University of Chester

‘The Functionality of “Ogi” – a fermented cereal gruel made in Nigeria, in the Management of Gastrointestinal Disease’

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy

By

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Acknowledgements

To the Immortal, Invincible, Only Wise, True and Good God, who cannot be seen but whose works are evident in the whole universe and more specifically, in my life, I would like to express my gratitude. I could never thank you enough but Lord, please take all the praise.

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Abstract

Fermentation is an ancient food processing technique which has over the years had significant impact on food availability, production, economy and health. Fermented corn, 'ogi', is a popular meal in South and West African countries and is also traditionally administered to people, especially children, during the incidence of diarrhoea as it is believed to enhance gut health. Various authors have supported the advantageous health functions of fermented corn but have focussed on the possible health benefits that may be derived from the action of the fermentation organisms by investigating their probiotic potentials. Furthermore, in vivo studies have investigated effective functionality of fermented foods and where studies have suggested positive effect of the fermented food intervention, the component of the complex food matrix that may be responsible is often unknown. Though hypothesised, there has been little information on the possible effects of the end products of the fermentation on gut health. Therefore, this thesis aimed to explore the functionality potentials for 'ogi', to enhance gut health by investigating the mechanisms through which it may be able to confer the health benefits when consumed.

An independent meta-analysis was undertaken to explore and review the analysis of the effectiveness of fermented foods to significantly reduce the duration of diarrhoea. The first experimental study was a comparison between spontaneously fermented vs controlled method of 'ogi' production with the aim to explore the possibility of achieving similar end products. The probiotic potential of fermentation organisms and antimicrobial effect of fermentation organisms against *E. coli* were also investigated. Colonic short chain fatty acids (SCFAs) are known to enhance immune function in the gut. The second experimental study explored the production of SCFAs in fermented corn with the theory that fermented corn may be a dietary source of SCFA and may produce a comparable effect in the gut as the colonic SCFA. It is established that one of the functions of endogenously synthesised nitric oxide (NO) in the body is to enhance immune functions. Therefore, the potential for 'ogi' to enhance immune response in the gut by being a dietary source of Nitric Oxide (NO) was explored in the third experimental study.

The outcomes of this thesis suggest that it may not be possible to mimic the exact spontaneous fermentation process in a controlled setting. *Weissella confuse*, *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* were the lactic acid bacteria (LAB) and yeast isolated from spontaneously fermented corn using 16S rRNA sequencing. The isolated organisms along with *Lactobacillus plantarum* were used as starter culture for controlled fermentation. There was a significant difference between the end products of both fermentations. All organisms except *W. confuse* showed probiotic potential. Acetate was produced during both fermentations but butyrate was produced during spontaneous fermentation only. There was however higher concentration of NO in controlled fermented corn than spontaneous fermentation with nitrate values of 30.54 µM and 23.21 µM and nitrite values of 17.50 µM and 10.11 µM respectively. These suggest that fermented corn may have probiotic potential and may be able to further enhance immune health in the gut by being a dietary source of SCFAs and NO.

Declaration

“The material being presented for examination is my own work and has not been submitted for an award of this or another HEI except in minor particulars which are explicitly noted in the body of the thesis. Where research pertaining to the thesis was undertaken collaboratively, the nature and extent of my individual contribution has been made explicit.”

Signed:…….

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Chapter 1

Introduction

A review of cereal fermentation – processes and benefits

1.1 The history of food fermentation

Fermentation is the oldest method of food preservation after drying (Prajapati & Nair, 2008; Sekar & Mariappan, 2007). There are no records stating precisely when the process of food fermentation first began but, there are speculations that the process has been since pre-biblical times (Anukam & Reid, 2009) **(Table 1.1)**. Fermentation is postulated to have originated from the Indian subcontinent with indications that the process was also utilised during ancient times in Egypt and the Middle East from where it spread to Africa, America and Europe (Prajapati & Nair, 2008).

Antony Van Leeuwenhoek was the first person to use a microscope to confirm the existence of microorganisms (Bamforth, 2009) however Louis Pasteur between 1850 and 1860, showed the involvement of microorganisms in the process of food fermentation (Prajapati & Nair, 2008; Shurtleff & Aoyagi, 2008). Understanding the processes of fermentation since Pasteur's discovery has resulted in the technological evolution of food, leading to a significant increase in the number and types of available fermented food products. It has been postulated that fermentation started accidentally when leftover food and drink were contaminated by microorganisms in ancient times but the process later developed into a controlled, cheap and effective method of food preservation and processing (Sekar & Mariappan, 2007).

1.2 Biochemistry of fermentation

Fermentation is the biochemical conversion of organic substances into smaller or simpler compounds for energy by complex nitrogenous substances such as enzymes produced by living cells (Marshall & Mejia, 2011; William & Akiko, 2007). During aerobic respiration, organic compounds are converted to pyruvate which is further converted to Acetyl CoA and then fed into the Citric or Krebs cycle. During this process, oxygen serves as the electron acceptor, travelling through the electron transport chain with water as a by-product. Anaerobic respiration utilises weaker inorganic electron acceptors such as sulphate and nitrate compounds. The process uses a non-oxygen electron transport chain and the by-products from the electron acceptors are gases such as nitrogen oxide, in place of water.

Table 1.1 Milestones in the History of Fermented Foods

Milestone	Development/Location
ca.10,000 B.C. to Middle Ages	Evolution of fermentation from salvaging leftover food and drinks, probably by pre-Aryans.
ca. 7000 B.C.	Cheese in Iraq and bread making practiced
ca. 6000 B.C.	Wine and beer making in the Near East
ca. 5000 B.C.	Fermentation of milk and beverages in Babylon
ca. 3500 B.C.	Bread making in Egypt using yeast
2000 B.C.–1200 A.D.	Preparation of various fermented milk products such as yoghurt, sour cream in Europe, Asia and Arab world
ca. 1500 B.C.	Preparation of meat sausages by ancient Babylonians
500 B.C	Mouldy soybean curd used as antibiotic in China
ca. 300 B.C.	Preservation of vegetables by fermentation by the Chinese
500–1000 A.D.	Development of cereal-legume based fermented foods
1276 A.D.	First whisky distillery established in Ireland
1881	Published literature on koji and sake brewing
1907	Publication of book Prolongation of Life by Eli Metchnikoff describing therapeutic benefits of fermented milks
1900–1930	Application of microbiology to fermentation, use of defined cultures
1953	Nisin marketed in UK and since approved for use in over 50 countries
1970–present	Development of products containing probiotic cultures or friendly intestinal bacteria

Adapted from: Prajapati, J., & Nair, B. (2008). History of Fermentation. In E. R. T. Farnworth (Ed.), Handbook of fermented functional foods (pp. 2-22). Boca Raton: CRC press.

