to increase their folate content through biofortification. The application of these starter cultures could potentially be patented and used to commercialise high folate fermentations.

The microbiomes of the sorghum fermented with *L. plantarum* alone were also analysed through 16S rRNA gene sequencing with the aim of establishing a base microbiome and determining whether these microbiomes share any similarities. The microbiomes gave insight into the microbial communities of these fermentations and revealed that they were dominated by *Lactobacillus*. *Lactobacillus* was responsible for the fermentation process and hence would serve well as starter cultures for these fermentations.

In addition, the microbiomes of two South African and two Nigerian fermentations were analysed with the aim of identifying a potential starter culture and to create a reference microbiome for future studies. The microbiome of sorghum spontaneously fermented for 24h was successfully determined and can be used as a reference microbiome in future studies. The sorghum fermentations produced in a laboratory (South Africa) were dominated by *Paenibacillus* after 24h and *Clostridium* sensu_stricto_11, and other unidentified genera, after 72h. The microbiomes of the maize and sorghum fermentations sourced from a Nigerian market were dominated by *Lactobacillus* and appeared similar. *Lactobacillus* was present in all fermentations and therefore there is potential in the use of *Lactobacillus* as a starter culture in sorghum fermentations.

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List of Abbreviations

α	Alpha
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
ADP	Adenosine Diphosphate
ALAB	Amylolytic Lactic Acid Bacteria
ANCOM	Analysis of Composition of Microbiomes
ANOVA	Analysis of Variance
API	Analytical Profile Index
ATP	Adenosine Triphosphate
BC	Before Christ
CFU	Colony Forming Unit
CPD	Cyclic Dipeptide
DADA	Divisive Amplicon Denoising Algorithm
df	Degrees of Freedom
DFE	Dietary Folate Equivalent
DHF	Dihydrofolate
DHP	7,8-dihydropteroate
DHPPP	6-hydroxymethyl-7,8-dihydropterin pyrophosphate
DNA	Deoxy Ribonucleic Acid

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EMP	Emden-Meyerhof-Parnas
FAO	Food and Agricultural Organisation
FAOSTAT	Food and Agricultural Organisation Corporate Statistical Database
g	Gram
gDNA	Genomic Deoxy Ribonucleic Acid
GRAS	Generally Regarded as Safe
GTP	Guanosine Triphosphate
h	Hour
HTS	High Throughput Sequencing
L	Litre
LAB	Lactic Acid Bacteria
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation – Time of Flight
ml	Millilitre
min	Minute
MRS	DeMan, Rogosa and Sharpe
MY	Mid-Year
n	Number
NAD/NADH	Nicotinamide Adenine Dinucleotide
ng	Nanogram
nm	Nanometre
No	Number
NTD	Neural Tube Defect
OTU	Operational Taxonomic Unit
p-ABA	Para-Amino Benzoate
PBS	Phosphate Buffered Saline

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PERMANOVA	Permutational Multivariance Analysis of Variance
pH	Power of Hydrogen
PCoA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
PCR-DGGE	Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis
PD	Phylogenetic Diversity
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
RDA	Recommended Daily Allowance
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
SA	South Africa
SANBS	South African National Blood Service
Sig.	Significance
ssp.	Subspecie
TA	Titratable Acidity
THF	Tetrahydrofolate
USA	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organisation

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CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Many countries in sub-Saharan Africa are developing countries where nutrient deficiencies are an unfortunate reality. Most South Africans have limited food access and variety which leads to the inadequate intake of all nutrients, except carbohydrates (Oldewage-Theron and Kruger, 2011). Minerals and vitamins are micronutrients that are responsible for a multitude of functions in the human body (Eitenmiller, Ye and Landen, 2007; LeBlanc et al., 2011). Group B vitamins are important for their synergetic roles in metabolic processes such as energy production and red blood cell formation (LeBlanc et al., 2011). Folate (vitamin B9) is especially important as it plays a role in preventing NTD's in foetuses during pregnancy (Molloy, 2005). Folate deficiencies may be caused by a lack of a diverse diet or its destruction during cooking and processing. It is for these reasons that mandatory fortification with folic acid, the synthetic form of folate, was implemented in South Africa in 2003 for wheat and maize meal products (SA. Department of Health, 2003). This fortification is however not mandatory for sorghum, a cereal commonly fermented and consumed in South Africa.

Even though mandatory fortification of folic acid can prevent NTD's (Molloy, 2005), concerns have been raised regarding excess intake of fortified folic acid. Excess folic acid in the blood plasma can mask the early haematological manifestations of vitamin B12 deficiency (LeBlanc et al., 2011). In order to avoid this, alternative food sources with natural folate need to be explored. While folate is naturally present in many different foods, microorganisms such as *Lactobacillus plantarum* are also capable of producing folate derivatives. Sorghum products fermented with folate producing strains would serve as an alternative source of dietary folate.

The microbial communities of fermented food products are complex, and it is a research field that still needs to be explored. Analysing the microbiome of a fermentation could give insight into the genotypical characteristics of the fermentations and enhance the understanding of the role of strains capable of producing folate derivates in the microbiome.

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In this chapter sorghum, lactic acid fermentations, folate and the microbiome of food fermentations are explored.

1.2 SORGHUM

Sorghum is a grain crop, indigenous to Africa, belonging to the grass family Poaceae (commonly known as Gramineae) (Figure 1). It is the 5th most important grain, preceded by wheat (*Triticum aestivum*), maize (*Zea mays*), paddy rice (*Oryza sativa*), and barley (*Hordeum vulgare*) (Taylor, 2019). The most recent data shows that the world sorghum yield in 2018 was 59.3 million tonnes of which Africa contributed 29.7 million tonnes (FAOSTAT, 2020). This was an increase from 2017, where the world sorghum yield was 57.7 million tonnes of which Africa contributed 27.3 million tonnes. In South Africa alone sorghum production increased in the 2018/19 mid-year (MY) to 166,000 tonnes after the 2017/18 MY only produced 115,000 tonnes due to drought conditions (Esterhuizen, 2019).

Sorghum plays a role in the food security of many countries in sub-Saharan Africa as it is readily available and used in the production of many foods such as breads, porridges and syrups (Coulibaly et al., 2014).

Sorghum types may vary in colours, from white, lemon yellow, various shades of red, brown and black due to the expression of different levels and classes of phenolic compounds in the pericarp of the grain (Duodu and Awika, 2019). Sorghum may also be broadly classified as tannin and non-tannin types. Tannin sorghum types may undergo an inactivation process to inactivate the tannins for the production of opaque beer, while non-tannin sorghums are more widely used in the production of porridges, beverages, syrup and baked goods (Coulibaly et al., 2014; Taylor and Duodu, 2019).

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Figure 1 Typical appearance of *Sorghum bicolor*. (SA. Department of Agriculture, Forestry and Fisheries, 2010).

1.2.1 SORGHUM FERMENTATION

Fermented cereals, particularly maize, wheat and sorghum, are commonly consumed in Africa. These cereals are fermented to enhance safety, nutrition and sensory qualities as well as to prolong its shelf life at ambient temperature (Holzapfel, 2002; Blandino et al., 2003; Olotu, Oyetayo and Adebolu, 2009). Fermented cereal foods are consumed in many communities and are often prepared through spontaneous fermentation at household level or small-scale industries. While opaque beer is probably the most common fermented sorghum product in South Africa, fermented beverages and porridges are also consumed on a day to day basis either as breakfast or a snack. They are also served at traditional events or used as a weaning food (Mukisa et al., 2012). A variety of fermented sorghum products that are consumed in Africa can be seen in Table 1. Cereal fermentations are typically lactic acid fermentations carried out by lactic acid bacteria (LAB) and are non-alcoholic. However, they may also be alcoholic if they were fermented predominantly by yeasts through alcoholic fermentation (Blandino et al., 2003).

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Table 1 Types of fermented sorghum products available in Africa.

Product Name	**Microorganisms Responsible for Fermentation**	**Type**	**Region**	**Reference**
Motoho	Unkown	Non-alcoholic beverage	Lesotho	Gadaga, Lehohla and Ntuli, 2013; Taylor, 2019
Mahewu	*L. delbruekii*, *L.bulgaricus*	Non-alcoholic beverage	South Africa	Nyanzi and Jooste, 2012; Taylor, 2019
Ogi	*L. plantarum*	Porridge	Nigeria	
Ting	*Lactobacillus* spp.	Porridge	Botswana	Sekwati-Monang and Gänzle, 2011
Obushera	LAB and *Saccharomyces cerevisiae*	Non-alcoholic beverage	Uganda	Mukisa et al., 2012
Kisra	Unknown	Flatbread	Sudan	Blandino et al., 2003
Injera	*Candida guilliermondii*	Flatbread	Ethiopia	

1.2.2 SORGHUM MALT

Malting (or sprouting) of cereals is a traditional processing technique which involves the sprouting of the germ (Taylor and Kruger, 2019). It is used to improve the sensory, technological, and nutritional properties of foods and starts with the steeping of clean grains. The grains are drained and spread out to keep damp where after they are left to sprout for 48h, or up to a week. They are then dried by a process often referred to as kilning and milled into a flour for utilisation (Coulibaly et al., 2014; Taylor and Kruger, 2019). During the malting process enzymes, such as amylases and proteases, are synthesised which hydrolyse and modify the grain components and structure (Taylor and Kruger, 2019). These changes not only improve the inherent properties of the malt, it also improves the application of malt in food products (Tou et al., 2007; Mukisa et al., 2012).

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Malt is commonly associated with beer as malting is usually the first step in the production of traditional sorghum beer (Coulibaly et al., 2014). It is also common practice to add malt to fermented cereals to act as a source of amylase. This enzyme hydrolyse some of the starch into sugars which ultimately results in a product with a reduced viscosity. (Tou et al., 2007; Mukisa et al., 2012; Taylor and Duodu, 2019). Other chemical changes in malt include the activation of enzymes, a decrease in antinutritional factors, and an increase in the bioavailability of minerals (iron and zinc), content of essential amino acids (especially lysine, tryptophan and methionine) and the digestibility of macronutrients (Coulibaly et al., 2014; Taylor and Kruger, 2019).

Fermented foods are popular as weaning foods due to their low viscosity, but unfortunately, they have low nutrient density. Not only do cereal malts reduce the viscosity of the product due to enzymes, it can also help to initiate lactic acid fermentation as the fermentable sugars in the malt will be utilised by LAB for growth (Gadaga, Lehohla and Ntuli, 2013). The use of malt in fermented cereal foods can however significantly increase the processing time (3 to 5 days) and contribute to low or variable amylolytic activity. Spoilage and pathogenic microflora in cereals malts can also pose quality and safety risks in case of slow fermentation (Tou et al., 2007).

Amylolytic lactic acid bacteria (ALAB) have been isolated from various traditional fermented cereal products (Agati et al., 1998; Sanni, Morlon-Guyot and Guyot, 2002) and these bacteria have similar effects on the viscosity of cereal fermentations as malt (Mukisa et al., 2012). Amylolysis is expected to increase the availability of energy sources for other associated non-amylolytic LAB, to contribute to a rapid pH decrease, and to impart favourable rheological properties (Sanni, Morlon-Guyot and Guyot, 2002). Microbial fermentation with ALAB leads to a decrease in the level of carbohydrates as well as some non-digestible poly- and oligosaccharides which leads to improved starch digestibility (Kohajdová and Karovičová, 2007). ALAB and/or sorghum malt can therefore be used to produce low viscosity and energy and nutrient dense porridges that will be especially beneficial to infants.

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1.3 LACTIC ACID BACTERIA

LAB refers to a group of bacteria that are Gram-positive, non-motile, non sporeforming rods or cocci. Most of them are aerotolerant anaerobes that are catalase- and oxidase-negative (Huang et al., 2018). They are mesophilic, but some can grow at temperatures as low as 5°C or as high as 45°C. Most strains grow in a pH range of 4.0-4.5, but exceptions are active at pH 9.6 and pH 3.2 (Caplice and Fitzgerald, 1999). Important genera of LAB include, but is not limited to, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, *Bifidobaterium* and *Streptococcus*. They are present in a wide range of ecological niches, including food and the gastrointestinal tract of animals and humans (LeBlanc et al., 2011). The name 'lactic acid bacteria' was given to this group because they derive their cellular energy from the fermentation of carbohydrates to produce lactic acid. A variety of organic acids and other metabolites may be produced and depends on the method of fermentation (Caplice and Fitzgerald, 1999).

1.3.1 LACTIC ACID BACTERIA FERMENTATION

LAB have been used in the production of fermented foods since ancient times and fermentation methods for meats, vegetables and milk have been reported from as early as 6000 BC (Holzapfel, 1997; Caplice and Fitzgerald, 1999). These foods became popular due to their unique sensory characteristics and their preservative effect. Preservation of foods through fermentation is dependent on the oxidation of carbohydrates into acids, alcohols and carbon dioxide. These metabolites are not only responsible for preservation but also contribute to the sensory qualities such as flavour, aroma and texture (Caplice and Fitzgerald, 1999).

LAB produces lactic acid from carbohydrates, specifically hexoses, and can be divided into homofermentative (ex. *Pediococcus, Streptococcus, Lactococus* and some lactobacilli) or heterofermentative LAB (ex. *Weisella, Leuconostoc* and some lactobacilli) based on the pathways these hexoses are metabolised by (Caplice and Fitzgerald, 1999).

Lactate is produced as virtually a single product from homofermentative fermentation of glucose. This happens through the Emden-Meyerhof-Parnas glycolytic pathway

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(EMP) (Figure 2a). Glucose is phosphorylated and isomerised before the enzyme aldolase cleaves it into glyceraldehyde-3-phosphate. ATP is then produced by substrate level phosphorylation at two sites during the conversion of glyceraldehyde-3-phosphate into pyruvate. This conversion gives an overall yield of two ATP molecules for every glucose molecule fermented. During this conversion two NAD+ molecules were consumed. In order to regenerate NAD+, two molecules of NADH is used to reduce pyruvate into lactate. (Caplice and Fitzgerald, 1999; Gänzle, 2015)

Heterofermentative fermentation of glucose produces roughly equal amounts of lactate, ethanol/acetate, and carbon dioxide. Heterofermenters lack the enzyme aldose and rather transforms glucose into a pentose through a series of oxidation and decarboxylation reactions known as the phosphoketolase pathway (Figure 2b). This pentose is cleaved into triose phosphate and acetyl phosphate by the enzyme phosphoketolase, hence the name of the pathway. The triose phosphate is then subsequently converted into lactate by the same series of reactions of glycolysis, producing one molecule of ATP. The fate of acetyl phosphate depends on electron acceptors available. If no electron receptors are available, acetyl phosphate is reduced to ethanol which regenerates two NAD+ molecules from NADH. Acetyl phosphate may be converted to acetate in the presence of oxygen (Caplice and Fitzgerald, 1999; Gänzle, 2015).

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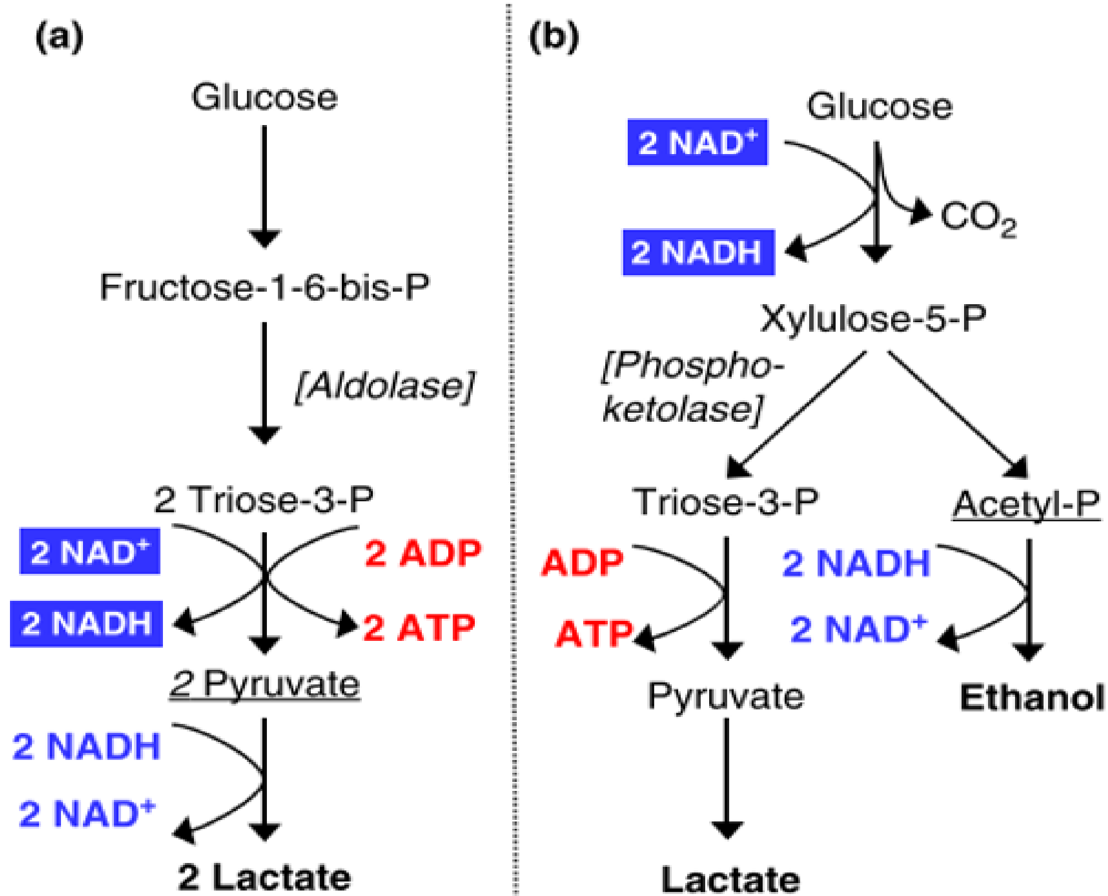

Figure 2 Overview on homofermentative and heterofermentative carbohydrate fermentation of lactic acid bacteria. (a) Homofermentative metabolism via the Emden–Meyerhoff pathway. (b) Heterofermentative metabolism via the phosphoketolase pathway. Adapted from Gänzle (2015).