On the other hand, fermentation processes do not involve the use of the electron transport chain but utilises organic compounds as electron acceptors. (Jay, 1997). Both prokaryotic cells e.g. bacteria and eukaryotic cells e.g. yeast undergo fermentation process as an alternative energy source when oxygen is not available (Harden & Macfarlane, 1930; White, 2007). The fermentation process is the incomplete oxidation of the parent organic compound, with the release of a smaller amount of energy when compared to energy derived from aerobic respiration process (Michal & Schomburg, 2013) (**Figure 1.1**). Alcohol, organic acids and gases are end products of fermentation (White, 2007). Unlike aerobic respiration, oxygen is not a requirement for the process of fermentation, hence the process can occur with or without oxygen (Amore & Faraco, 2013) and is most often undertaken by anaerobic and facultative anaerobic microorganisms (White, 2007).

The response to the presence of oxygen during fermentation process varies amongst microorganisms. When oxygen is present, some organisms utilize it for respiratory activities, producing carbon dioxide, water and energy which are channelled towards cell multiplication (van Dijken, Weusthuis, & Pronk, 1993; Visser, Scheffers, Batenburg-van der Vegte, & van Dijken, 1990). Facultative anaerobic organisms subsequently shift to fermentation when there is a depletion in the amount of oxygen present. Although, yeast cells are able to undergo aerobic and anaerobic fermentation. Aerobic fermentation occurs through a process called 'The Crabtree Effect' (De Deken, 1966). During anaerobic fermentation, the fermentative capacity depends not only on quantity of substrate but also on the availability of a limited amount of oxygen for multiplication (Visser et al., 1990). Depending on the type of organism, oxygen may be a requirement to synthesize sterols and unsaturated fatty acids which in turn are used for growth and maintenance of the cell wall, during anaerobic fermentation, particularly when there is limited accessible substrate (De Deken, 1966; Jakobsen & Thorne, 1980; Snoek & Yde, 2007; Visser et al., 1990). Nevertheless, substantial production of fermentation output only occurs after available oxygen has depleted (Amaral-Phillips, 2013).

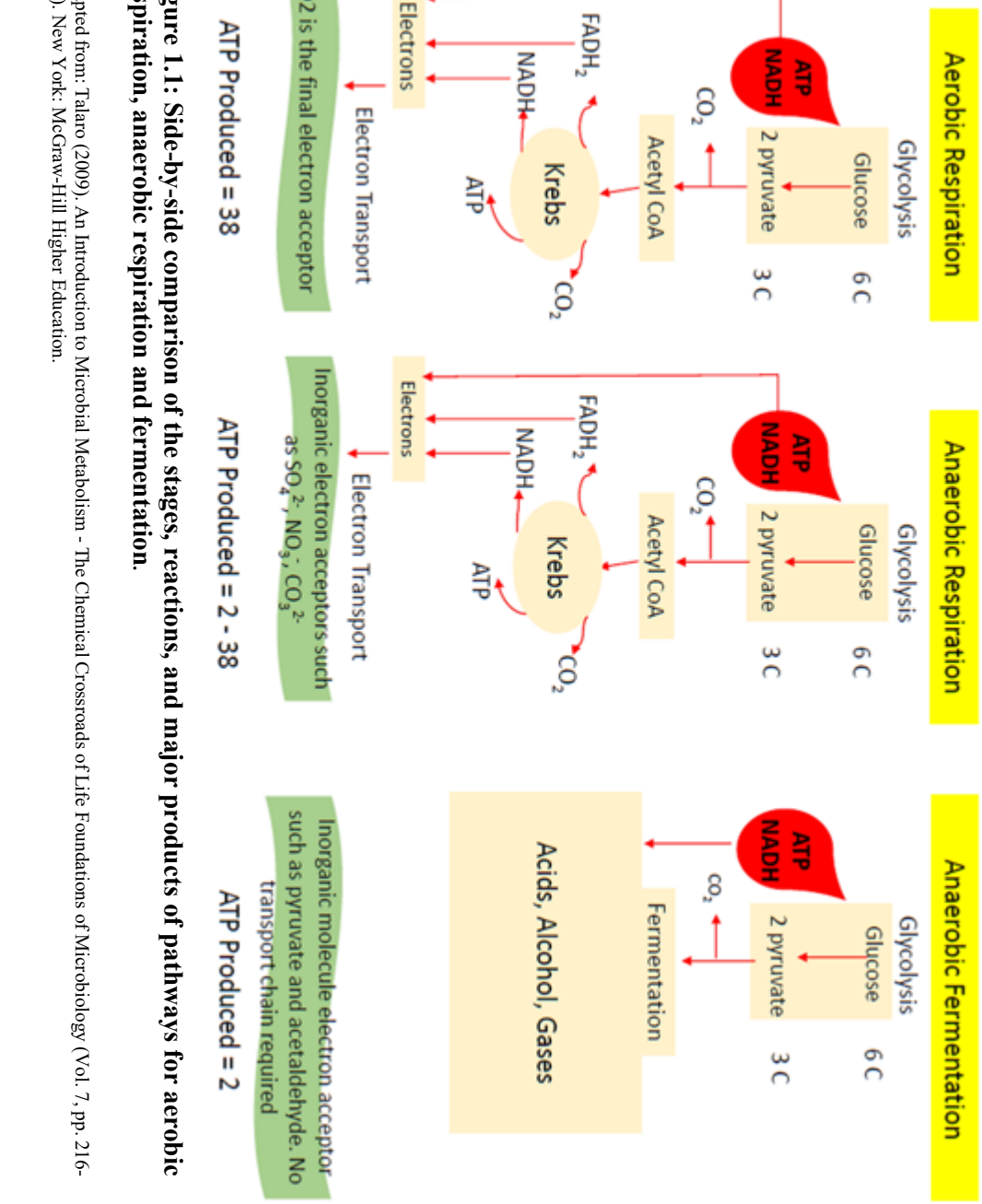


Figure 1.1: Side-by-side comparison of the stages, reactions, and major products of pathways for aerobic respiration, anaerobic respiration and fermentation.