Pyruvate is an intermediary compound in many sugar fermentation pathways and is almost always converted into lactic acid. Pyruvate metabolism is dependent on carbohydrate availability and external electron acceptors. The limitation of these factors can lead to the generation of many other metabolites such as acetate, ethanol, diacetyl, and acetaldehyde (Caplice and Fitzgerald, 1999). These volatiles contribute to characteristic flavours of fermented foods such as sourdough (determined by the lactate/acetate ratio), kefir and koumiss (ethanol), butter and buttermilk (diacetyl), and yoghurt (acetaldehyde) (Leroy and De Vuyst, 2004).

Figure 3 shows the formation of these important metabolites. Four pyruvate metabolism pathways are available namely the pyruvate oxidase pathway, the pyruvate dehydrogenase pathway (aerobic metabolism), diacetyl/acetoin pathway and

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the pyruvate-formate-lyase system (active in anaerobic environment). The desirability of the production of metabolites depends on the product being produced: diacetyl is desirable in wine and dairy products but seen as spoilage in beer; excessive acetate formation is considered as spoilage in the production of alcoholic beverages but may improve the flavour of baked goods (Gänzle, 2015).

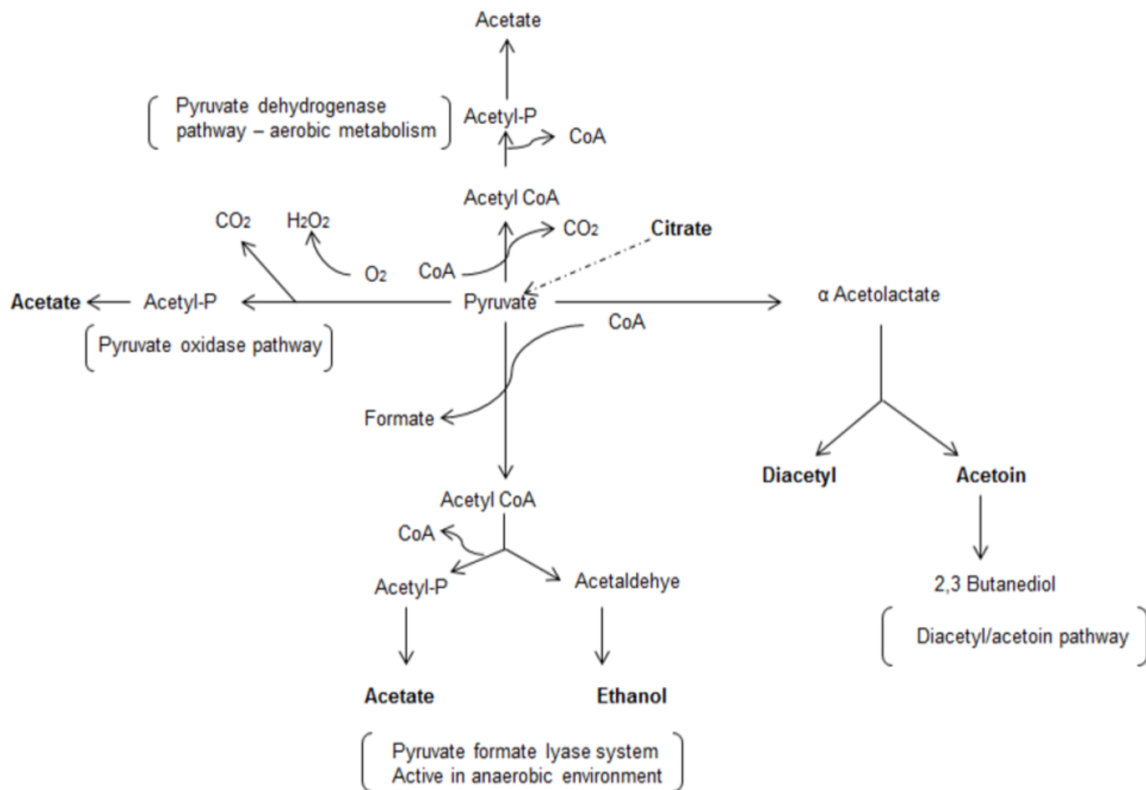

Figure 3 Generalized scheme for the formation of important metabolic products from pyruvate in lactic acid bacteria. Brackets indicate the four different pyruvate metabolism pathways. (Caplice and Fitzgerald, 1999)

1.3.2 STARTER CULTURES

In many African communities fermented food is achieved through spontaneous fermentation which is difficult to control when carried out at a large scale. Even though this could lead to variation in characteristic sensory, quality and safety properties, these fermented products are still popular due to the inaccessibility of commercially processed foods, inconsistent electricity supply used for refrigeration and the high

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population of consumers being low-income earners (Olotu, Oyetayo and Adebolu, 2009).

Large-scale spontaneous fermentations can be unhygienic and can lead to contamination with pathogens leading to foodborne-illnesses and even death (Olotu, Oyetayo and Adebolu, 2009). In order to ensure that the fermentations are controlled one can make use of pure starter cultures.

Holzapfel (1997) defines a starter culture as 'a preparation containing high numbers of viable microorganisms, which may be added to bring about desirable changes in a food substrate'. This allows for accelerated fermentation and control during the fermentation process. In modern day starter cultures are used in the dairy, wine and beer industry, and variations in products can depend on different types, mixes and application of starter cultures (Holzapfel, 1997).

1.3.3 HEALTH BENEFITS OF FERMENTED FOODS

It is known that microorganisms serve a technical function in fermented foods such as lowering the pH for safety purposes and producing flavour compounds that contributes to the sensory qualities of the product. In addition to this they can also be beneficial for humans after ingestion. These types of microorganisms are classified as probiotics and are defined by the FAO and WHO as 'live microorganisms that confer a health benefit to its host if it is administered in adequate amounts' (FAO/WHO, 2006).

Probiotics used for human consumption are typically from the genera *Lactobacillus* or *Bifidobacterium* (Rossi, Amaretti and Raimondi, 2011; Herbel et al., 2013). Extensive research has been done on the health benefits attributed to specific probiotic strains. Health benefits include protection against pathogens, prevention and treatment of diarrhoea, improving lactose intolerance and the production of important group B vitamins (Collins, Thornton and Sullivan, 1998; LeBlanc et al., 2011; Herbel et al., 2013). Some studies only have preliminary evidence of health benefits and it is too early to make definitive clinical conclusions, while others provide strong evidence of health benefits (FAO/WHO, 2006).

One of the most common benefits of probiotics known is the prevention and treatment of diarrhoea caused by pathogenic bacteria and viruses. The prophylactic use of

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Lactobacillus GG significantly reduced the risk of diarrhoea in infants, especially gastroenteritis from rotavirus infections (Szajewska et al., 2001). The FAO and WHO found multiple other studies where the use of probiotic strains could not only treat diarrhoea but also prevent it through the inhibition of growth and adhesion of a range of enteropathogens (FAO/WHO, 2006).

During fermentation, probiotics may eliminate toxic substances. A study done by Holzapfel (2002) showed that when cassava was fermented with *L. plantarum* starter cultures it significantly reduced the content of cyanogenic glucoside, an agent that can cause acute poisoning.

Probiotics also have beneficial effects on indigestible compounds. Cereals and legumes contain oligosaccharides such as stachyose, verbascose and raffinose. These oligosaccharides are indigestible and cause digestion problems and diarrhoea. Some LAB strains, *L. fermentum*, *L. plantarum*, *L. salivarius*, *L. brevis* etc., can degrade the α-D- galactosidic bonds of the oligosaccharides and improve their digestibility (Holzapfel, 2002).

Probiotics can aid in lactose metabolism (Kechagia et al., 2013). This is especially beneficial to lactose intolerant individuals who develop gastrointestinal symptoms after the consumption of dairy products. These individuals are deficient in the enzyme lactase which breaks down lactose into glucose and galactose. This causes lactose to enter the small intestine which is subsequently broken down into short-chain fatty acids and gas. Probiotics such as *Bulgaricus*. ssp, *L. delbrueckii* and *S. thermophilus* are well adapted to milk as a substrate and can ferment lactose (Holzapfel et al., 2001; Kechagia et al., 2013).

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1.3.4 GENERAL OVERVIEW OF *LACTOBACILLUS PLANTARUM* AND ITS HEALTH BENEFITS

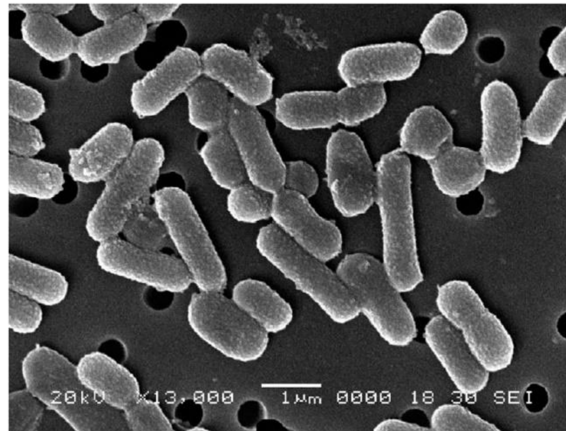

Figure 4 Micro-morphological image of *Lactobacillus plantarum* strain. (Arasu et al., 2016)

L. plantarum falls under the group LAB (Holzapfel, 1997; Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004). They are microaerophilic, mesophilic and grow at 20-42°C. They can occur as single cells, in pairs or in short chains and are straight rods with rounded ends, 0.9–1.2 x 3.0–8.0 µm in size (Figure 4) (Machielsen et al., 2010; Arasu et al., 2016).

L. plantarum, *L. pentosus*, and *L. paraplantarum* have 99.7–99.9% sequence similarity and cannot be distinguished by 16S rRNA gene sequence analysis. They form a closely related group known as the '*Lactobacillus plantarum* group' and show similar fermentation patterns (Corsetti and Valmorri, 2011). In 1990-1995 the whole genome of *L. plantarum* WCFS1 was sequenced. It contained a single circular chromosome of 3 308 274 base pairs and is considered one of the largest genome sizes known for LAB to date (Kleerebezem et al., 2003). This genome encoded all enzymes required for the glycolysis and phosphoketolase pathways, meaning *L. plantarum* is classified as a facultative heterofermentative lactic acid bacterium. Micro analysis of the genome revealed two large regions in the chromosome with unusual base compositions. This could indicate horizontal gene transfer and it is hypothesised that this part of the *L. plantarum* genome is where adaption to specific environmental niches may take place (Corsetti and Valmorri, 2011).

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L. plantarum has been frequently isolated from milk and milk products and has therefore been identified as useful for dairy technology (Caplice and Fitzgerald, 1999; Corsetti and Valmorri, 2011). Apart from dairy products, *L. plantarum* strains have been isolated from numerous traditional fermented products including cereals and vegetables (Table 2).

Table 2 Non-dairy fermented foods with *Lactobacillus plantarum*.

Food Product		**Substrate**	**Reference**
Cereals	Busa	Maize	Blandino et al., 2003
	Seketeh	Maize	
	Ogi	Maize, Sorghum, Millets	
	Kishk	Wheat	
	Tapuy	Rice	
	Uji	Maize, Sorghum or Millets	Franz et al., 2014
	Kenkey	Maize	
	Bensaalga	Millets	
	Ting	Sorghum	
	Obushera	Sorghum and Millets	Mukisa et al., 2012
Vegetables	Kimchi	Cabbage	Behera, Ray and Zdolec, 2018
	Fermented Sauerkraut	Sauerkraut	Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004
	Fermented pickles	Pickles	

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Table 2 continued

Food Product		Substrate	Reference
Vegetables	Fermented olives	Olives	Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Pessione et al., 2015
Legumes	Fermented soymilk	Soybean	Behera, Ray and Zdolec, 2018
Roots	Gari	Cassava	Caplice and Fitzgerald, 1999; Franz et al., 2014
	Fufu	Cassava	Franz et al., 2014
Seafood	Jeotgal	Fish and Shellfish	Park et al., 2014

The use of *L. plantarum* has been extensively studied and because it is 'generally regarded as safe' (GRAS) it is applied as a probiotic and/or starter culture in the food industry (Behera, Ray and Zdolec, 2018). *L. plantarum* produces organic acids (lactic acid, acetic acid etc.) during fermentation which causes a reduction in pH (Nout and Motarjemi, 1997). These acids act on the cytoplasmic cell membrane which negatively affects the membrane potential and inhibits active transport. This increases the stability and shelf life of the fermentation as it inhibits microorganisms such as unwanted yeasts, moulds and bacteria (Caplice and Fitzgerald, 1999). Lactic acid directly contributes to the sensory characteristics of the fermentation as acidic tastes are often desirable and associated with fermented foods.

Other than organic acids, *L. plantarum* can also produce a multitude of enzymes such as α-amylase, ester hydrolases and lipases (Behera, Ray and Zdolec, 2018). α-Amylase catalyses the hydrolysis of starch and is beneficial in processes that involve the fermentation of cereals, e.g. breadmaking. Ester hydrolases produce a range of phenolic alcohols and fatty acids which have a broad range of application in the food industry. Lipases are used for meat degradation and to contribute to sensory qualities (Behera, Ray and Zdolec, 2018). *L. plantarum* can produce antimicrobial cyclic dipeptides (CDP's). These peptides were verified to have antimicrobial activity against

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multidrug-resistant bacteria, pathogenic fungi, and influenza A virus (Behera, Ray and Zdolec, 2018).

L. plantarum is well known for its ability to produce or synthesise group B vitamins (Corsetti and Valmorri, 2011; LeBlanc et al., 2011; Behera, Ray and Zdolec, 2018). Riboflavin producing *L. plantarum* strains used in the production of bread had an enhancing effect on the final riboflavin content (LeBlanc et al., 2011). *L. plantarum* strains can also produce folate (LeBlanc et al., 2011; Rossi, Amaretti and Raimondi, 2011). In a study done on several *Lactobacillus* strains, it was found that all strains consumed folate with the exception of *L. plantarum*, which was also the only *Lactobacillus* strain with the ability to produce folate without supplementation of precursors required for *de novo* folate synthesis (Rossi, Amaretti and Raimondi, 2011).

In addition to the functional properties and health promoting agents produced by *L. plantarum,* these strains are also able to survive the low pH of gastric acids and can colonise the gut (de Vries et al., 2006). All these factors, including critical factors such as adaptability, metabolic flexibility and its biosynthesis ability, make *L. plantarum* strains useful as starter cultures and probiotics in fermented foods (Behera, Ray and Zdolec, 2018). However, these factors are strain specific and careful selection of strains should be considered if specific results are desired.

1.4 FOLATE

Vitamins are important micronutrients that are essential for all living organisms. Folate (B9) falls under the group B vitamins, which also includes thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), pantothenic acid (B5), biotin (B7) and cobalamin (B12) (Eitenmiller, Ye and Landen, 2007). Folate is a generic term used to refer to both the natural form of the vitamin (food folate) and the monoglutamate form (folic acid), which is used in fortified foods and supplements. For the purpose of this study reference will be made to folate only as the natural form and folic acid as the synthetic form.

Folate functions as a one-carbon source in the metabolism of nucleic and amino acids and plays a role in cellular pathways including DNA, RNA and protein methylation as

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well as DNA synthesis and maintenance (LeBlanc et al., 2011; Crider et al., 2012). It can be found in foods such as yeast extract spread, liver, legumes, cereals and green leafy vegetables (Table 3). The synthetic form, folic acid, can also be used to fortify foods such as wheat flour, white bread and maize meal (SA. Department of Health, 2003) and performs the same function in the body as folate.