Adapted from: Talaro (2009). An Introduction to Microbial Metabolism - The Chemical Crossroads of Life Foundations of Microbiology (Vol. 7, pp. 216-251). New York: McGraw-Hill Higher Education.

The presence of oxygen is toxic to some fermentation organisms and they are described as obligate anaerobes (Brooks, Carroll, Butel, Morse, & Mietzner, 2007). These organisms use fermentation as their energy source throughout the process (White, 2007). Facultative anaerobic organisms that undergo fermentation use enzymes, namely superoxide dismutase, catalase and peroxidase, which either assist the depletion of oxygen available or support the cells to continue with fermentation processes negating the effect of oxygen toxicity (Baron, 1996; Pedersen, Gaudu, Lechardeur, Petit,

& Gruss, 2012). Apart from organism species (**Figure 1.2**) and availability of oxygen (O_2), the metabolic pathway and final products of a fermentation process are also dependent on nutritional and environmental factors including temperature, pH, nature and composition of substrate, dissolved carbon dioxide (CO_2), water and time (Amore & Faraco, 2013; Bouthyette, 2008; Chisti, 1999; Epp, 2008).

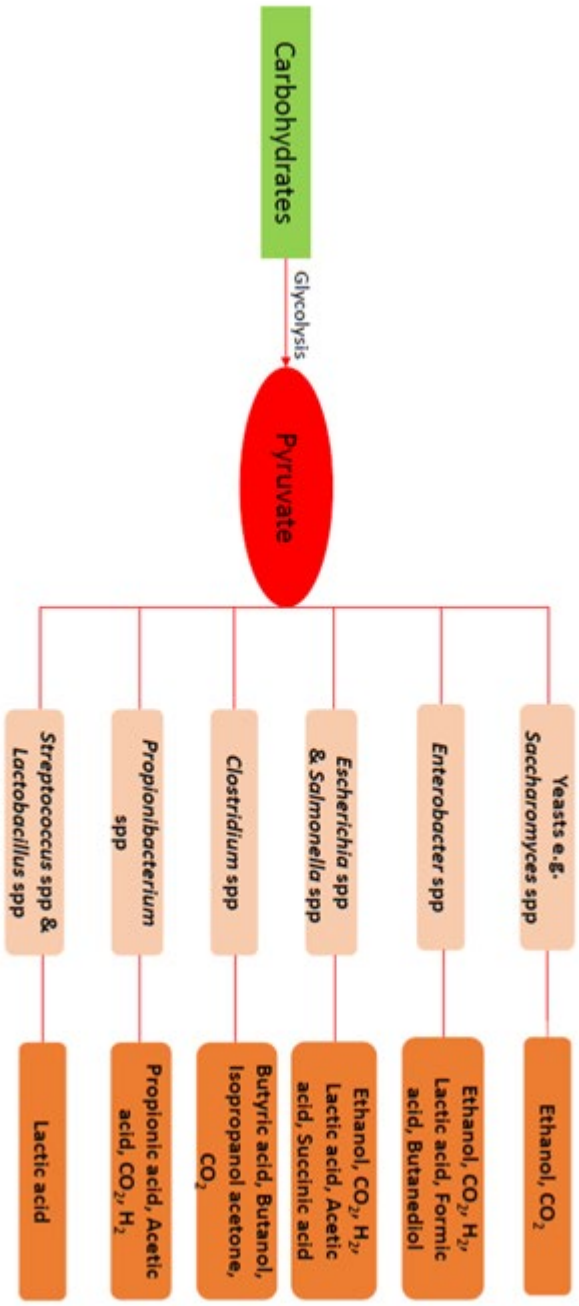


Figure 1.2: Products of fermentation by various organisms with carbohydrates as the organic substrate.

Bouthyette, P.-Y. (2008). Fermentation - Fermentation throughout the ages. Retrieved from http://www.chemistrywithdrb.com/files/Fermentation.pdf

1.3 Process of Food fermentation

Food fermentation processes begin with the availability of a substrate (**Figure 1.3**). Fermentation can either be spontaneous also described as natural fermentation or controlled. Controlled process is the deliberate inoculation of a substrate with live starter organisms.

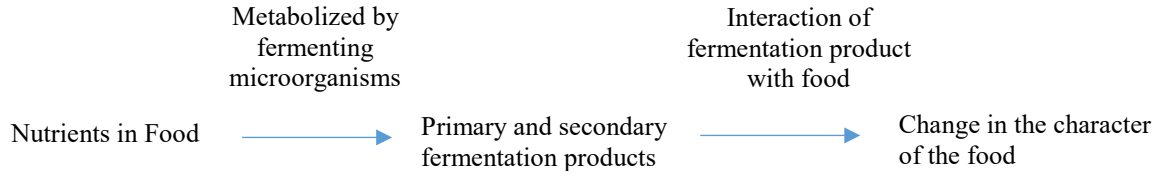


Figure 1.3: Summary of the metabolic process of fermentation.

Adapted: Murano (2003). *Understanding Food Science and Technology*. Wadsworth, Cengage Learning. USA. Pg 202

Traditional and low scale food fermentation as well as some industrial processes such as sauerkraut fermentation are often started spontaneously (Giraffa, 2004). Spontaneous fermentation processes rely on the indigenous or natural microbiota present in and on the food and the physical environment (Charalampopoulos, Pandiella, & Webb, 2002; Katongole, 2008). End products of spontaneously fermented foods could be unpredictable (Giraffa, 2004) but it is possible to manipulate the process to encourage the growth of desired or preferred organisms and/or inhibit the growth of undesired ones. This could range from adding salt or sugar for control of water concentration by creating a hypertonic solution, to addition of fruit juice to lower pH (Chisti, 1999; Giraffa, 2004). Spontaneous fermentation often involves submerging the substrate in water at ambient temperature. The addition of water activates dormant indigenous organisms to produce enzymes which then result in various stages of metabolic changes of the substrate (Katongole, 2008). Microorganisms generated for the fermentation process may be influenced by temperature, pH, geographical location and composition of substrate, dissolved carbon dioxide (CO_2), dissolved oxygen, water and time (Bessmeltseva, Viiard, Simm, Paalme, & Sarand, 2014; Katongole, 2008). In a controlled fermentation process, the sterile substrate is inoculated with a known starter culture in large and sometimes known inoculum size. Starter cultures may be described as an inoculant containing large quantities of viable microorganisms which may be of the same or varied species (Holzapfel, 2002). Controlled fermentation processes are often undertaken during the industrial production of fermented foods especially when specific properties such as appearance, aroma, texture and taste are desired (Chisti, 1999; Markov, 2012). Starter culture could be single strain culture, containing 1 strain of a species; multi-strain, containing multiple strains of the same species; multi-strain mixed cultures containing different strains of various species (Vogel et al., 2011) depending on the desired outcome. For example, *Lactobacillus lactis* ssp. *cremoris* and

Lactococcus lactis subsp. *lactis* are able to rapidly convert lactose to lactic acid and are therefore called acid producers while *Lc. lactis* ssp. *lactis biovar diacetylactis* and *Lb mesenteroides* subsp. *cremoris* are known for their efficient and sufficient production of diacetyl, an aromatic flavour (Niamsiri & Batt, 2009). These organisms are therefore used as starter culture for the production of cultured buttermilk (Niamsiri & Batt, 2009).

During fermentation, the dynamics of the fermenting microbiota evolve according to the changes in the environmental conditions which occur as the process progresses (Bautista-Gallego et al., 2014). Characteristically, fermentation involves the activities of more than one microbial species acting concurrently and sequentially. In controlled fermentation however, it is possible to have a single species fermentation after the substrate has been sterilized to kill unwanted microorganisms. Typically, bacteria dominate the early stages of fermentation processes, owing to their relatively high growth rate, followed by yeasts in substrates that are rich in fermentable sugars (Holzapfel, 2002).

1.4 Submerged Fermentation

Submerged Fermentation (SmF), involves the use of a free-flowing liquid where substrates can either be liquid in nature e.g. milk, molasses or broth or solid but suspended in a large mass of liquid to form a slurry, suspension or solution (Chisti, 1999). SmF is the technique used for the production of yoghurt, alcohol and some cereal based fermented beverages such as 'ogi' and 'akamu' which are popular in West African countries. This fermentation technique is best suited for microorganisms, usually bacteria which require high moisture content. SmF is predominantly used in the extraction of secondary metabolites that a soluble in the chosen liquid (Subramaniyam & Vimala, 2012). There are 3 types of submerged fermentation which include:

A) **Batch Fermentation**

A closed culture system process, with an initial limited amount of nutrients (Stanbury, Whitaker, & Hall, 1999). Fermentation proceeds for a specified period of time, after which end products are harvested. Fermentation time depends on substrate and the desired end product (Chisti, 1999). Batch fermentation could either be spontaneous or controlled. Microbial growth during this close system is in different phases which are lag, log or exponential, deceleration, stationary and death phases (Brennan, 2006; El-Mansi, Bryce, Demain, & Allman, 2006).

- **Lag phase:** Inoculated (in controlled fermentation) and activated (in spontaneous fermentation) microorganisms adapt to the new environment, therefore there is limited

microbial growth. There may be an increase in biomass but without a change in the microbial population.

- **Log/exponential phase:** Microorganisms begin to grow utilizing nutrients available in the substrate to produce energy. There is a growth balance as all cells grow at the same rate. Nutrients in the substrate are converted to acids, alcohol and gases which are also called growth inhibitors.
- **Deceleration phase**: There is a depletion of one or more essential nutrients, accumulation of by-products of fermentation (or primary metabolites) toxic to the microorganisms and 'crowding' i.e. increased population of microbial cells resulting in decreased growth rate of the microorganisms.
- **Stationary phase**: Total cell mass may remain constant, but number of viable cells decrease. Net growth rate is zero as growth rate is equivalent to death rate. Death is as result of factors previously mentioned in deceleration phase. Secondary metabolites such as gibberellic acid are formed during this phase.
- **Death phase**: there is an increased death rate of the microbial cells. There is more production of secondary metabolites.

B) **Fed Batch Fermentation**

Involves the addition of a substrate to the fermentation batch thereby increasing the volume of the fermenting broth. This system if carefully controlled, extends the exponential phase of batch fermentation (Stanbury et al., 1999). Addition of a sterile substrate not only encourages the continuous growth of pre-existing microbiota and the production of primary metabolites but it may also solve the problem of by-product toxicity (Li, Srivastava, Suib, Li, & Parnas, 2011) when secreted metabolites begin to inhibit growth or cause the death of microorganisms

C) **Continuous Fermentation**

Repeated fed-batch process, during which the medium is periodically withdrawn and substituted with fresh medium, such that a constant volume of the fermenting broth is maintained (Chisti, 1999). This process is known to enhance the productivity of microbial fermentation as it saves time for cleaning, sterilization, seed culture and inoculation processes between batches (Qu et al., 2013).