Table 3 Folate content of selected foods. (USDA, 2018)

Food		Folate Content (µg/100 g)
Cereals	Rice, (white, short-grain, raw, enriched)	231
	Rice bran, crude	63
	Rice, brown, long-grain, raw	23
	Oats	56
	Rye grain	38
	Sorghum grain	20
Fruits	Avocados, raw, California	89
	Mangos, raw	43
	Oranges, raw, California, valencias	39
	Papayas, raw	37
	Kiwifruit, gold, raw	34
	Strawberries, raw	24
	Raspberries, raw	21
Vegetables	Spinach, raw	194
	Turnip greens, raw	194
	Cabbage, raw	43
	Cowpeas (blackeyes), immature seeds, raw	168

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Table 3 continued

Food		Folate Content (µg/100 g)
Vegetables	Soybeans, green, raw	165
	Mustard spinach, (tendergreen), raw	159
Spices and herbs	Parsley, fresh	152
	Bay leaf	180
	Dill weed, fresh	162
	Peppermint, fresh	114
	Rosemary, fresh	109
	Spearmint, fresh	105
Legumes	Yardlong beans, mature seeds, raw	658
	Cowpeas, catjang, mature seeds, raw	639
	Mung beans, mature seeds, raw	625
	Beans, kidney, all types, mature seeds, raw	394
	Chickpeas, mature seeds, raw	557
	Lentils, raw	479
	Beans, French, mature seeds, raw	399
Dairy and egg products	Egg, yolk, raw, fresh	146
	Cheese, brie	65
	Egg, whole, raw, fresh	47
	Yoghurt, plain	12
	Yeast extract spread (Marmite)	793
	Chicken, liver, all classes, cooked, pan-fried	560
	Chicken, broilers or fryers, giblets, cooked, fried	379

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The recommended dietary allowance (RDA) for folate is 400 µg/day dietary folate equivalents (DFE's) for both men and women, while pregnant women must ensure they increase their daily intake to 600 µg/day (Institute of Medicine (USA) Standing Committee, 1998).

Folate intake varies between populations and several studies have been done to determine the effect folate intake has on preventing folate deficiency-related defects. Human studies varied widely in terms of study design, timing of exposure, folate intake etc. which unfortunately leads to varying findings. However, in general the evidence suggests that a low folate intake leads to low DNA methylation which results in an increased risk for cancer (Crider et al., 2012). Folate deficiency has also been directly linked to NTD's (Molloy, 2005). These defects can affect the spine (spina-bifida) and the brain (anencephaly) and is caused by lower plasma and red blood cell folate concentrations in the mother of affected foetuses during pregnancy.

Due to the importance of folic acid in the prevention of NTD's during pregnancy, many countries have implemented mandatory fortification of folate. According to the Minister of Health (SA. Department of Health, 2003), the regulations for mandatory food fortification were legally implemented in South Africa on 7 October 2003 under Act No 54 of 1972 Foodstuffs, Cosmetics and Disinfectants. These regulations apply to any person or company who manufactures, imports, or sells maize meal, wheat flour or foodstuffs which contain 90% of either maize meal or wheat flour.

Currently, mandatory folic acid fortification does not apply to any sorghum products. Even though fortification of maize and wheat flour has been implemented in South-Africa, rural populations are still prone to deficiency in all nutrients, except carbohydrates due to a lack of diversity in their diet (Oldewage-Theron and Kruger, 2011). While supplements could provide a solution for folate deficiencies, it has been suggested that an excess of folic acid could mask a vitamin B12 deficiency (LeBlanc et al., 2011). It is therefore necessary to consider other dietary sources or alternative supplementations of folate in order to avoid deficiencies.

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1.4.1 THE CHEMISTRY OF FOLATE

The folic acid molecule consists of a para aminobenzoate (p-ABA) molecule linked to a pteridine ring and a glutamic acid molecule (Figure 5A). Folate (Figure 5B) exists in the form of various derivatives that contains additional glutamates making them polyglutamate. They also differ in substituents linked at the N_5 and/or N_{10} positions which results in variation in biological activity (Table 4) (Saini, Nile and Keum, 2016).

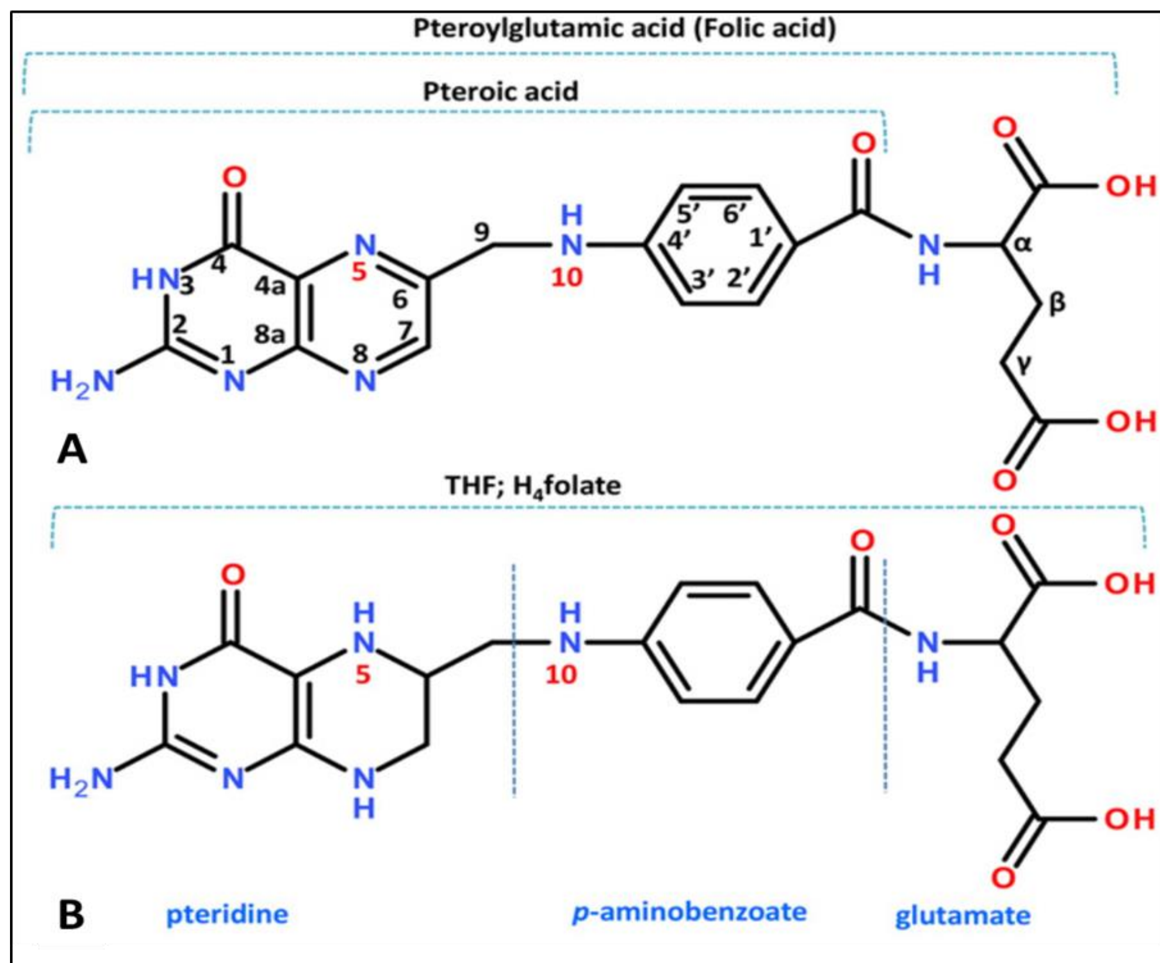

Figure 5 Chemical structure of A) folic acid and B) H4-folate. (Saini, Nile and Keum, 2016)

The main circulatory form of folate in humans is 5-methyl-tetra-hydrofolate. Both food folate and folic acid is converted into 5-methyl-tetra-hydrofolate through two enzyme dependent reactions after consumption. This folate derivative donates its methyl group to methionine synthase, a B12-dependent enzyme that converts homocysteine into

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methionine via the methionine synthase pathway. It is for this reason that folic acid supplements usually also contain vitamin B12. Methionine plays a role as a substrate, a cofactor and methyl group donor for various methylation reactions including DNA- and RNA methylation, neurotransmitters, etc. (Crider et al., 2012).

Table 4 Different forms of folate and their substituents linked to the N5 and N10 position. (Eitenmiller, Ye and Landen, 2007)

Name	**Abbreviation**	**Position**	
		N_5	**N_{10}**
Pteroylglutamic acid	Folic acid	—	–H
7,8-Dihydrofolate	H_2 folate	–H	–H
5-Methyl-5,6-dihydrofolate	*	$-CH_3$	–H
5,6,7,8-Tetrahydrofolate	H_4 folate	–H	–H
5-Methyltetrahydrofolate	5-CH_3–H_4 folate	$-CH_3$	–H
5-Formyltetrahydrofolic acid	5-CHO–H_4 folate	–CHO	–H
10-Formyltetrahydrofolate	10-CHO–H_4 folate	–H	–CHO
5,10-Methenyltetrahydrofolate	5,10-CH+=H_4 folate	=CH- ± bridge	—
5,10-Methylenetetrahydrofolate	5,10-CH_2H_4 folate	$-CH_2$- bridge	—
5-Formiminotetrahydrofolate	5-CHNH–H_4 folate	–CHNH	–H

* No abbreviation

— No substituent

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1.4.2 *LACTOBACILLUS PLANTARUM* AND FOLATE PRODUCTION

Folate is naturally present in many different plant foods and can also be present in fermented foods. This is because some microorganisms such as yeasts and bacteria can produce folate during fermentation. Examples of folate producing microorganisms include *S. cerevisiae*, *Streptococcus thermophilus*, *St. macedonicus*, *L. lactis* and *L. plantarum* (LeBlanc et al., 2011; Park et al., 2014; Laiño et al., 2015; Shibata et al., 2020).

As previously mentioned, the folate molecule consists of a p-ABA molecule linked to a pteridine, which originates from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP). The *de novo* biosynthesis of folate requires both these precursors and can be seen in Figure 6 (Rossi, Amaretti and Raimondi, 2011).

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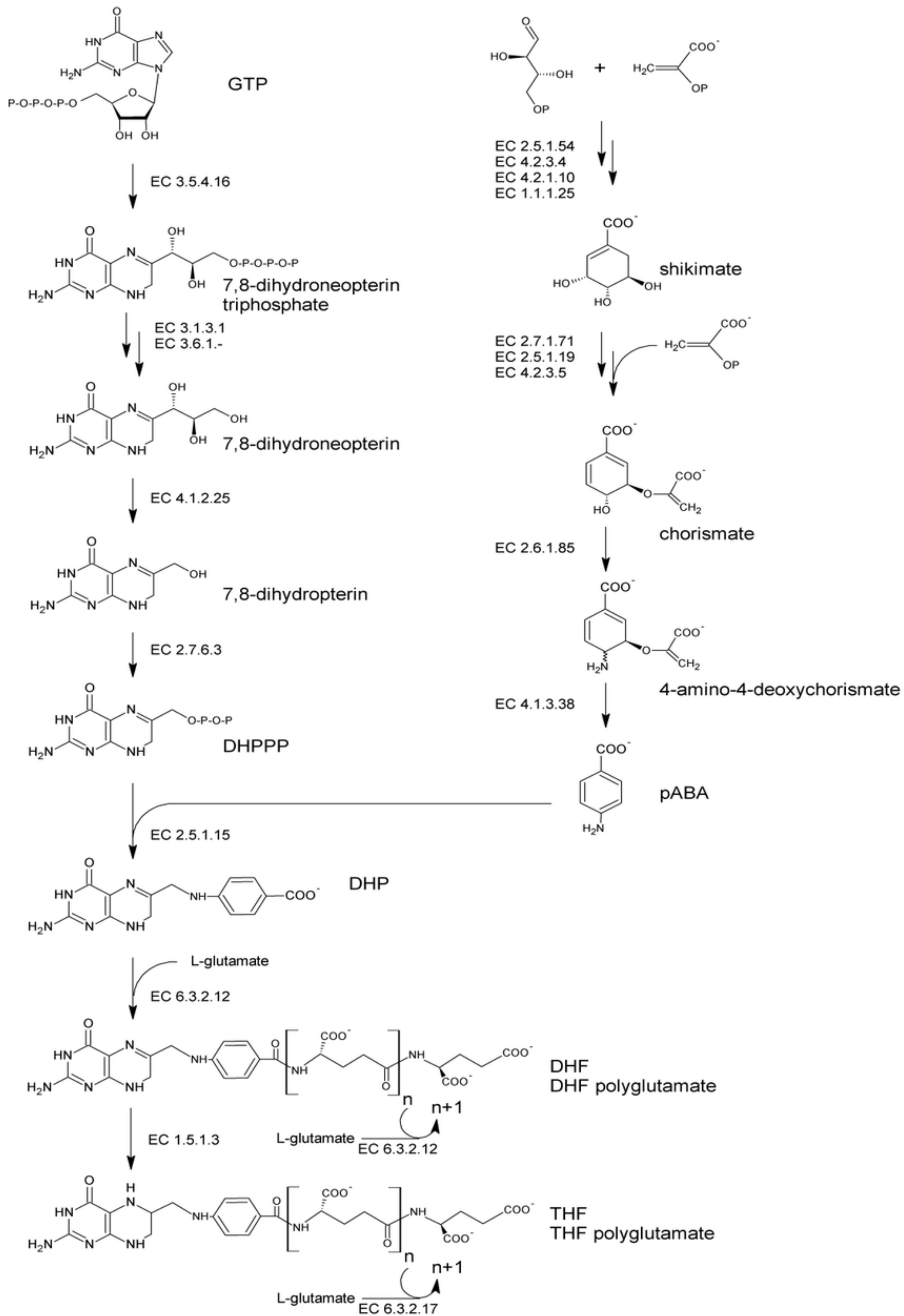

Figure 6 Pathway of *de novo* bacterial biosynthesis of folate. (Rossi, Amaretti, and Raimondi, 2011)

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In plants and bacteria p-ABA can be produced via the pentose phosphate pathway. Phosphoenoylpyruvate and erythrose-4-phospahate react in the shikimate pathway and form chorismate. Chorismate is converted to 4-amino-4-deoxychorismate by aminodeoxychorismate synthase where after pyruvate is cleaved by 4-amino-4-deoxychorismate lyase to give p-ABA (Rossi, Amaretti and Raimondi, 2011).

DHPPP is synthesised in four consecutive steps during the conversion of guanosine triphosphate (GTP). First a pterin ring structure is formed through Amadori rearrangement during the transformation of GTP. This first step is catalysed by GTP cyclohydrolase I. It is followed by dephosphorylation, where after the pterin molecule undergoes aldolase and pyrophosphokinase reactions which results in the production of activated pyrophosphorylated DHPPP (Rossi, Amaretti and Raimondi, 2011).

A condensation reaction, catalysed by dihydropteroate synthase, joins DHPPP and p-ABA together with the formation of a C-N bond. During this condensation reaction 7,8-dihydropteroate (DHP) is formed. Dihydrofolate synthase glutamylates DHP and gives dihydrofolate (DHF). The reduction of DHF by DHF reductase results in the biologically active cofactor tetrahydrofolate (THF). THF is subjected to the addition of multiple glutamate moieties by folylpolyglutamate synthase to yield THF polyglutamate. (Rossi, Amaretti and Raimondi, 2011).

The addition of polyglutamates may occur before the reduction step by DHF synthase or in many bacteria by a bifunctional enzyme responsible for DHF synthase and folylpolyglutamate synthase. This THF polyglutamate is one of the biologically active forms of folate (Rossi, Amaretti and Raimondi, 2011).

Folate production is strain specific and LAB strains that have been reported to synthesise folate include: *L. plantarum*, several *Bifidobacterium* strains and *St. thermophilus* (Crittenden, Martinez and Playne, 2003; LeBlanc et al., 2011; Rossi, Amaretti and Raimondi, 2011). As previously mentioned, the synthesis of folate requires p-ABA as a precursor. An analysis of the genome sequences for predictable metabolic pathways found that the enzyme converting chorismate into the important p-ABA precursor is absent in *Lactobacillus* and therefore this genus would not be capable of *de novo* folate synthesis in the absence of these precursors. From the 21 *Lactobacillus* strains reported, an exception to the ability to synthesise p-ABA is *L. plantarum*, which consists over a complete shikimate pathway with all relevant

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enzymes (Rossi, Amaretti and Raimondi, 2011). This shikimate pathway is responsible for the conversion of erythrose 4-phosphate and phosphoenolpyruvate into p-ABA through multiple reactions, which plays an integral role in folate synthesis. *L. plantarum* is therefore capable of folate synthesis and can be used as a folate producer in fermented cereals in the presence or absence of p-ABA.

1.5 MICROBIOMES OF FERMENTED FOODS

The production of traditional fermented foods through natural fermentation processes has a long history. These fermented foods are popular not only for their unique sensory qualities, but also for their good storage properties and many health benefits (Holzapfel, 2002). In order to commercialise these products a more controlled fermentation technique is needed. This depends on the understanding of the fermentation process and characterisation of the microorganisms involved (Mohammed, Steenson and Kirleis, 1991). The correct identification of microorganisms is therefore crucial especially if they are to be used in food products. Different methods of identification are available, and they are either culture-dependent or culture-independent (Temmerman, Huys and Swings, 2004).

A culture-dependent approach is a common way to determine microorganisms present in fermentations and they generally rely on phenotypic characteristics for identification. Specific techniques include protein profiling (MALDI-TOF), biochemical characterisation (API kits) and physiological (growth characteristic tests) and morphological analysis (microscopic analysis) (Temmerman, Huys and Swings, 2004). In an attempt to identify the microorganisms in kisra, a Sundanese fermented sorghum product, samples were plated in selective media (Mohammed, Steenson and Kirleis, 1991). Subsequent culture-dependent tests allowed for the identification of bacteria (*L. brevis*, *L. confuses*, *Erwinia ananas*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*), yeasts (*Candida intermedia* and *Debaryomyces hansenii*), and molds (*Aspergillus* ssp., *Penicillium* ssp., *Fusarium* ssp., and *Rhizopus* ssp.) (Mohammed, Steenson and Kirleis, 1991). Although these methods proved to be useful, they are labour intensive and may underestimate the microbial diversity and,

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in some cases, even fail to identify the dominant microorganism(s) (Temmerman, Huys and Swings, 2004; Mukisa et al., 2012).

The most recent and comprehensive culture-independent method for studying the microbial communities within foods is the meta-analysis of the microbiome. The microbiome refers to all the microorganisms and their genomes in a specified environment (Ercolini, 2013). The microbiome of a food fermentation will therefore refer to all the microorganisms present in the specific fermentation and give insight to the composition and diversity of the microbial community.

Culture-independent methods such as Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR–DGGE) based on 16S rRNA gene amplification are available. Nucleic acids are extracted directly from the matrix followed by rRNA amplicon sequencing (Ercolini, 2013). The use of these methods is known as the high-throughput-sequencing (HTS) approach and have been widely used to study the dynamics and microbial diversity of food fermentations (Mukisa et al., 2012; Ercolini, 2013). Culture-independent methods were used to successfully study the microbial communities of fermented sorghum, maize and pearl millet slurries (Humblot and Guyot, 2009; Oguntoyinbo et al., 2011). Studies were also done on other cereal fermentations such as wheat and rye sourdough. The PCR-DGGE method based on 16S rRNA amplification used in those studies is still one of the most popular methods used to study microbial communities in food fermentations. It shows the proportion of the different taxa present and gives tremendous insight to the occurrence and abundance of the microorganisms and genes present in the studied environment (Ercolini, 2013).

According to Temmerman (2004) the advantages of culture-independent methods by far outweigh the disadvantages. While these methods require a need for bioinformatic skills and may be more expensive than culture-dependent methods, they allow for the screening of multiple samples at the same time, result in reliable identification of microorganisms, allow for safer bench work and give insight regarding the taxonomy and activities of the microbiome.

Microbiome studies are dominated by the medical field with several studies focussing on the human gut microbiome (Cani, 2018), while microbiome studies in other fields are still emerging. This limitation makes it difficult to find comparable data for food

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fermentations. Hopefully, the necessity for microbiome studies in the field of food science will be realised and extensive research will be available in a few years.

1.6 CONCLUSIONS

The fermentation of sorghum is popular in many countries across sub-Saharan Africa. It is consumed daily, either as a drink or a porridge, and is a good source of carbohydrates. These communities do not have a wide variety in their diets which leads to deficiencies. One of these deficiencies is folate (vitamin B9), a micronutrient important for the cell metabolism of all living organisms. Folate prevents NTD's and is especially important in pregnant women and they are therefore urged to take a supplement. These supplements are produced with folic acid, the synthetic form of folate, and excess levels can potentially mask a vitamin B12 deficiency. While fortification of folic acid has been implemented in many different cereals in South Africa, it is not mandatory for sorghum. Alternative dietary folate sources need to be explored for populations that consume sorghum frequently. Since many different microorganisms, including *L. plantarum*, have proved to produce folate, they can potentially be applied as starter cultures for the biofortification of folate in sorghum fermentations.

The use of malt in cereal fermentations is common. They are added for their functional properties as they contain enzymes such as α-amylase that aids in reduction of viscosity. Not only does this make it more suitable as a weaning food, it also increases the available energy sources for LAB to grow and produce metabolites on.

The microbial communities of food fermentations are complex, and the analysis of the microbiome gives insight to the presence and diversity of the different microorganisms. The meta-analysis of the microbiome is a culture-independent approach and can serve as a fingerprint of the fermentation which allows for view of the fermentation on a microbial level. Microbiome studies of food fermentations are still limited, and research in various fields are needed.

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1.7 HYPOTHESES AND OBJECTIVES

1.7.1 HYPOTHESES

Hypothesis 1

The use of folate producing *L. plantarum* strains as a starter culture for the fermentation of sorghum will increase the folate levels of the fermented product after 24h of fermentation. Folate production is strain specific and therefore it is believed that different folate levels will be observed. LAB is dominant in most spontaneous cereal fermentations and many studies have isolated *L. plantarum* from sorghum fermentations (Blandino et al., 2003; Mukisa et al., 2012; Franz et al., 2014). *L. plantarum* is capable of *de novo* folate production (Rossi, Amaretti and Raimondi, 2011).

Hypothesis 2

The addition of sorghum malt will increase the growth of the starter culture *L. plantarum* and in return lead to increased folate levels of the final fermented sorghum product. It is expected that the sorghum fermentations with malt will have higher folate contents than those fermented without malt. Malt contains enzymes, such as amylase, that break complex sugars, such as starch, down to simple sugars. These simple sugars are more readily available for the *L. plantarum* to consume and in return grow and produce folate (Sanni, Morlon-Guyot and Guyot, 2002).

1.7.2 OBJECTIVES

- To determine the effect of pre-selected folate producing *L. plantarum* strains on the folate levels of sorghum fermented, with and without sorghum malt, with the aim of increasing the folate levels of these fermentations.
- To determine the microbiomes of spontaneous sorghum fermentations and sorghum fermented with *L. plantarum* strains with the aim of establishing a base microbiome and determining whether these microbiomes share any similarities.

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CHAPTER 2: RESEARCH PHASE 1 - DETERMINING THE MICROBIAL COMPOSITION OF AFRICAN FERMENTED CEREALS THROUGH 16S rRNA AMPLICON SEQUENCING

Abstract

Fermented foods consist of complex microbial communities which are referred to as their microbiomes. These microbiomes give a comprehensive look into what microorganisms are responsible for fermentation and the identification of these microorganisms can aid in the development of optimum starter cultures. In this study the microbiomes of four spontaneous cereal fermentations, two from the lab (sorghum) and two sourced at a Nigerian market (sorghum and maize), were analysed through 16S rRNA sequencing. Cereal fermentations sourced from the market were dominated by *Lactobacillus* while lab fermentations were dominated by *Paenibacillus* and other unknown microorganisms. *Lactobacillus* was found in both lab fermentations and therefore *Lactobacillus* strains will be able to serve as starter cultures for the production of specialised sorghum fermentations.

2.1 INTRODUCTION

Fermented foods have been popular in the diet of communities worldwide for many years (Campbell-Platt, 1994). Africa has an especially rich variety of traditional fermented foods with raw materals such as cereals (maize, sorghum and millet), starchy roots (cassava) and animal proteins (dairy) (Holzapfel, 1997; Olasupo, Odunfa and Obayori, 2010; Oguntoyinbo et al., 2011). These fermentations are either non-alcoholic or alcoholic, and are typically consumed as a beverage or a cereal, depending on their sensory characteristics (Holzapfel, 1997). The varying sensory characteristics of these fermented foods are typically caused by differences in raw materials and fermentation processes (Oguntoyinbo et al., 2011). Cereal

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fermentations such as mawe, obushera and ogi are popular African cereal fermentations. Even though the dominant microorganisms in these type of cereal fermentations tend to be LAB, many different sensory characteristics and safety properties have been observed in these fermentations. These sensory and safety characteristics have been directly linked to the microbial communities of the fermentation (Holzapfel, 1997; Olasupo, Odunfa and Obayori, 2010).

Understanding the microbial communities of food fermentations will not only allow us to assess the quality of these fermentations, but it could also assist in the development of optimum starter cultures. The use of these starter cultures can provide a fermentation of consistent quality and safety (Holzapfel, 1997).

Culture-dependent methods are popular for the analysis of the microbial communities of fermented products, however, these methods pose some limitations. They can be labour intensive and have a low throughput; it is for these reasons that the use of culture-independent methods have increased in recent years (Oguntoyinbo et al., 2011; Cocolin et al., 2013). These methods are more reliable and have been used in several studies regarding fermented cereal foods (Oguntoyinbo et al., 2011; Cocolin et al., 2013).

In this study the microbiomes of four different spontaneous cereal fermentations from Africa was analysed using culture-independent methods with the aim of determining whether their microbiomes share any similarities on a genus level. The microbiome of traditional South African sorghum fermentation was also determined with the aim of identifying a potential starter culture(s) and creating a reference microbiome for future studies.

2.2 MATERIALS AND METHODS

2.2.1 FERMENTATIONS

In this study, three spontaneous sorghum fermentations and one maize fermentation were analysed (Table 5). Two of the sorghum fermentations were prepared in the laboratory by adding 70 g Sorghum flour (King Korn, Tiger Brands, Bryanston) to 1 L distilled water. The gruel was boiled for 10 minutes whereafter it was left to cool down

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to ambient temperature. The gruel was then divided into five 100 ml portions and incubated at 42°C. Sample S1 was incubated for 24 h and sample S2 for 72 h. After incbation the samples were put in the fridge (8°C) until ready for analysis.

The third sorghum fermentation and the maize fermentation was sourced at a market in Lagos, Nigeria. Sample S3 is sorghum ogi, while sample S4 is yellow maize ogi. Both ogi fermentations were produced by the same method. Whole grains were washed and then soaked in a generous amount of clean water. The grains were stored in the water for 24 h, whereafter the grains were washed and put in clean water again. After 48 h the grains were washed again and grounded/blended with a generous amount of water, in a heavy-duty grinder, till smooth. The mixture was then strained through a chiffon cloth with water to remove the chaff/husks which were later disposed of. The strained mixture was left to settle for 3 hours, whereafter the water was carefully decanted. The leftover product was poured into a cotton muslin bag. The water was thoroughly drained through the bag and the content of the bag was left overnight to sour. The ogi was then cut into single-use pieces, wrapped in plastic and sold at the market.

Table 5 Summary of spontaneous fermentations.

Sample code	Product name	Raw material	Fermentation group	Country	Production conditions
S1	Fermented sorghum	Sorghum	Cereal	South Africa	Laboratory
S2	Fermented sorghum	Sorghum	Cereal	South Africa	Laboratory
S3	Ogi	Sorghum	Cereal	Nigeria	Artisanal
S4	Ogi	Yellow maize	Cereal	Nigeria	Artisanal

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2.2.2 MICROBIOME ANALYSIS

The microbial diversity of sorghum fermentations were analysed at the University of Ghana, Accra.

PRE TREATMENT

In order to improve the yield of DNA extracted, a pre-processing treatment was done. This pre-treatment separated the microbial cells from large solid particles present in the sample and reduced the background DNA of the food matrices. Without this pre-treatment the amount of bacterial DNA could be overestimated during the applicaton of high-throughput techniques. First, 20 g of the sample were mixed with 10 ml of cold ultrapure H2O and centrifuged at 800 x g for 1 min at 4°C to remove solid particles. The supernatant was retained and 10 ml H_2O was added. This process was repeated three times to obtain a final volume of approximately 30 ml supernatant. To harvest cells from the supernatants the samples were centrifuged at 3000 x g for 20 min at 4 °C. The supernatant was then discarded and the pellet was washed three times with 1 ml PBS buffer. After centrifugation at 14,000 x g for 2 min, the pellet was resuspended in 978 µl sodium phosphate buffer and 122 µl MT buffer and incubated for 1 h at 4°C (Diaz et al., 2019).

DNA EXTRACTION

The FAST Spin DNA Kit (MP Biomedicals, UK) for soil was used to extract the bacterial DNA. This kit uses mechanical lysis to break the cells and is normally recommended for samples dominated by Gram-positive bacteria.

Using the solutions provided in the kit, 0.5 g of fermented sample were mixed with 978 µl sodium phosphate buffer and 122 µl MT buffer. The samples were then incubated for 1 h at 4°C followed by homogenisation for 60 s at 6.5 m/s, using a FastPrep-24 instrument (MP Biomedicals, UK). This was repeated three times and samples were kept on ice for 5 min between each homogenization step. Apart from homogenisation, the DNA extraction was performed according to the manufacturer's instructions. The extracted DNA was resuspended in 50 µl elution buffer (Diaz et al., 2019).

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DNA QUANTIFICATION

The DNA extracted from the fermented samples was quantified fluorometrically by a Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA) using the Qubit dsDNA BR Assay Kit (Invitrogen) for samples S1, S3 and S4, and the Qubit dsDNA HS Assay Kit (Invitrogen) for sample S2 due to its DNA concentration being <10 ng/µl (Table 6). Bacterial DNA was quantified by quantitative PCR (qPCR) of the 16S rRNA gene using a SensiFAST SYBR No-ROX Kit (Bioline, UK) and a ViiA 7 Real-Time PCR System (Applied Biosystems, USA). The reaction consisted of 2x SensiFAST SYBR No-ROX Mix, 0.4 µM primers 515F and 806R and 0.1 ng DNA as template. A calibration curve (R2 > 0.99) for calculation of bacterial DNA quantity was generated based on gDNA extracted from *L. plantarum* FI11116 (isolated from ogi, a traditional fermented maize) (Diaz et al., 2019).

Table 6 DNA concentrations of spontaneous cereal fermentation samples.

Sample code	DNA concentration (ng/µl)
S1	10.7
S2	8.6
S3	502
S4	434

ILLUMINA HIGH-THROUGHPUT SEQUENCING

16S rRNA gene PCR amplification and sequencing was performed by Novogene Company Limited (Hong Kong). The V4 hypervariable region of the 16S rRNA gene was amplified using specific primers 515F and 806R28. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA). The libraries generated with NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, England) were sequenced using paired-end Illumina sequencing (2 × 250 bp) on the HiSeq2500 platform (Illumina, USA).

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SEQUENCE ANALYSIS:

Sequencing data were analysed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) 2018.8 software29 (Table 7). The demultiplexed paired-end reads were filtered of substitution and chimera errors and merged using Divisive Amplicon Denoising Algorithm 230 (DADA 230). Bacterial taxonomic assignment was performed at 97% similarity using a Naive Bayes classifier trained on the Silva version 132 99% operational taxonomic units (OTU's) database31, where the sequences have been trimmed to only include 250 bases from the V4 region bound by the 515F/806R primer pair. Alpha diversity was analysed using observed OTU's and Shannon phylogenetic diversity indexes.

Table 7 Number of reads per sample after each filtering step.

Sample Code	Sequence Count	Filtered	Denoised	Merged	Non-Chimeric	Non-Background DNA
S1	80091	72462	72462	71119	68837	68748
S2	84950	78859	78859	75296	60689	50148
S3	93400	87568	87568	84947	73746	73473
S4	84943	79578	79578	77385	67803	67649

2.2.3 STATISTICAL ANALYSIS

Significant differences in alpha diversity between groups were calculated using the alpha-group-significance script in QIIME2, which performs the Kruskal-Wallis test. Differences in beta diversity between groups were analysed using Permutational Multivariate Analysis Of Variance (PERMANOVA) including pairwise test. Significant differences ($p<0.05$) in the bacterial community structure amongst the groups were evaluated by Analysis of Composition of Microbiomes (ANCOM).

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2.3 RESULTS

Alpha Diversity

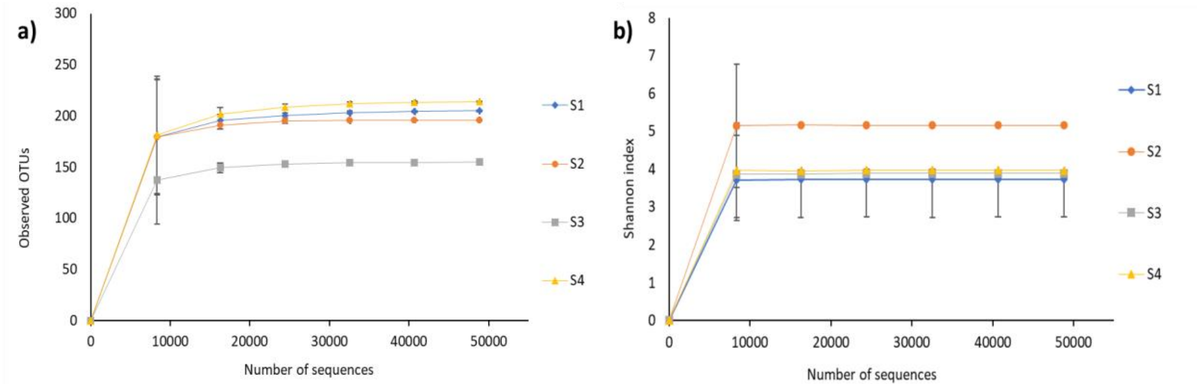

Figure 7 Rarefaction plots of a) Observed OTU's and b) Shannon index for each sorghum fermentation (S1, S2, S3 and S4).

The rarefaction plots in Figure 7 both reached a plataeu, indicating that there was sufficient coverage to capture the majority of the microbial diversity. Figure 7a show that sample S4 had a higher number of observed OTU's than the other fermentation samples. Figure 7b shows that sample S2 had a higher Shannon index while the other samples were all close together.