1.5 Solid-State Fermentation

Solid-state or solid-substrate fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials without the presence of free liquid (Cannel & Moo-Young,

1980; Pandey, Soccol, Rodriguez-Leon, & and Nigam, 2001). The substrate usually possesses enough moisture to support growth and metabolism of the microorganism (Chisti, 1999; Singhania, Patel, Soccol, & Pandey, 2009) but in an absorbed state or in a complex with solid matrix (Bhargav, Panda, Ali, & Javed, 2008). Fungal and yeast cultures have been described as the most suitable microorganisms for SSF processes, due to their lower water activity requirement (Thomas, Larroche, & Pandey, 2013). Mienda, Idi & Umar (2011) stated that filamentous fungi are the most significant group of microorganisms used in SSF process due to their physiological, enzymological and biochemical characteristics. Apart from fungal tolerance for low water activity, hyphal mode during growth and tolerance for high osmotic pressure conditions make them most efficient for bioconversion of solid substrates. Bacterial cultures require significant availability free water which makes them less suited for the process. Nevertheless, bacterial cultures with some tolerance of low water activity such as *Bacillus, Staphylococcus, Clostridium* and some *Lactobacillus* spp could be manipulated for SSF processes (Adelekan & Nwadiuto, 2012; Mienda, Idi, & Umar, 2011; Pandey, 2003; Subramaniyam & Vimala, 2012). For example, according to Nampoothiri and Pandey (1996) maintaining moisture content at maximum of 90% and fortifying the medium with 10% of glucose was effective in using *Brevibacterium* spp for solid state fermentation in the production of L-glutamic acid. However Mienda et al. (2011) explained that metabolic processes and output quantities during bacterial SSF would differ and may not be comparable to fungal cultures.

SSF is a common fermentation practice in Africa, Asian and Europe. Examples of food produced from this process include ‘iru’ or ‘dadawa’ made from locust beans using *Bacillus*, *Leuconostoc* and *Staphylococcus* spp (Amao, Abel, & Agboola, 2013; Antai & Ibrahim, 1986), ‘koji’ made from soybean along with rice and wheat bran using *Aspergillus oryzae*, ‘tempeh’ made from soybean using *Rhizopus oligosporus*, veining of blue cheese using *Penicillium roqueforti* and Chinese wine, soya sauce and vinegar (Couto & Sanromán, 2006). SSF processes are also used in biomass energy conservation and solid waste treatment (Pandey, 1992).

Table 1.2: Examples of fermented foods & beverages

Food Group	Example of Food/Beverages	Regions of consumption
Cereal Maize/Corn, Rice, Wheat, Millet, Sorghum	Ogi, Togwa, Anarshe, Busa, Chongju Mantou, Sour Bread Busa, Burukutu, Bushera	East, West and Southern African countries, India, Indonesia, China, Korea, Philippines, Arabian countries, European countries
Roots Cassava	Lafun, Fufu, Gari, Cossettes, Kanyanga, Mapanga, Makopa, Chickwangue, Kocho	African countries
Dairy Milk	Sour cream; Cheese, Yoghurt, Kefir	Asian and European countries
Vegetables Cabbage Cucumber/Olives	Kimchi, Sauerkraut, Pickles	European countries and USA Asian countries, USA and Canada
Fruit Grapes	Alcoholic fruit wines	International
Legumes and Pulses Soyabean Locust beans	Tempeh Ugba, Dawadawa/ Soumbala/Netetu Ogiri	China, Japan Nigeria
Meat	Chorizo, Salami	Southern and Central Europe, USA
Fish	Fish sauce,	Asian countries

Adapted from: Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Review: diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology*, 7.

1.6 Types of fermented food products

There has been a rapid increase in consumption of fermented foods over the years (Tamang et al., 2020; Kok & Hutkins, 2018). Over the years, the range of fermentable substrates has widened, from being just cereal and milk products, to fermented seeds, meat and fish (**Table 1.2**). Heterogeneity of tradition and culture, different geographical locations, staple foods and available substrates have also contributed to the significant variation of fermented food and drinks across the globe (Marshall & Mejia, 2011). The type of substrate, method of fermentation and microbial species involved determine the end product of the metabolic process (Divya, Varsha, Nampoothiri, Ismail, & Pandey, 2012; Marsh, Hill, Ross, & Cotter, 2014). The four major processes of fermentation include i) alcoholic fermentation which is predominantly undertaken by species of yeasts with ethanol or other forms of alcohol as the major end product (Soni & Sandhu, 1990) ii) lactic acid fermentation involving the use of cereals, milks and starches as substrate and lactic acid as the main the end products (Blandino et al, 2003) iii) acetic acid fermentation involving anaerobic *Acetobacterium* and *Clostridium* strains for the production of acetic acid (McKay & Baldwin, 1990) which can also be formed by aerobic *Acetobacter* strains via oxidation of alcohol (ethanol) during alcohol fermentation (Hromatka & Ebner, 1949; Potter & Hotchkiss, 1995) iv) alkaline fermentation involves proteolytic activities of microorganisms on the substrates resulting in the production of compounds with high (up to 9) pH values (Parkouda et al., 2009). This thesis however aims to focus on lactic acid fermentation.

1.7 Significance of Cereal Fermentation

Although there are various fermentable food substrates, this review will focus on cereal crops, particularly on maize (*Zea mays* subsp. *mays*) fermentation. Cereals are important agricultural crop with an estimated global production volume of 2500 million tonnes in 2013 (Global Information and Early Warning System & FAO, 2014). They are cultivated on approximately 73% of the world agricultural soil and their contribution to food crop produce in the world is up to 60% (Charalampopoulos et al., 2002). Maize, wheat and rice account for approximately 80% of total cereal production (Global Information and Early Warning System & FAO, 2014). Major reasons for the successful world cultivation of cereals are adaptability, high yield and ease of harvest and storage (Lafiandra, Riccardi, & Shewry, 2014). Cereal produce are inexpensive and easily affordable sources of carbohydrates and calories (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003).

1.7.1 Food preservation

Preservation was the sole reason for fermentation in the early years (Bouthyette, 2008). During this time, there were no advanced food storage technologies which resulted in spoilage and food waste. Even though some agricultural produce such as cereal grains have lower moisture compared to fruits and vegetables, thereby having extended longer shelf life, availability of these crops is periodic due to seasonal variations (Zilberman & Kim, 2011). Through fermentation, seasonality constraints of agricultural produce was managed (Simango, 1997). The production of organic acids during fermentation reduces the pH to below 4.0 making survival of spoilage microorganisms difficult (Blandino et al, 2003). With a fast growing world population, which has been estimated to increase to over 9.6 billion by 2050 (United Nations, 2013), there will be an increased demand and pressure on global food resources. Indigenous fermentation technology may be a useful alternative for food production, reducing dependence on meat and other unsustainable food resources (van Hijum, Vaughan, & Vogel, 2013).