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Observed OTU's

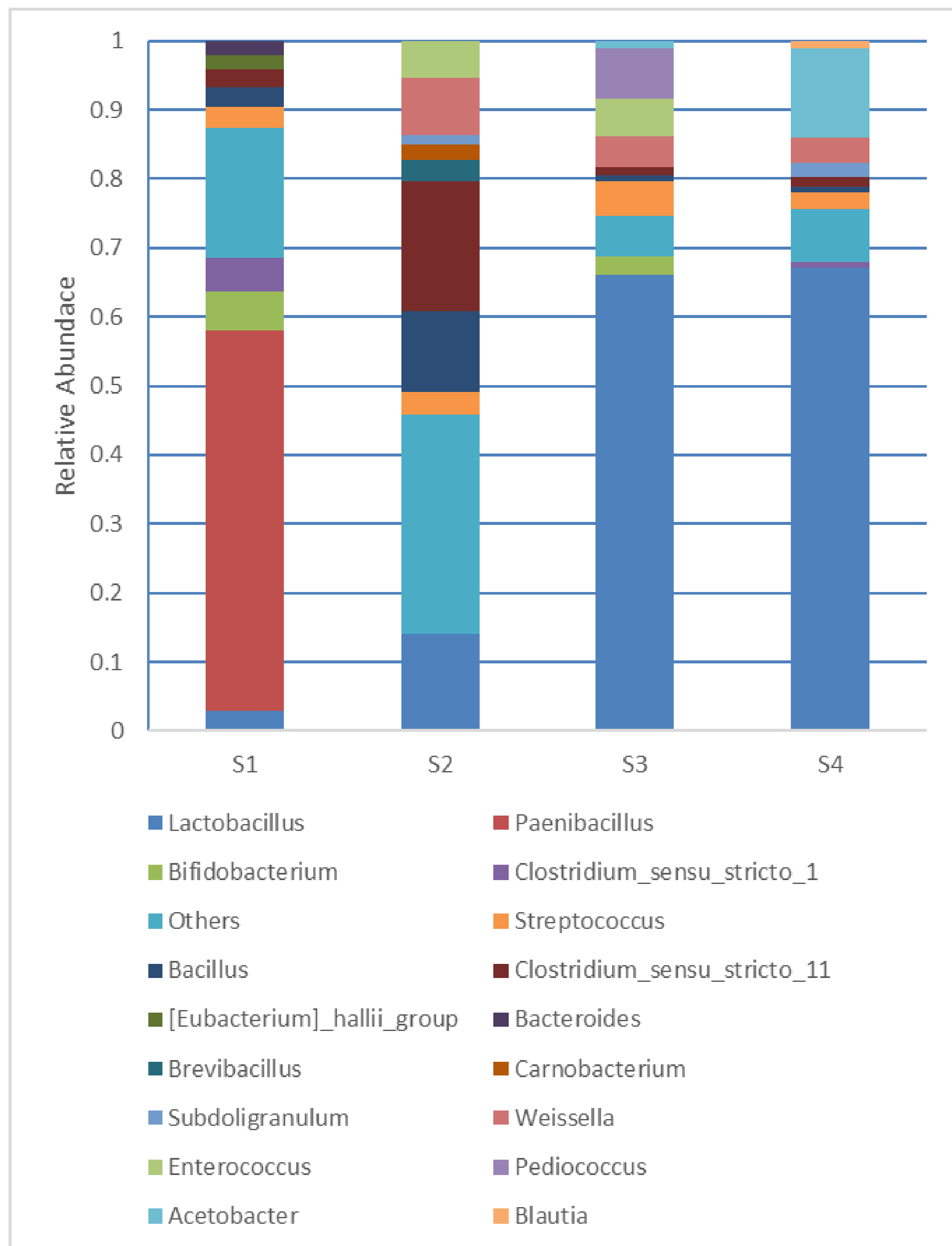

Figure 8 Taxonomic composition of the top 10 OTU's of bacterial communities at genus level in spontaneous cereal fermentations. Sample S1 is sorghum fermented in laboratory for 24 h, sample S2 is sorghum fermented in laboratory for 72 h, sample S3 sorghum ogi from Nigerian market and sample.

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The top 10 OTU's in natural cereal fermentations can be seen in Figure 8. *Lactobacillus* dominated traditional fermentations S3 (0.66) and S4 (0.67) while *Paenibacillus* dominated fermentation S1 (0.55). *Clostridium* sensu_stricto_11 and other unknown OTU's dominated fermentation S2 (0.19 and 0.15 respectively). *Lactobacillus*, *Streptococcus*, *Bacillus* and *Clostridium* sensu_stricto_11 were found in all four fermentations in varying abundancies.

2.4 DISCUSSION

The microbiomes of spontaneous cereal fermentations tend to share OTU's as they are typically lactic acid fermentations where *Lactobacillus* dominates the microbiome (Humblot and Guyot, 2009; Chaves-Lopez et al., 2016; Diaz et al., 2019). This was seen in both fermentations S3 and S4 where *Lactobacillus* was the dominant OTU. This was expected as these fermentations types are similar and were produced in the same environment (Houngbédji et al., 2018).

Limited studies were available to explain the abundant presence of *Paenibacillus* in fermentation S1. One study isolated *Paenibacillus* strains from sorghum rhizospheres (Rodrigues et al., 2008), while another successfully isolated *Paenibacillus* from environmental air (Sáez-Nieto et al., 2017). It is therefore possible that the *Paenibacillus* could have originated from the sorghum flour itself or the laboratory environment. It is clear that the *Paenibacillus* drastically lowered between fermentations S1 (24 h) and S2 (72 h). This could be explained by the increase of other OTU's such as *Lactobacillus* and unidentified OTU's as they became more adapted to the environment and experienced exponential growth.

According to Houngbédji et al. (2018) *Lactobacillus* was present in commercial sorghum mawe before fermentation and *Lactobacillus* counts increased over time as the fermentation took place. The relative abundance of *Lactobacillus* in sample S2 is higher than in sample S1 which was expected as sample S2 was incubated for a longer time. *Lactobacillus* is naturally present in sorghum (Oguntoyinbo et al., 2011), and when allowed to ferment the amount of *Lactobacillus* increased over time (Mohammed, Steenson and Kirleis, 1991 and Houngbédji et al., 2018).

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When comparing the relative abundance of *Lactobacillus* in samples S1 and S2 with samples S3 and S4, it is clear that the samples produced in the laboratory (S1 and S2) had a much lower level than those produced locally in Nigeria (S3 and S4). Studies have shown that microorganisms, such as *Lactobacillus*, is not only inherantly present in cereals, but also introduced by equipment such as utensils during the production of cereal fermentation (Cocolin et al., 2013). The lower abundance of *Lactobacillus* in the lab fermentations can therefore potentially be explained by the use of sterile equipment (Diaz et al., 2019). The effect of sterile conditions were also clear as the concentration of DNA isolated in the lab fermentations were much lower than that of the local fermentations.

Generas such as *Streptococcus* and *Bacillus* were found in all fermentations and was part of the top ten observed OTU's, while other generas such as *Weisella*, *Pediococcus* and *Lactococcus* were also found in all fermentations, but not necessarily in the top 10 observed OTU's (not shown). These observations agree with previous studies describing African cereal fermentations (Hozapfel, 1997; Humblot and Guyot, 2009; Oguntoyinbo et al., 2011; Mukisa et al., 2012; Houngbédji et al., 2018).

It is important to keep in mind that not all bacteria are favourable in the end product. Some microorganisms such as *Clostridium* and *Bacillus* are potential pathogens and their presence highlights the fact that the fermentation methods used were uncontrolled (Oguntoyinbo et al., 2011). The isolation of the *Lactobacillus* present in fermentation samples S1 and S2 could aid in the development of starter cultures for South African sorghum fermentations. Not only can starter cultures aid in the safety of the product, it can also add in the production of a consistent quality product (Holzapfel, 1997).

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2.5 CONCLUSIONS

The microbiomes of spontaneous cereal fermentations were succesfully determined using 16S rRNA amplicon sequencing. The microbiomes of the natural fermentations obtained from Nigeria were similar, with *Lactobacillus* being the dominating OTU. The microbiomes of the fermentations produced in the laboratory were different, not only from each other, but also from the natural Nigerian fermentations. Differences are contributed to different processing methods, times and environments. The S1 microbiome was succesfully determined and can serve as a reference for future studies on the same fermentation. From this microbiome *Lactobacillus* was identified as a potential starter culture for South African sorghum fermentations. Further research is however needed in order to identify specific *Lactobacillus* strains to be used as starter cultures

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CHAPTER 2: RESEARCH PHASE 2 - INCREASING THE FOLATE CONTENT OF FERMENTED SORGHUM WITH *L. PLANTARUM* AND ANALYSING THE BACTERIAL COMMUNITIES THROUGH 16S rRNA APPROACH

Abstract

Fermented foods are popular around the globe for various reasons. They form part of the daily diet of many communities in sub-Saharan Africa due to their extended shelf life and unique sensory properties. The microorganisms responsible for these fermented products can often confer a variety of nutritional and health beneficial effects, including the production of vitamins. Folate (vitamin B9) is an important vitamin responsible for the prevention of NTD's in foetuses. For this reason, folic acid, the synthetic form of folate, is taken as a supplement by pregnant women. However, excessive levels of folic acid could potentially mask a vitamin B12 deficiency. The availability of a natural source of dietary folates could therefore eliminate or reduce the need for folic acid as a supplement. In this study the effect of folate producing *L. plantarum* strains (FS2, B411, S7 and S49), with and without 10% sorghum malt, on the folate content of fermented sorghum gruel was determined. The microbiomes of the fermentations without sorghum malt were also analysed. It was found that all the strains, alone and in combination with malt, significantly increased the folate content after 24 h of fermentation. The highest folate content was obtained when *L. plantarum* B411 and sorghum malt was used in combination. This combination is considered the best option to use for the development of a starter culture for the commercialisation of fermented sorghum products biofortified with folate. The analysis of the microbiomes gave detailed insight into the fermentations on a microbial level and showed that all fermentations inoculated with *L. plantarum* were similar.

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2.6 INTRODUCTION

Fermented cereal products are popular in the diets of people in Africa (Nyanzi and Jooste, 2012). These fermentations are obtained mainly through natural and uncontrolled fermentation processes which may cause irregularities in the quality and stability of the products (Mukisa et al., 2012). Studies have identified the microorganisms responsible for many different fermented products (Leroy and De Vuyst, 2004). These microorganisms can be used as starter cultures in order to have better control of the fermentation process and ultimately the end product (Holzapfel, 1997).

Malt has a history of being added to cereal fermentations due to its functional properties (Mukisa et al., 2012). Amylase, an enzyme produced during the processing of malt, allows for better fermentation and results in products with reduced viscosity (Taylor and Duodu, 2019). The presence of amylase contributes to the initiation of lactic acid fermentation as the fermentable sugars in the malt will be utilised by LAB for growth (Gadaga, Lehohla and Ntuli, 2013).

The microorganisms in a fermentation are not only responsible for the fermentation process itself; they also affect the sensory, nutritional and functional properties of the fermentations (Leroy and De Vuyst, 2004; Gänzle, 2015). In order to commercialize or develop a fermented sorghum product it is important to understand what fermentations look like on a microbial level. A microbiome refers to all the microorganisms and their genomes in a specified environment and the most recent and comprehensive culture-independent method for studying these microbial communities within foods is through meta-analysis (Ercolini, 2013). Meta-analysis is achieved through 16S rRNA gene sequencing and allows for a detailed insight into fermentations on a microbial level.

L. plantarum strains are well studied and are commonly present in a variety of fermentations including fermented sorghum (Corsetti and Valmorri, 2011; Mukisa et al., 2012). Research has shown that *L. plantarum* is capable of producing substances such as antimicrobial compounds, vitamins etc., and/or have a direct beneficial health effect when consumed (LeBlanc et al., 2011; Behera, Ray and Zdolec, 2018). One of these vitamins is folate which plays a crucial role in the prevention of NTD's (Molloy, 2005). Humans cannot produce folate themselves and therefore mandatory

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fortification with folic acid, the synthetic form of folate, has been implemented for some foods in South Africa (SA. Department of Health, 2003). There are however still some concerns regarding its long-term use since an excess amount of folic acid can potentially mask a vitamin B12 deficiency (LeBlanc et al., 2011). Alternative dietary sources of folate are therefore needed.

The present study was thus undertaken to determine whether the use of folate producing *L. plantarum* strains as starter cultures will increase the folate content of fermented sorghum gruels. Sorghum malt was added as a source of amylase with the aim of increasing the growth of *L. plantarum* which in return could result in higher production of folate. The microbiomes of these fermentations were also analysed.

2.7 MATERIALS AND METHODS

2.7.1 SORGHUM FERMENTATION PROCESS

Sorghum fermentations were made by mixing 70 g sorghum flour (King Korn, Tiger Brands, Bryanston) with 1 L distilled water and cooked for 30 minutes on an electric hotplate. The gruel was continuously stirred to prevent lumps from forming. After the sorghum gruel cooled down to ambient temperature it was divided into 4 portions of 90 g each and inoculated with 10 ml 10^6 CFU/ml of selected *L. plantarum* strains (FS2, B411, S7 and S49). The *L. plantarum* strains were selected from the study 'Contribution of cereal-based fermented foods to folate intake in European and African countries' (Fayemi and Buys, 2017). A separate 100 g portion of the sorghum gruel was used for the spontaneous fermentation. The fermentation flasks were incubated for 24 hours at 42˚C. For the malt treatments, gruels were prepared from 7 g sorghum malt (King Korn, Tiger Brands, Bryanston) added to 63 g sorghum flour (King Korn, Tiger Brands, Bryanston) to obtain a final malt concentration of 10% and this was followed by the fermentation process as described above.

2.7.2 LACTIC ACID BACTERIA AND YEASTS AND MOULD COUNTS

After 24 h of fermentation 10 g of each fermentation was added to 90 ml peptone buffer. These solutions were stomached for 2 minutes where after dilutions were made. LAB, and yeasts and mould counts were determined by plating dilutions (10^{-1}

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to 10^{-8}) on MRS (OXOID) and Potato Dextrose Agar (OXOID) respectively and incubating them at 30°C for 48 hours.

2.7.3 PH AND TITRATABLE ACIDITY

The pH of each fermentation was measured with a digital benchtop pH meter (Crison, Barcelona, Spain) before and after 24 h of fermentation. Titratable acidity (TA) was measured before and after 24 h of fermentation by titrating 0.1 N NaOH against 9 ml of the fermentation with 10 drops phenolphthalein indicator solution (FAO/SIDA, 1997). The TA was expressed as a percentage lactic acid and calculated by dividing the millilitres needed for titration by 10.

2.7.4 FOLATE EXTRACTION AND QUANTIFICATION

An adaptation of the microbiological folate assay methods described by Kariluoto (2013) and Greppi et al. (2017) was used.

Preparation of solutions: Amylase was prepared by adding 20 g of α-amylase (Sigma-Aldrich) to 1 ml 1% ascorbic acid (Sigma Aldrich). Protease was prepared by adding 4 mg protease (Sigma Aldrich) to 2 ml 1% ascorbic acid. These solutions were prepared in advance and stored at –20°C until ready to use.

Extraction: After 24 h fermentation, 4.5 ml of each fermentation was twice diluted with Hepes buffer (pH 7.85) in small Erlenmeyer flasks. The flasks were flushed with nitrogen, whereafter they were heated at 100˚C for 10 minutes in a covered waterbath and vortexed twice while heating. The flasks were cooled on ice and the pH was adjusted to 4.9 with glacial acetic acid. Human plasma (2 ml) (SANBS) and α- amylase (1 ml) (Sigma-Aldrich) was added to each flask before they were incubated at 37°C for 3 hours in a covered shaking waterbath. Afterwards the pH was adjusted to 7 with 50% NaOH, 2 ml protease (Sigma-Aldrich) was added and the flasks were flushed with nitrogen. The flasks were once again incubated at 37°C for 1 hour in a covered shaking waterbath, followed by heating to 100°C for 10 minutes and cooled on ice. Each flask were then filled to 25 ml with a 0.5% sodium ascorbate buffer and a 1 ml aliquot from each flask was centrifuged for 10 minutes at 13 000 rpm in Eppendorf tubes. Afterwards, 100 µl aliquots from each tube was added to folic acid assay media (Sigma-Aldrich) with *Streptococcus faecalis* as the indicator microorganism. These tubes were incubated at 37˚C for 16-18 h.

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Quantification: After incubation the tubes were kept at 7°C for 30 minutes to stop growth of the indicator microorganism and ensure accurate readings. Turbidimetric readings were then read at 620 nm, thereafter folate levels were calculated with the use of a standard curve.

A standard curve was set up for each separate assay. This is because the autoclaving and incubation conditions that influence the standard curve readings cannot always be duplicated. The standard curve was obtained by using folic acid (Sigma-Aldrich) at levels of 0, 2, 4, 6, 8 and 10 ng per 10 ml assay tube.