Bacteriocin and other antibacterial compounds produced during fermentation have not only been reported to improve food safety through inhibition of pathogens or removal of toxic compounds (Epp, 2008; Prajapati & Nair, 2008) but they have also been said to provide health benefits to consumers of the fermented product (Anukam & Reid, 2009; Franz et al., 2014). Bacteriocins are ribosomally synthesized peptides with antimicrobial properties, produced by bacteria and archaea during primary phase of growth (Dobson, Cotter, Ross, & Hill, 2012; Zacharof & Lovitt, 2012). For examples, *Lb plantarum* produce bacteriocins called plantaricins (Zacharof & Lovitt, 2012). Antibiotics such as acidolin produced by *Lb acidophilus* (Hamdan & Mikolajcik, 1974) are secondary metabolite produced during fermentation able to kill or inhibit the growth of other bacteria (Zacharof & Lovitt, 2012).

1.7.2 Enhanced sensory properties

Fermentation is a food processing technique that enhances the sensory properties including taste, appearance, aroma and texture of the food substrate (Sekar & Mariappan, 2007; Simango, 1997; Ng'ong'ola-Manani, Mwangwela, Schüller, Østlie & Wicklund, 2014). These new sensory properties are the result of the combination of aroma and flavour compounds - organic acids, alcohols, ketones, aldehydes, esters, lactones and pyrazines – produced during the process (Deshpande, 2000; Divya et al., 2012).

1.7.3 Enhanced nutrient bioavailability

Cereals have low protein content which is deficient in lysine, an essential amino acid and therefore incomplete and of lower quality in comparison to milk and eggs (Webb, 2012). They also contain nutrient inhibitors which compromises the bioavailability of micronutrients they contain (Hotz & Gibson, 2007; Hurrell & Egli, 2010). Nutrient inhibitors bind with micronutrients, making them unavailable for absorption (Blandino et al., 2003). Several processing methods, such as cooking, sprouting, soaking, milling and fermentation, aimed at improving the nutritional properties of cereals have been explored (Gibson, Perlas, & Hotz, 2006; Sokrab, Mohamed Ahmed, & Babiker, 2012), but fermentation has been reported to be the most effective of these methods (Mattila-Sandholm, 1998) which may be associated with the activities of microbial community during the process. It has been reported that fermentation could decrease the effect of nutrient inhibitors by over 90% consequently increasing the bioavailability of micronutrients such as iron, calcium and B vitamins (ELKhier & Abd-ALRaheem, 2011; Gibson et al., 2006; Hotz & Gibson, 2007). There have been inconsistent conclusions on the effect of fermentation of protein quality and quantity in cereals (Blandino et al, 2003) but Gibson et al. (2006) suggested that improvements in protein quality and quantity may be better achieved with blended mixtures of plant-based complementary foods based on cereals and legumes. Fermentation also decreases the quantity of complex carbohydrates in cereals which include non-digestible polysaccharides and oligosaccharides (Blandino et al, 2003). These complex carbohydrates serve as nutrients for microorganisms and are broken down to simple, nontoxic substances such as organic acids that give fermented product desirable sensory properties (Divya et al., 2012).

1.7.4 Social, cultural and economic significance

Apart from food preservation, enhanced flavour, food security and nutrient availability, fermented foods are also of social, cultural and economic significance. Fermented beverages and alcoholic drinks are served across the globe during social functions and celebrations such as weddings (Marshall & Mejia, 2011). In developing countries, indigenous food fermentation is often carried out by women (Battcock, 1998) employing spontaneous fermentation processes using maize, sorghum and millet as the main cereals (Nyanzi & Jooste, 2012). These fermented food products have the potential to contribute to the livelihood of these women not only by promoting food security, but also but as a form of income generation, through small scale business opportunities (FAO, 2011; Nyanzi & Jooste, 2012). Fermented foods are consumed all over the world, including the rural and urban regions of developing countries. Westernisation in the urbanized regions has nevertheless resulted in reduced popularity of some locally fermented food products. Hugenholtz (2013) reported that availability of efficient and rapid storage systems in Europe and North America

has resulted in decreased demand for most fermented foods except fermented meat and dairy products, giving rise to increased preferences for fresh agricultural products. It is also believed that there is often a shift from indigenously fermented foods to readymade and processed foods as soon as the latter become affordable to the household (Holzapfel, 2002; Watson, Ngesa, Onyang'o, Alnwick, & Tomkins, 1996). This however is not the case in regions where fermented foods are of cultural significance (Marshall & Mejia, 2011). Fermented milk products, alcoholic drinks and condiments with traditional importance, have ready markets (Marshall & Mejia, 2011). In the last ten years, there has been considerable increased interest in fermented food, giving rise to various innovative fermented products in the market such as fermented fruit drinks. This increased interest has been associated with the 'naturalness' with minimum level of processing and absence of artificial food additives such as sulphite and nitrite compounds, sustainability (Hugenholtz, 2013) and diversity of fermented products (Clydesdale, 2004). Large scale fermented product companies have as a result emerged in many countries in the world generating employment opportunities in the urban and rural communities (Adesulu & Awojobi, 2014). According to Battock and Azam-Ali (1998) as stated by Quave & Pieroni (2014), food fermentation is important in "providing food security, enhancing livelihoods and improving the nutrition and social wellbeing of millions of people around the world, particularly the marginalized and vulnerable.''

Food fermentation technology generates multiple products. Fermented cereal wastes and by-products from corn ethanol production have the potential to be used for the production of livestock feed (Makkar, 2012). Microorganisms employed for the fermentation process could be used as biomass generating biofuels, giving biofuel refineries the capacity to be a part of agribusiness, linking agriculture to the energy sector (Zilberman & Kim, 2011). Some secondary metabolites derived from food fermentation including antibiotics (Pereira et al., 2013), enzymes, statins and antihypertensive agents (Mienda et al., 2011; Sekar & Mariappan, 2007; Subramaniyam & Vimala, 2012) are used for therapeutic purposes. There is however limited information about the possibilities and effectiveness of using anti-microbial by-products produced during fermentation in lieu of artificial preservatives (Giraffa, 2004). So far, only nisin, a type of bacteriocin produced during fermentation, is being used as a natural preservation in food production, extending shelf life of highly perishable refrigerated foods (Guizani & Mothershaw, 2006).

1.8 Fermentation microorganisms

A wide spectrum of microorganisms is able to breakdown nutrients in food substrates, however, not all microbial activities in food result in flavour or nutrient enhancement. Generally, organisms that dominate food substances, depending on the substrate are able to undergo proteolytic, lipolytic

and fermentation activities (Potter & Hotchkiss, 1995). Whilst all organisms are able to undergo all three activities, they are usually dominant in one action (Potter & Hotchkiss, 1995). Proteolytic activities, which involves catabolism of protein and nitrogenous compounds by the microorganisms, cause the production of substances that give putrid and rotten flavour. Lipolytic activities including breaking down of fat and fat related compounds, give rise to production of a rancid and fishy flavour (Potter & Hotchkiss, 1995). If dominant proteolytic and lipolytic activities, often result in food spoilage (Chelule, Mokoena, & Gqaleni, 2010).

On the other hand, food fermentation is mostly carried out by lactic acid bacteria (LAB) such as, *Leuconostoc, Streptococcus, Lactobacillus, Enterococcus, Aerococcus* and *Pediococcus* species (*spp*), yeasts such as *Saccharomyces, Candida, Kluyeromyces and Debaryomyces spp* and moulds, including *Penicillium, Mucor, Geotrichium,* and *Rhizopus spp* (Battcock, 1998; Holzapfel, 2002; Tripathi & Giri, 2014; Wouters, Ayad, Hugenholtz, & Smit, 2002). Most organisms involved in food fermentation are Generally Recognized As Safe (GRAS) and/or 'Qualified Presumption of Safety' (QPS) by Food Drug Association (FDA) and European Food Safety Association (EFSA) (Holzapfel, 2002; Papadimitriou, Pot, & Tsakalidou, 2015). LAB and yeast strains are significant in cereal fermentation (Nwosu & Oyeka, 1998; Todorov & Holzapfel, 2014) as mould is reported to be lost soon with a decrease in pH (Omemu, 2011). Newer strains of LAB and yeast are constantly being isolated from indigenously fermented foods, but some of them are yet to be identified (Franz *et al*., 2014).

1.9 Lactic Acid Bacteria (LAB)

1.9.1 End product of LAB fermentation

LAB, majorly *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*, have been identified as the main microorganisms associated with cereal fermentation (Nyanzi & Jooste, 2012; Omemu, 2011; Oyedeji, Ogunbanwo, & Onilude, 2013) and they are regarded as the major contributor of desirable characteristics observed in fermented foods, (Divya et al., 2012; Leroy & De Vuyst, 2004). LAB are fast growing gram positive, non-spore forming organisms (Saeed & Salam, 2013). They are either anaerobic or facultative anaerobic and have lactic acid as the main or only end product of sugar fermentation (Saeed & Salam, 2013). LAB are divided into three groups based on their fermentation outputs. Obligate homofermentatives LAB which are unable to ferment pentoses, but undergo homolactic process of fermentation, converting hexoses almost exclusively to lactic acid (about 85% from one molecule of hexose sugar) (Saeed & Salam, 2013). Obligate heterofermentative LAB undergo heterolactic fermentation process converting hexoses and pentoses to lactic acid (about 50% from a molecule of hexose sugar), acetic acid, ethanol and CO_2

in different proportions (Saeed & Salam, 2013). Facultative heterofermentative LAB undergo both homolactic and heterolactic processes (Divya et al., 2012; James, 2000; Mikelsaar et al., 2002).

During spontaneous fermentation of cereals, a population of wild naturally existing microorganisms are activated, the majority of which are LAB. Once activated, LAB are known for their fast growth rate and ability to initiate fermentation process with a characteristic high acidification rate (Leroy & De Vuyst, 2004). Acidification during fermentation process inhibits the growth of spoilage bacteria (Ionescu, Zara, Aprodu, Vasile, & Istrate, 2007) and decreases the chances of food spoilage instead of fermentation. Back-slopping, is traditionally used to accelerate fermentation. It is the inoculation of raw substrates with a portion of the already fermented product (Chelule et al., 2010). Continuous back-slopping results in stability of the microbial ecosystem with best adapted strains dominating the fermentation process (Leroy & De Vuyst, 2004; Papadimitriou et al., 2015; Todorov & Holzapfel, 2014; Weckx et al., 2010). These wild dominant strains are isolated, propagated and stored up for use as industrial pure or mixed starter culture (Todorov & Holzapfel, 2014). It is important to note that continuous propagation, as it is in continuous back-slopping, may result in a skewed microbial ecosystem, causing the disappearance of some strains which though less dominant and less adaptive, may be important for unique flavour development of the fermented product (Leroy & De Vuyst, 2004). While starter LAB are effective for accelerated and controlled fermentation process, non-starter LAB strains have been associated with enhanced flavour intensity during fermentation (Leroy & De Vuyst, 2004).