2.7.5 CALCULATION OF PERCENTAGE CHANGE IN FOLATE CONTENT BETWEEN AND WITHIN FERMENTATIONS

% Increase in folate content between fermentations

This value indicates the % increase in folate content between the spontaneous fermentation without malt and any other fermentation.

$$\%\ Increase\ between\ fermentations = \frac{F\ fermentation - F\ spontaneous\ without\ malt}{F\ spontaneous\ without\ malt} \times 100$$

F fermentation = folate content of any fermentation

F spontaneous without malt = folate content of spontaneous fermentation without malt

% Increase in folate content within fermentations

This value indicates the % increase between the fermentation without malt and the fermentation with malt of 2 fermentations inoculated with the same *L. plantarum* strain.

$$\%\ Increase\ within\ fermentations = \frac{F\ with\ malt - F\ without\ malt}{F\ without\ malt} \times 100$$

F with malt = folate content of fermentation with malt

F without malt = folate content of fermentation without malt

2.7.6 ANALYSIS OF BACTERIAL COMMUNITIES IN FERMENTATIONS

DNA EXTRACTION

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DNA from the sorghum fermentations (24 h) was extracted using a ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, 2019) following the prescribed protocol with 'soil' as the selected sample type. First, 250 mg of the fermentations were mixed with 750 µl of ZymoBIOMICS™ Lysis Solution through vortexing on maximum speed for 5 minutes. The sample solids were then removed by centrifuging the ZR BashingBead™ Lysis Tubes at 10 000 × g for 1 minute. After the solids were removed, 400 µl of the supernatant was filtered with further centrifugation at 8 000 × g for a minute in a filter-fitted collection tube. DNA binding buffer solution (800 µl) was added to the filtrate and mixed whereafter duplicate 800 µl aliquots of the mixture were centrifuged, one at a time, at 10 000 × g for 1 min in a collection tube. The flow-through from both aliquots were then discarded. The pellet was then washed twice with 700 µl and 200 µl of the DNA wash buffer, separately, and the flow-through was discarded after centrifuging at 10 000 × g. The extracted DNA was then suspended in 100 µl DNase Free water for 1 minute which was eluded by centrifugation a 10 000 × g for 1 min. The eluded DNA was finally filtered into a clean microcentrifuge tube at exactly 16 000 × g for 3 minutes. The DNA was stored at -20°C until molecular analysis could take place.

TAXONOMIC COMPOSITION AND 16S RRNA SEQUENCE DIVERSITY ANALYSIS THROUGH QIIME SOFTWARE

The isolated DNA was sent to Mr DNA lab (Shallowater, TX, USA) for 16S rRNA amplification and sequencing. The microbiome data (16S rRNA) of the sorghum fermentation samples were processed through the software package "QIIME2" version 2018.11. (QIIME2, 2018). The raw paired-end FASTQ reads were imported and demultiplexed using demux command of QIIME2. The Divisive Amplicon Denoising Algorithm 2 (DADA2) was used to de-noise, quality filter, trim, and merge the data (Table 8). Chimeric sequences were removed using the consensus method. A feature classifier in QIIME2 trained with the Greengenes 99% OTU database was used to assign taxonomy to all ribosomal database project classifier algorithm in QIIME. Contaminating mitochondrial and chloroplast sequences were filtered out of the resulting feature table. A total of 247066 valid sequences were generated from the fermented food DNA samples, and after filtering and trimming, 196134 high-quality reads were obtained. A sequencing depth cut-off value of 1782 sequences from each sample was set and designed into OTU's. The demultiplexed sequence count summary graph, and the alpha rarefaction graph is given in Figure 9. The alpha

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diversity (microbial diversity within samples) and richness indices of the samples were measured by observed species OTU's and the Shannon diversity index.

Table 8 Quality filtering and denoising the sequences through Divisive Amplicon Denoising Algorithm 2 (DADA2).

Sorghum fermented with *L. plantarum*	**input**	**filtered**	**denoised**	**merged**	**non-chimeric**
B411	56886	52610	52610	45923	45246
FS2	60591	56190	56190	48675	48032
S49	66677	61796	61796	54456	53887
S7	62912	58308	58308	49593	48969

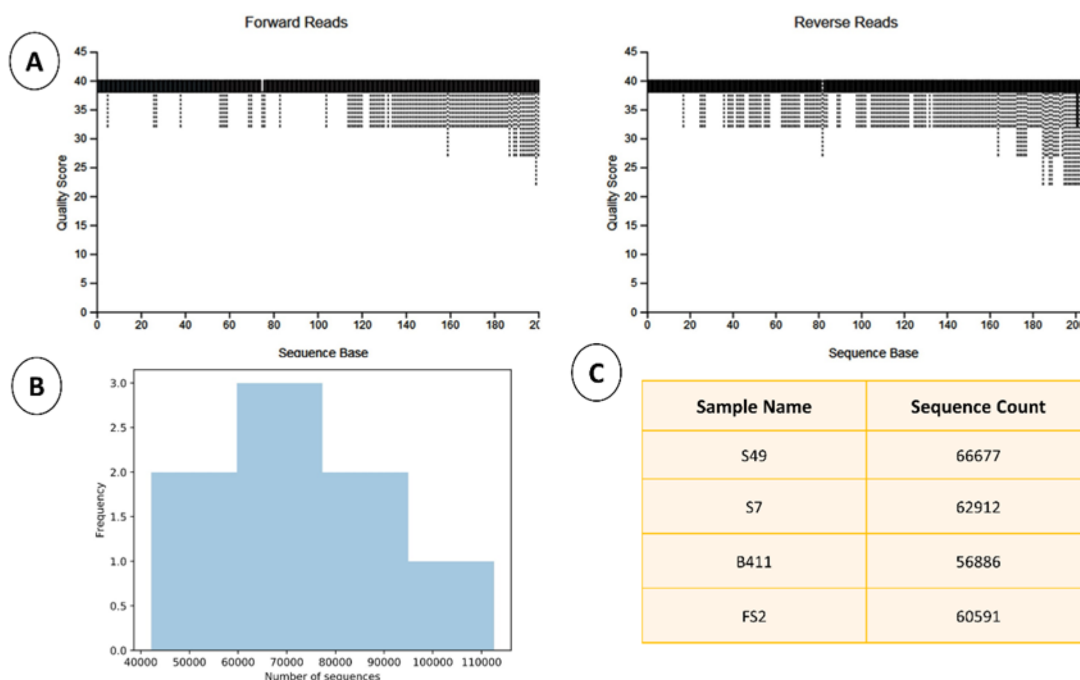

Figure 9 The raw data was demultiplexed through Qiime 2. A indicates the quality score of forward and reverse reads of the sample sequences. The sequences are of good quality since the score is more than 20. B indicates the frequency of sampling in regards of sequence counts obtained from the 16S rRNA data of the samples.

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2.7.7 STATISTICAL ANALYSIS

A two-way ANOVA (Analysis of Variance) was used in SPSS Statistics software for the difference in folate contents between sorghum fermentations. Microsoft Excel 2010 was used for assimilation of data and graphs. Significant differences were tested at 95% confidence level for all tests. For the sequencing data the alpha diversity was determined with Shannon index using QIIME2 software.

2.8 RESULTS

2.8.1 STATISTICAL ANALYSIS

Table 9 Analysis of variance of the effect of fermentation treatment (*L. plantarum* strains and with or without malt) on the lactic acid bacteria counts, yeast and mould counts, pH and titratable acidity of sorghum gruel fermented for 24 h (p<0.05).

Treatment	df	Sig.
Fermentation (Spontaneous and *L. plantarum* FS2, B411, S7 and S49)	4	0.000
Malt (With or Without)	1	0.000
Fermentation * malt	4	0.000

Treatment	Lactic Acid Bacteria Counts		Yeast and Mould Counts		pH		Titratable Acidity	
	df	Sig.	df	Sig.	df	Sig.	df	Sig.
Fermentation (Spontaneous and *L. plantarum* FS2, B411, S7 and S49)	4	0.000	4	0.000	4	0.000	4	0.000
Malt (With or Without)	1	0.496	1	0.404	1	0.000	1	0.000
Time (0 h and 24 h)	1	0.000	1	0.000	1	0.000	1	0.000
Fermentation * Malt * Time	13	0.000	13	0.000	13	0.000	13	0.000

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Table 10 Analysis of variance of the differences between the folate contents of sorghum gruel fermented spontaneously or with *L. plantarum* strains, with or without malt, for 24 hours ($p<0.05$).

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2.8.2 LACTIC ACID BACTERIA COUNTS

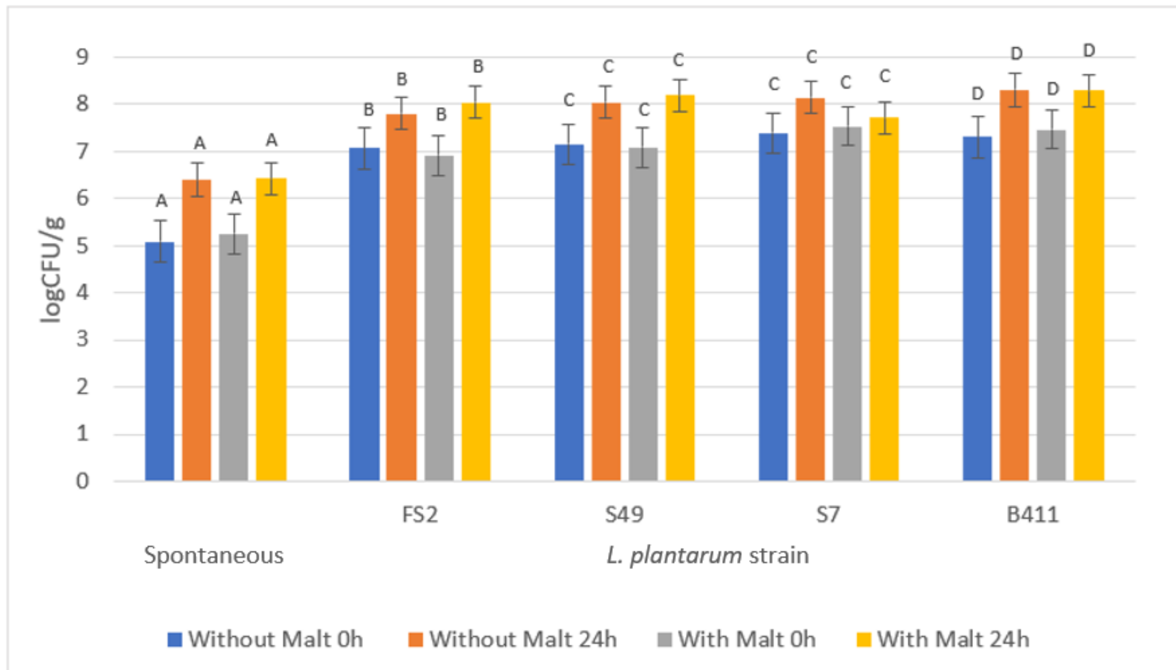

Figure 10 Lactic acid bacteria counts of sorghum gruel fermented spontaneously or with *L. plantarum* strains before and after 24 hours of fermentation, with or without malt to increase the folate content. Fermentations with different superscripts differ significantly ($p<0.05$, Table 9).

Figure 10 shows the LAB counts in sorghum fermented with *L. plantarum* strains. For all the fermentation treatments the lactic acid bacteria counts significantly increased during 24 h of fermentation ($p<0.05$, Table 9). These significant differences are not indicated on the graph since there were only two groups (0 h and 24 h), meaning that the significant difference can only be between these two groups. The mean values for the spontaneous, FS2, and B411 fermentations were all significantly different from each other and the S7 and S49 fermentations. The addition of malt had no significant effect on the LAB growth. The spontaneous sorghum fermentation without malt had the lowest count of 5.09 logCFU/g before fermentation. After fermentation it increased to 6.40 logCFU/g. The spontaneous fermentation with malt had an initial count of 5.25 logCFU/g and increased to 6.43 logCFU/g after fermentation. A similar trend can be seen throughout for the other fermentations. The sorghum fermentation with *L. plantarum* B411 without malt had the highest count of 8.31 logCFU/g after fermentation.

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2.8.3 YEAST AND MOULD COUNTS

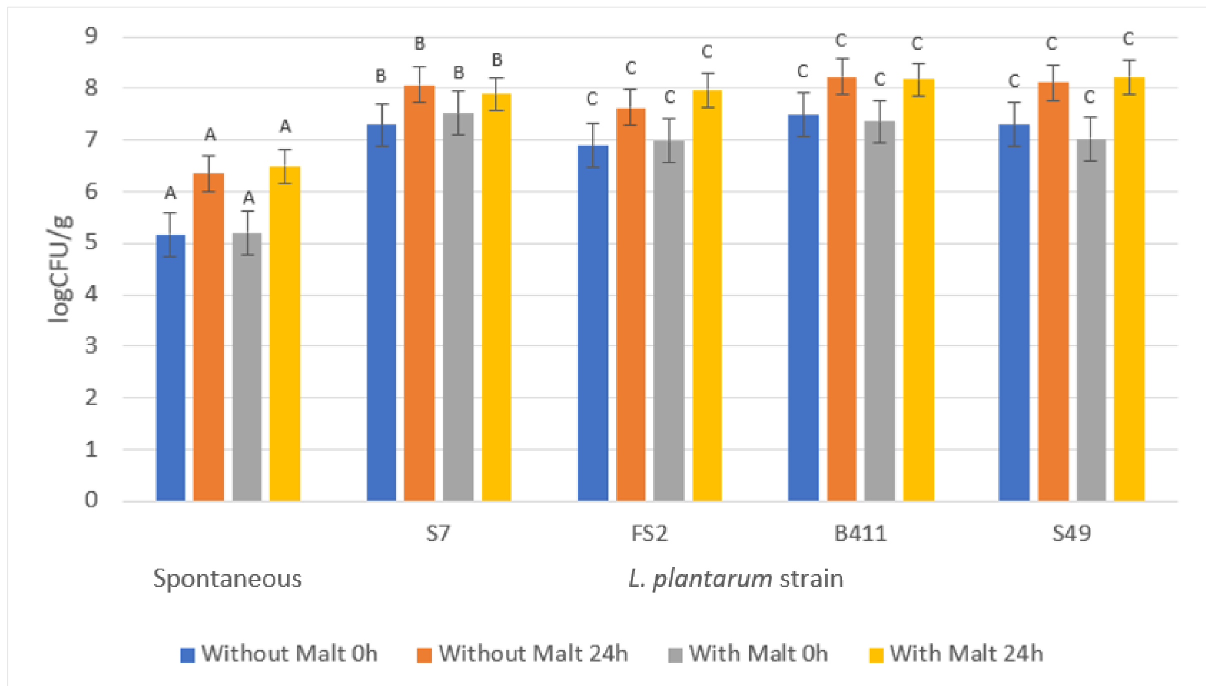

Figure 11 Yeast and mould counts of sorghum gruel fermented spontaneously or with *L. plantarum* strains before and after 24 hours of fermentation, with or without malt. Fermentations with different superscripts differ significantly ($p<0.05$, Table 9).

Figure 11 shows the yeast and mould counts of sorghum fermented with *L. plantarum* strains with and without malt. The *L. plantarum* strains used during fermentation significantly affected the yeast and mould counts during fermentation of the sorghum ($p<0.05$, Table 9). The mean values for the spontaneous and FS2 fermentations were significantly different from each other and the B411, S7 and S49 fermentations. For all the fermentation treatments the lactic acid bacteria counts significantly increased during 24 h of fermentation ($p<0.05$, Table 9). These significant differences are not indicated on the graph since there were only two groups (0 h and 24 h), meaning that the significant difference can only be between these two groups. The yeast and mould counts were not affected differently by the malt treatments. The spontaneous sorghum fermentation without malt had the lowest count before fermentation of 5.18 logCFU/g and increased to 6.36 logCFU/g after fermentation. The spontaneous sorghum fermentation with malt had an initial count of 5.20 logCFU/g before fermentation and increased to 6.49 logCFU/g after fermentation. The highest count (8.22 logCFU/g) was

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seen with both the *L. plantarum* B411 without malt, and *L. plantarum* S49 with malt treatments after fermentation.

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2.8.4 PH AND TITRATABLE ACIDITY

Table 11 Effect of spontaneous fermentation and inoculation with *L. plantarum* (FS2, B411, S7 and S49) on the pH and titratable acidity (TA) of sorghum gruel fermented for 24 h, with and without malt.

Fermentation	**Without Malt**				**With Malt**			
	0 h		**24 h**		**0 h**		**24 h**	
	pH	**TA (%)**	**pH**	**TA (%)**	**pH**	**TA (%)**	**pH**	**TA (%)**
Spontaneous	6.32^{C} ± 0.64	0.05^{a} ± 0.01	5.43^{C} ± 0.25	0.07^{a} ± 0.01	6.24^{C} ± 0.14	0.05^{a} ± 0.01	4.47^{C} ± 0.05	0.10^{a} ± 0.01
S7	6.03^{B} ± 0.00	0.05^{b} ± 0.01	4.20^{B} ± 0.00	0.09^{b} ± 0.01	5.96^{B} ± 0.01	0.06^{b} ± 0.00	4.03^{B} ± 0.01	0.14^{b} ± 0.00
FS2	6.05^{AB} ± 0.02	0.05^{c} ± 0.01	4.15^{AB} ± 0.04	0.11^{c} ± 0.01	6.08^{AB} ± 0.07	0.06^{c} ± 0.00	3.68^{AB} ± 0.01	0.17^{c} ± 0.01
B411	5.99^{A} ± 0.01	0.06^{c} ± 0.01	4.16^{A} ± 0.08	0.10^{c} ± 0.01	6.05^{A} ± 0.01	0.06^{c} ± 0.01	3.61^{A} ± 0.04	0.20^{c} ± 0.01
S49	6.06^{AB} ± 0.04	0.05^{d} ± 0.01	4.20^{AB} ± 0.04	0.10^{d} ± 0.01	6.12^{AB} ± 0.01	0.06^{d} ± 0.01	3.49^{AB} ± 0.00	0.23^{d} ± 0.01

Mean values with different superscripts in different columns differ significantly ($p<0.05$)

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Table 11 shows the effect of lactic acid fermentation (spontaneously or with *L. plantarum* strains) with and without malt on the pH and TA of sorghum gruels. The spontaneous fermentation, with and without malt, had pH values of 6.24 and 6.32 respectively at 0 h. After fermentation, the pH of the spontaneous fermentation with malt decreased to 4.47 while the spontaneous fermentation without malt only decreased to 5.43. All pH values at 24 h of the fermentations made with malt were significantly lower than that of the fermentations made without malt ($p<0.05$, Table 9). Both spontaneous fermentations had a TA of 0.05% at 0 h. The spontaneous fermentation without malt increased to 0.07% and the spontaneous fermentation with malt increased to 0.10%. This was expected and corresponds with the pH values as pH and TA are inversely related to each other. All TA values at 24 h of the fermentations made with malt were significantly higher than the fermentations made without malt ($p<0.05$, Table 9).

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2.8.5 FOLATE CONTENT

Table 12 Effect of spontaneous fermentation and inoculation with *L. plantarum* (FS2, B411, S7 and S49) on the folate content (µg/100 ml) of sorghum gruels fermented for 24 hours, with and without malt.

Fermentation		Folate (µg/100 ml)		Fermentation		Folate (µg/100 ml)		%increase between fermentations[a]	%increase within fermentations
Spontaneous	-malt	14.57 ^{A}	± 1.45	Spontaneous	+malt	20.08 ^{A}	± 0.67	38	-
				L. plantarum FS2	+malt	23.35 ^{B}	± 3.55	60	44
					-malt	16.22 ^{B}	± 1.25	11	
				L. plantarum S49	+malt	20.89 ^{B}	± 0.36	43	10
					-malt	18.93 ^{B}	± 0.82	30	
				L. plantarum S7	+malt	30.39 ^{C}	± 3.52	109	39
					-malt	21.81 ^{C}	± 0.91	50	
				L. plantarum B411	+malt	31.82 ^{D}	± 0.50	118	38
					-malt	23.13 ^{D}	± 3.85	59	

Mean values with different superscripts in different columns differ significantly ($p<0.05$)

[a] % increase between fermentations calculated on spontaneous fermentation without malt

+malt is with 10% malt

-malt is without malt

Table 12 shows the effect of lactic acid fermentation (spontaneously or with *L. plantarum* strains), with or without malt, on the folate content of sorghum gruels. The spontaneous fermented sorghum gruel without malt had the lowest folate content of 14.57 µg/100 ml and was significantly lower ($p<0.05$, Table 10) than all the other fermentations, except the spontaneous fermentation made with malt. The spontaneous fermentation, without malt, was not inoculated with any starter culture and is representative of traditional sorghum fermentations available in South Africa. The folate content of this spontaneous fermentation, without malt, was used to calculate the % increase between fermentations.

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This % increase between fermentations indicated the percentage difference between any fermentation and the spontaneous fermentation without malt. The fermentation with the highest increase from the spontaneous fermentation without malt was the fermentation inoculated with *L. plantarum* B411 and malt. This fermentation showed an increase of 118% and had a folate content of 31.82 µg/100 ml. This fermentation was significantly different from all other fermentations ($p<0.05$, Table 10). The folate content of the fermentation made with *L. plantarum* S7 and malt showed a 109% increase from the folate content of the spontaneous fermentation without malt. This fermentation had the second highest folate content overall of 30,39 µg/100 ml. Sorghum fermentations made with *L. plantarum* S7 and B411 were significantly higher than sorghum fermented with FS2 and S49. The fermentations made with *L. plantarum* FS2 with malt, and *L. plantarum* S49 with malt, had folate contents of 23.35 µg/100 ml and 20.89 µg/100 ml, respectively. The folate contents of these two fermentations were not significantly different from each other but were indeed significantly higher than the spontaneous fermentation without malt ($p<0.05$, Table 10).

The % increase of folate within fermentations were calculated between any fermentation made with *L. plantarum* with malt and a fermentation made with the same *L. plantarum* strain but without malt. This % increase within fermentations were determined to observe what the effect of sorghum malt was on the folate content of each fermentation. Sorghum malt had the biggest effect on the *L. plantarum* FS2 fermentations where the folate content increased by 44% between the fermentations made with *L. plantarum* FS2 without malt and *L. plantarum* FS2 with malt. The % increase of folate within fermentations made with *L. plantarum* B411 were 38% while the % increase within the *L. plantarum* S7 fermentations were 39%. The fermentations made with *L. plantarum* S49 showed an increase of 10%. The folate contents of all the fermentations made with sorghum malt were higher than the same fermentations made without malt. This is represented by a positive % increase within all fermentations.

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2.8.6 ANALYSIS OF MICROBIOMES OF FERMENTED SORGHUM

ALPHA DIVERSITY

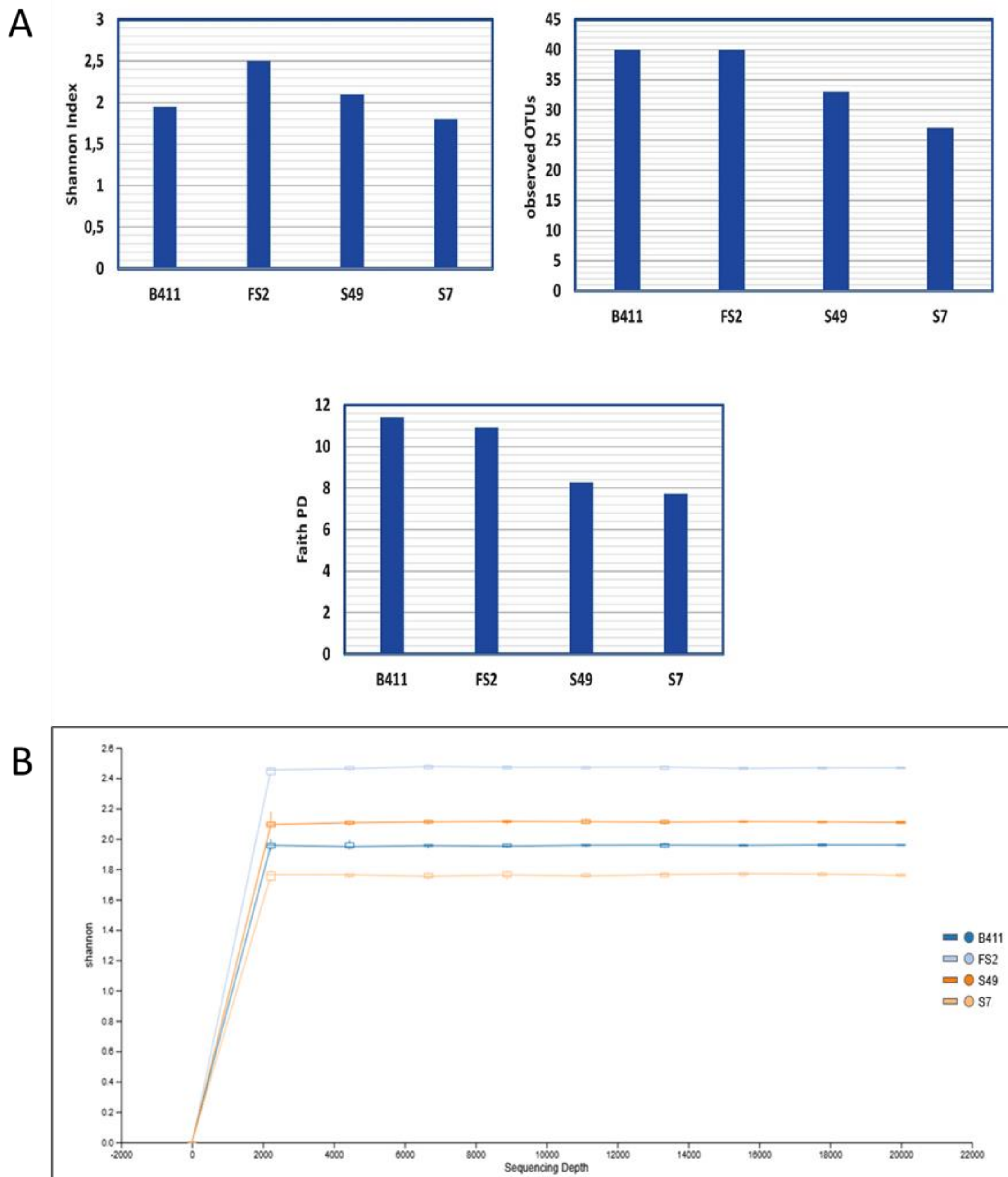

Figure 12 A) Shannon index, observed OTU's and Faith PD (phylogenetic diversity) of the bacterial communities of sorghum fermented with folate producing *L. plantarum* B411, FS2, S49 and S7 B) Shannon index of sorghum fermentations.

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Figure 12 shows the Shannon index of sorghum fermented with *L. plantarum* B411, FS2, S49 and S7. The Shannon index represents the alpha diversity (diversity within) of these fermentations and it can be seen in both Figure 12 A and B. The fermentation made with *L. plantarum* FS2 had the highest index of 2.5 followed by S49 (2.2), B411 (1.95) and S7 with the lowest value at 1.8. A higher Shannon index indicates more diversity, while a lower index shows less diversity. The observed OTU's in each fermentation sample can be seen in Figure 12A. This is an indication of specie richness, where a high number indicates more species. Both the sorghum fermented with *L. plantarum* B411 and FS2 had an observed number of 40, S49 had 33 and S7 had 27. The Faith PD can be seen in Figure 12A. Like observed OTU's, this is also an indication of the specie richness, however Faith PD incorporates the phylogenetic relationships between features of the OTU's. Faith PD is equal to the sum of the length of branches of the phylogenetic tree that span the members of the set. The sorghum fermented with *L. plantarum* B411 had the highest value of 11.4 and was the most specie rich followed by FS2 (10.9), S49 (8.3) and S7 (7.8). It is important to note that with alpha diversity you cannot compare the fermentations with each other. While one fermentation might be more specie rich that another, it does not mean it is more diverse. This can only be done with beta diversity.

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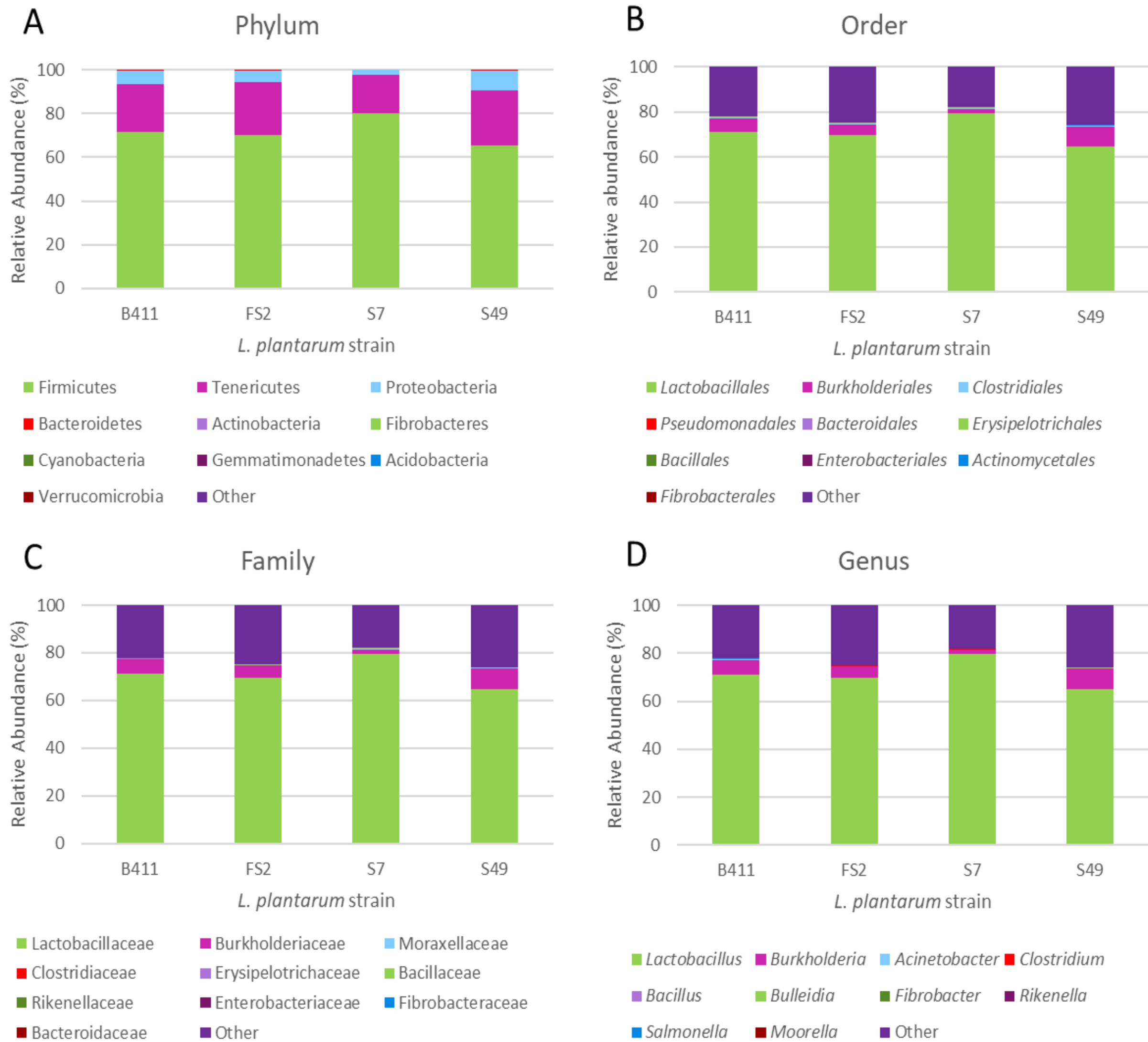

Figure 13 Relative abundance of bacterial taxa in sorghum fermented with *L. plantarum* B411, FS2, S49 and S7 at the A) phylum, B) order, C) family and D) genus levels. Only the top 11 taxa from each fermentation is shown.

The relative abundance of the bacterial taxa at the phylum, order, family and genus level can be seen in Figure 13. Figure 13A shows that all fermentations were dominated by the phylum Firmicutes (>60%), followed by Tenericutes (>17%). Proteobacteria were also present in all fermentations (>12%). The dominant order, family and genus in all fermentations were Lactobacillales, *Lactobacillaceae*, and *Lactobacillus* respectively (>64.9%) (Figure 13 A, B and C). Unidentified orders, families and genera made up approximately 20% in all fermentations, while the phylum Burkholderiales the family *Burkholderiaceae* and the genus *Burkholderia* were present in small amounts in all fermentations.

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0.0
0.8
1.6
2.4
3.2
4.0
log10 frequency

k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__cereus
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae;g__;s__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Acholeplasmatales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardioidaceae;g__Aeromicrobium;s__
k__Bacteria;p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;f__RFP12;g__;s__
k__Bacteria;p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter;s__
k__Bacteria;p__Tenericutes;c__RF3;o__ML615J-28;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;__;__;__
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HF | S7
HF | S49
LF | B411
LF | FS2

Figure 14 Heatmap showing the core bacterial microbiomes of sorghum fermented with *L. plantarum* FS2, B411, S49 and S7 and the log10 frequency of each identified specie.

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The heatmap of the bacteria present in sorghum fermented with *L. plantarum* B411, FS2, S49 and S7 is shown in Figure 14. The colour intensity of the blocks directly relates to the log10 frequency of the identified bacteria with lighter blocks having a higher frequency than those who are dark. It is clear that all fermentations were dominated by the family Lactobacillaceae as the corresponding blocks are very light in all fermentations (>4.0 log10). The genus *Burkholderia* is of importance as it had high log10 frequencies in all fermentations. *L. brevis* is present in all fermentations but at clear different frequencies. Sorghum fermented with *L. plantarum* S49 and S7 had higher log10 frequencies of *L. brevis* than B411 and FS2.

BETA DIVERSITY

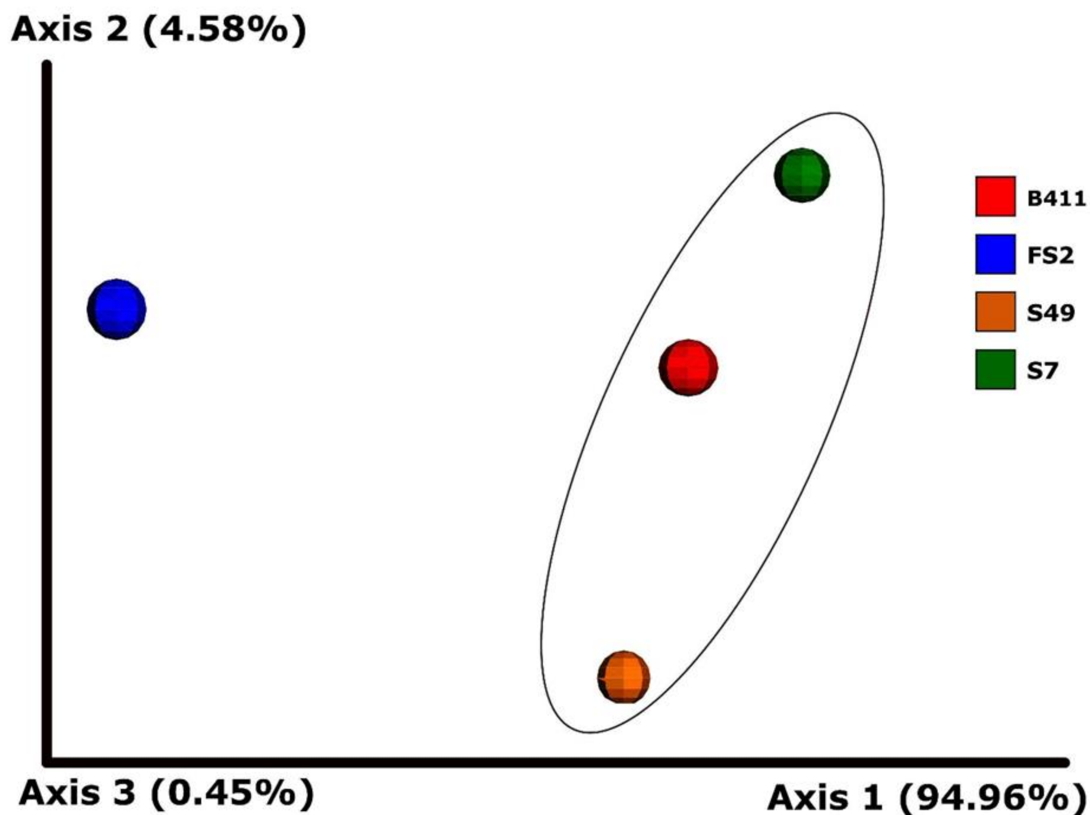

Figure 15 PCoA (Principle Coordinate Analysis) of Bray Curtis dissimilarity of sorghum fermented with *L. plantarum* B411, FS2, S49 and S7.

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In Figure 15 the PCoA of Bray Curtis of sorghum fermented with *L. plantarum* B411, FS2, S49 and S7 can be seen. Bray Curtis dissimilarity is an indication of beta diversity which allows us to see how different the microbial community of one fermentation is to another. It is clear that B411, S49 and S7 are located closer to each other than FS2. This means that the species population of the fermentations made with *L. plantarum* B411, S49 and S7 are similar.

2.9 DISCUSSION

The LAB counts significantly increased in all samples after 24 h of fermentation as expected. Previous studies on various *Lactobacillus* strains showed an increase and survival of strains cultivated for 24 h (Kneifel, Rajal and Kulbe, 2000; Nemska et al., 2019). This is a result of the bacteria's complex requirement for nutrients such as the fermentable carbohydrates present in the sorghum for growth. The starch content of sorghum is reported to be approximately 69.5% (Kulamarva, Sosle and Raghavan, 2009). *Lactobacillus* growth relies on the degradation of glucose through glycolysis and the pentose phosphate pathway (Salvetti et al., 2013; Gänzle, 2015). Even though the fermentation samples were inoculated with *L. plantarum*, except the spontaneous fermentations, LAB present in the samples are not only from the inoculum. The spontaneous fermentations also had LAB growth, meaning LAB were present naturally in all fermentation samples before inoculation.

The yeast and mould counts significantly increased in all fermentation samples after 24 h of fermentation. Previous authors confirmed the presence of yeasts and moulds, such as *S. cerevisiae* and *Candida* spp, in spontaneous cereal fermentations (Omemu et al., 2007; Ekwem and Okolo, 2017). Yeasts and moulds are able to utilize fermentable sugars present in the sorghum fermentation as nutritional substrates (Holzapfel, 1997).

The pH of all fermented samples decreased significantly after 24 h while the TA significantly increased. Charalampopoulos, Pandiella and Webb (2002) showed that *L. plantarum* produced both lactic acid and acetic acid, 1.99 and 0.29 g/l respectively, after 12 h fermentation in sorghum malt. Organic acids produced during fermentation caused a reduction in pH and an increase in the TA of the fermentation samples

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(Charalampopoulos, Pandiella and Webb, 2002). Ratnavathi and Ravi (1991) reported that, depending on the sorghum variety, the pH of sorghum gruel before and after spontaneous fermentation was approximately 6.3 and 4.3 respectively. In this study it was found that the spontaneous sorghum fermentation, without malt, had an initial pH of 6.32 while the final pH was 5.43. The higher final pH could possibly be explained by the slow growth rate of the LAB in this fermentation and/or the absence of malt.

The pH of the fermentations made with malt were significantly lower than those without malt after 24 h. Udeh, Duodu and Jideani (2018) reported that the pH of sorghum malt can range from 6.48 to 6.60 during a 96 h malting period, while Coulibaly, et al. (2014) reported a pH range of 5.05 to 6.05. During the malting process enzymes such as α-amylase are produced which are responsible for starch hydrolysis (Ratnavathi and Ravi, 1991). The presence of amylolytic enzymes in the fermentation samples with malt was responsible for the hydrolysis of starch into fermentable sugars, providing LAB and yeasts and moulds with nutrients that facilitated growth and in return produced organic acids that lowered the pH and increased the TA of these fermentations. The significantly lower pH of the fermentations with malt can therefore not be contributed to the pH of the sorghum malt itself, but rather to its amylolytic activity during fermentation.

The folate contents of the sorghum fermentations inoculated with *L. plantarum* were significantly higher than that of the spontaneous fermentations. This was expected as the *L. plantarum* strains used were selected based on their high folate production ability (Fayemi and Buys, 2017). Rossi, Amaretti and Raimondi (2011) reported that *L. plantarum* has a complete shikimate pathway. The shikimate pathway produces p-ABA, which in turn is used for folate production. This unique pathway allowed the *L. plantarum* strains to produce folate during the fermentation of sorghum. While *L. plantarum* could be naturally present in sorghum (Sekwati-Monang and Gänzle, 2011) the inoculation with *L. plantarum* significantly increased the folate content of the sorghum fermentations. Folate production is extremely strain specific and therefore the different levels of folate observed in the fermentations were expected (Rossi, Amaretti and Raimondi, 2011).

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Folate is formed by the condensation reaction between DHPPP and p-ABA. *L. plantarum* can produce DHPPP and p-ABA from GTP, and erythrose 4-phosphate and phosphoenolpyruvate respectively (Rossi, Amaretti and Raimondi, 2011). It has been reported that GTP is present in sorghum and concentrations vary between cultivars (Goodwin, Oliver and Hsiang, 2004). The specific cultivar of the sorghum malt used in this study is unknown and therefore the increased folate content of the fermentations with malt compared with those without malt could potentially be explained by higher GTP content in the sorghum malt used. The presence of p-ABA in sorghum could increase folate production by *L. plantarum* since the bacteria can immediately source it from the sorghum. It is unclear whether sorghum malt has higher levels of GTP and p-ABA than the flour from unmalted sorghum.

The folate content of sorghum flour has been reported as 20 µg/100 ml (Onyango et al., 2013; U.S. Department of Agriculture, 2018). This is similar to what was found in the spontaneous fermentations with and without malt. The addition of malt significantly increased the folate content of the fermentations. Although Onyango et al. (2013) found that the process of malting did not have any effect on the folate content of sorghum itself, it is possible that other metabolites/precursors produced during malting affected folate production by *L. plantarum* during fermentation.

The increased folate content in the fermentations with malt could also be explained by the microorganisms present in the malt. Sawadogo-Lingani et al. (2010) reported that the dominant microorganisms in sorghum malt include *L. fermentum*, *P. acidilactici*, *Weissella confusa* and *Lactococcus lactis* amongst a few. *Lactococcus* and *Streptococcus* species, with a few exceptions, are similar to *L. plantarum* as it possesses all the genes for both shikimate pathway and chorismate conversion into p-ABA (Rossi, Amaretti and Raimondi, 2011). It is therefore possible that other microorganisms capable of folate production were present in the sorghum malt and directly contributed to the folate content of the fermentations.

The microbiomes of sorghum fermentations inoculated with *L. plantarum* were successfully analysed. Differences were seen between the types and abundance of microorganisms present in the fermentations. All the fermentations showed abundance of *Lactobacillus*, which was expected as the fermentations were inoculated

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with *L. plantarum* isolated from cereal fermentations. Even though the fermentations were inoculated, indigenous microorganisms in the fermentations also contributed to the microbiomes. Previous studies that describe the microbial communities of traditional fermented cereals identified multiple strains dominating the fermentations. *L. reuteri*, *L. fermentum*, *L. harbinensis*, *L. plantarum*, *L. parabuchneri*, *L. casei* and *L. coryniformis* were found in ting, a fermented sorghum product from Botswana (Sekwati-Monang and Gänzle, 2011). *Enterococcus* spp., *L. plantarum*, *L. fermentum*, *L. delbrueckii*, *Lc. lactis*, *Leuconostoc lactis*, *St. infantarius*, *Pediococcus pentosaceus* and *Weisella confuse* were found in obushera, a fermented sorghum product from Uganda (Mukisa *et al.*, 2012). *L. delbrueckii* ssp. *bulgaricus, L. helveticus*, *L. fermentum*, *L. plantarum*, *L. pantheris*, *L. vaccinostercus*, *L. bifermentans*, *L. nantensis and C. perfringens* were found in ogi, a Nigerian fermented product made from maize or sorghum (Oguntoyinbo et al., 2011). These fermentations had different processing techniques and were made in different regions, therefore there were variations in the types of microorganisms responsible for the fermentations.

Variations in the folate contents of the sorghum fermentations were observed, which is primarily contributed by the different folate producing capabilities of the *L. plantarum* strains used for inoculation and the use of sorghum malt. However, the presence of indigenous microorganisms and their ability to produce or affect folate production cannot be ignored. Studies have found that microorganisms such as *Lc. lactis*, *L. delbrueckii* ssp. *bulgaricus*, *St. thermophilus*, *S. cerevisiae* and many others are capable of producing folate (LeBlanc et al., 2011; Park et al., 2014; Laiño et al., 2015; Shibata et al., 2020), while LeBlanc et al. (2011) has shown that microorganisms such as *L. delbrueckii* ssp. *bulgaricus*, may be folate consumers. The presence of these microorganisms may have a positive or negative effect on the folate content of the sorghum fermentations. While these microorganisms are not directly identified by the microbiomes in this study, it is clearly indicated that unidentified microorganisms, labelled in the results as ‘others’, contributed to the microbiomes. In conjunction with the different folate producing capabilities of *L. plantarum* strains used, the presence of other potential folate producers or consumers in the sorghum fermentations could also have contributed to variations in folate contents.

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It is important to note that the study of the microbiomes of fermented food products is still emerging and therefore limited literature were available to draw conclusions from. Further studies are still needed in order to expand knowledge in this field.

2.10 CONCLUSIONS

The addition of malt had no effect on the growth of LAB or yeasts and moulds, but it significantly affected the folate content of the fermentations. The use of folate producing *L. plantarum* strains as starter cultures alone or in combination with malt significantly increased the folate content of fermented sorghum. The highest increase was seen when *L. plantarum* B411 was used in combination with 10% malt and this combination would therefore be best when preparing starter cultures for the purpose of commercialising fermented products with high folate contents. The analysis of the microbiome of the fermentations gave tremendous insight into the fermentations on a microbial level as well as possible explanations for variations in the folate content of the sorghum fermentations. Further research is needed in order to determine the effect of sorghum malt on the microbiomes of the fermentation

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CHAPTER 3: GENERAL DISCUSSION

CRITICAL REVIEW OF METHODOLOGY

This study was aimed at determining the effect of *L. plantarum* on the folate content of fermented sorghum made with and without sorghum malt, as well as the microbiome(s) of these fermentations.

The use of 16S rRNA for the analysis of the microbiomes of sorghum fermentations allowed for accurate and fast results. Conventional methods are more common to use in research labs, but these methods are labour intensive, expensive and time consuming.

FAST DNA SPIN Kit for soil was used to extract DNA from the spontaneous fermented sorghum. This method was adapted to include a pre-processing step in order to separate microbial cells from larger solid particles. The pre-processing step included a sample size of 20 g being washed multiple times with cold ultrapure H_2O followed by centrifugation. The centrifugation step ensured that larger particles were removed from the supernatant. The pellet was then washed with PBS buffer and suspended in sodium phosphate and MT buffer whereafter DNA extraction as per the kit manual was followed. The pre-processing adaption was done with the analysis of a variation of African fermentations, including cereal fermentations, in mind. This method allowed for high DNA yield, which was ideal for the accurate meta-analysis of the microbiomes of the fermentations in this study.

While there is research available on sorghum and other cereal fermentations where 16S rRNA have been used, it is not as extensive as in the medical field. As far as we know, this study is the first in South Africa that uses 16S rRNA to determine the microbiome of spontaneous fermented sorghum and therefore it is considered to be novel in this matter.

The microbiome analysis of the sorghum fermentation made without *L. plantarum* inoculation served as a baseline for comparison with the other microbiomes in this study. Basic comparisons were made between the microbiomes of sorghum

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fermentations made with *L. plantarum*. However, statistical analysis of the microbiomes were difficult to achieve, but basic statistics could be done for the purpose of this study. Elaborate statistical analysis could have been done if more replicates of each sample were analysed. These analyses could include PCoA plots and Jaccard index which will allow for a better understanding of the similarities and differences between these sorghum fermentations.

The analysis of the microbiomes of the sorghum fermentations inoculated with *L. plantarum* allowed us to determine the effect of *L. plantarum* on the microbiome itself as well as identify other potential microorganisms that could have contributed to the elevated folate content of these fermentations. The microbiomes of the sorghum fermentations made with malt were not analysed. This would have been advantageous as it would have allowed us to determine the effect of not only *L. plantarum*, but also sorghum malt on the microbiome and folate levels of the sorghum fermentations.

In this study, a microbiological assay was used to quantify the total folate content extracted from the sorghum fermentations fermented with *L. plantarum* (FS2, B411, S7 and S49). This method was time consuming and tedious to use as folate is extremely light and heat sensitive. While this method was successful in the quantification of the total folate, we could not differentiate between different folate derivatives. In order to differentiate between the derivatives, HPLC could have been used. This method is fast, accurate and gives detailed results. However, a relevant folate standard was not available for this study, and in order to obtain one would have been expensive and time consuming. Determining the folate derivatives was not in the scope of this study.

RESEARCH FINDINGS

The microbiomes of fermented sorghum was successfully determined through 16S rRNA gene sequencing and showed that *Lactobacillus* dominated all fermentations inoculated with *L. plantarum*. The relative abundance of *Lactobacillus* in four sorghum fermentations fermented wit *L. plantarum* (FS2, B411, S7 and S49) were all more than 60%, while unidentified genera were present at about 20% or more. *Paenibacillus*

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initially dominated the spontaneous sorghum fermentation (> 50% at 24 h fermentation) but as time passed (72 h) the relative abundance of *Paenibacillus* significantly decreased and genera such as *Lactobacillus* and other unidentified genera started to dominate (> 50% together).

The objective 'to determine the effect of pre-selected folate producing *L. plantarum* strains on the folate levels of sorghum fermented, with and without sorghum malt, with the aim of increasing the folate levels of these fermentations' was achieved. This study showed that the use of four *L. plantarum* strains (FS2, B411, S7 and S49) as a starter culture, alone, and in combination with 10% malt, for the fermentation of sorghum significantly increased the folate content of the final fermentation. A range of 11 – 118% increase in folate from the spontaneous sorghum fermentation was seen. Sorghum fermented with *L. plantarum* B411 and malt increased the folate content with 118%, and sorghum fermented with *L. plantarum* S7 and malt increased the folate content with 109%. It was hypothesised that the use of folate-producing *L. plantarum* strains as a starter culture for fermentation of sorghum will increase the folate levels of the fermented product after 24h of fermentation. This was indeed the case and therefore this hypothesis can be accepted.

Many rural communities in South Africa do not have access or the financial means to support a varied diet and instead rely on fermented cereal foods for nutrients. Unfortunately, they are at high risk of many deficiencies, including folate which is important for normal foetus development during pregnancy. The *in situ* biofortification of sorghum fermentations with folate producing *L. plantarum* as starter cultures, with or without malt, is a potential solution or even prevention measure for folate deficiencies in these populations.

RECOMMENDATIONS

The bioavailability of the individual folate derivatives produced during fermentation should be investigated. Some folate derivatives are more bioavailable than others, which would greatly affect the nutritional quality of the sorghum fermentations made with *L. plantarum* (FS2, B411, S7 and S49).

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Spontaneous sorghum fermentations are consumed in many South African populations, and sensory studies are needed to determine whether the characteristic properties of fermented sorghum are still present if *L. plantarum* is used as a starter culture.

While the use of *L. plantarum*, with and without malt, as a starter culture resulted in a significant increase in the folate content of a 100ml sorghum fermentation, it does not reach the daily folate intake recommended as by South African guidelines. This calls for further studies on other folate-producing *L. plantarum* strains and their capabilities to increase the folate content of fermented sorghum. The isolation and application of the natural folates produced during fermentation as folate supplements also needs to be investigated.

Ultimately this study can result in the production of a patent or commercialised product. The best fermentation combinations to consider for patents would be the *L. plantarum* B411 with malt, and *L. plantarum* S7 with malt, as these combinations resulted in the highest folate levels. Patents can include products such as commercial *L. plantarum* starter cultures (B411 and S7) for the production of fermented cereal products, a high folate fermented product or even the application of these strains as starter cultures in the production of a high folate fermented cereal product.

This study was carried out on a small scale and unforeseen changes may affect fermentation at a larger scale. It is therefore recommended that this study is replicated at industrial scale to confirm results if the product is to be commercialised.

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