1.9.2 Nutritional requirement of LAB

Though LAB undergo a vast range of metabolic activities, they have fastidious metabolic requirements. Microbial growth, metabolic performance and fermentation outputs depend on LAB strain, biochemical and biophysical environments (Mohammadi, Sohrabvandi, & Mohammad, 2012; Saeed & Salam, 2013) such as nutrients and temperature respectively. It can thus be inferred that a strain of LAB will behave differently if cultured in different media (Wegkamp, Teusink, De Vos, & Smid, 2010). All LAB strains require carbohydrates as the main carbon and energy source (Camien, Dunn, & Salle, 1947), though they are also able to source carbon from proteins and fat (Gänzle, Vermeulen, & Vogel, 2007; Saeed & Salam, 2013). Glucose supports the growth of many LAB strains, however all strains have a preference for which sugar they metabolize optimally (Camien et al., 1947; Charalampopoulos et al., 2002). The second most important nutrient for LAB growth is nitrogen as it amounts to about 12% of total cell dry weight (Manca de Nadra, 2007). LAB usually source nitrogen from organic proteins present in the food substrate (Saeed & Salam, 2013) which is broken down to nitrogenous substances such as purine and pyrimidine needed to

synthesize nucleic acids and amino acids necessary for growth (Saeed & Salam, 2013; Snell & Mitchell, 1941). These proteolytic activities of extracellular and intracellular proteinases and peptidases in LAB (Guizani & Mothershaw, 2006) also contribute flavour development in fermented foods (Guizani & Mothershaw, 2006; Manca de Nadra, 2007). As with sugar requirements and preferences, protein and amino acid requirements of LAB are strain specific (Saeed & Salam, 2013). Although cystine has generally been seen to enhance the growth of a large number of strains, it is not regarded as a growth factor as it has not been identified as essential, where its absence may inhibit the growth of the LAB strains (Snell, 1945). B vitamins with the exception of thiamine and cobalamin (Saeed & Salam, 2013; Snell, 1945), minerals such as manganese and magnesium and fatty acids such as oleic acids encourage the growth of a large number of LAB strains, but with varied degree of strain responses (Saeed & Salam, 2013). Whilst some of these nutrients are essential, some are mainly stimulatory, enhancing strain growth. Where a required but non-essential nutrient is absent, LAB are sometimes able to replace or synthesize them using other available nutrients (Saeed & Salam, 2013; Snell, 1945; Wegkamp et al., 2010). LAB strain's nutritional requirement and substrate's nutritional composition will determine the suitability as a starter or non-starter culture for the fermentation of the food substrate. Therefore, some species are usually associated with specific food substrates (**Table 1.3**). Dominant wild strain/strains isolated and identified as stable microbial ecosystem after 5 to 10 days of continuous back-slopping fermentation (Bessmeltseva et al., 2014) of the substrate may therefore be ideal for use as starter and/or main fermentation culture for food substrates.

Table 1.3: Examples of LAB found in known fermented foods

Fermentable substrate	**Examples of Associated dominant LAB**
Legumes	*Bacillus* spp
Cereal (maize)	*Lb plantarum, Lb paraplantarum, Lb fermentum*,
Milk	*Lb delbrieckii* subsp *bulgaricus, Streptococcus thermophilus, Lactococcus lactis* subsp *lactis, Lactococcus lactis* subsp *cremoris, Lb casei, Lb acidophilus, Lb johnsonii*
Meat	*Lb sakei, Lb curvatus, Pedicoccus pentosaceus, P. acidilactici*
Vegetables	*Leuconostoc mesenteroides, Lb plantarum*
Alcoholic wine	*Oonecoccus oeni*

Adapted from: Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Review: diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology*, 7.

1.10 Yeasts

About 70 yeast species have been documented in the inventory of microbial food culture (MFC) as species associated with food fermentation (Bourdichon et al., 2012). Those commonly found in fermented cereals foods primarily belong to *Saccharomyces* and *Candida* genera (Blandino et al., 2003) and species that are known to be involved in indigenous maize fermentation include *Geotrichum fermentans, Geotrichum candidum, Rhodotorula graminis, Saccharomyces cerevisiae, Candida krusei,* and *Candida tropicalis* (Omemu, Oyewole, & Bankole, 2007). Yeast is an eukaryotic, unicellular, facultative anaerobic organism (Broach, 2012). They are rare causes of food borne illnesses but they behave as opportunistic pathogens with the potential to cause illness particularly in people with compromised immune function (Fleet, 2011). Yeasts are used in food fermentation producing ethanol, CO_2 and flavour enhancing compounds such as higher alcohols, organic acids, glycerol, esters, aldehydes, ketones and sulphur volatiles at the end of the fermentation process (Broach, 2012; Fleet, 2011; Kennedy & Rhymer, 2012; Snoek & Yde, 2007). Yeast cells are not as fastidious as LAB as they are have a wider variety of compounds they can use as carbon and energy source for growth (Broach, 2012). They however more readily ferment glucose and fructose compared to other carbon and energy sources (Broach, 2012). Nitrogen is the second most important nutrient required for yeast growth, which can be synthesized from organic and inorganic nitrogenous compounds for protein synthesis (Fleet, 2011). Other nutrients such as sterols and fatty acids, B vitamins, phosphate, magnesium, calcium and iron are also used for growth, most of which are either stimulatory or can be synthesized by yeast (Fleet, 2011; Joslyn, 1951; Nelson, Fulmer, & Cessna, 1921).

1.11 Advantages of starter culture

The use of a starter culture increases the rate of acidification further enhancing safety of fermented food (Holzapfel, 2002; Ionescu et al., 2007). Starter culture can compose of bacteria, yeast or mould strains, nevertheless, LAB are most used due to their ability to undergo a wide spectrum of metabolic activities, their potential to survive varied storage conditions (Saeed & Salam, 2013) and their long and safe history of application (Leroy & De Vuyst, 2004). The addition of a starter culture not only improves safety of the fermented food product (Bourdichon et al., 2012) and accelerates the initiation of fermentation by facilitating control over the initial phase of the fermentation process (Holzapfel, 2002), but also helps to catalyse and achieve *in situ* production of the desired properties of the food product (Ray & Joshi, 2014). The use of a functional starter culture - culture that are able to exhibit desirable properties in addition to rapid acid production - for industrial food fermentation over the years has gained recognition in fermented food production (Hati, Mandal, & Prajapati, 2013; Leroy & De Vuyst, 2004). Leroy and De Vuyst (2004), described a functional

starter as a starter with at least one of food safety/preservation, organoleptic, technological, nutritional and health functional properties. Some functional organisms may not be suitable as starter culture but may be able to exhibit the listed functional properties when they are co-cultured with starter organisms (Tripathi & Giri, 2014).

1.12 The ecosystem in cereal fermentation

1.12.1 Microbe interactions

Food substrates are most often fermented by a consortium of microorganisms (Blandino et al., 2003). Mixed culture fermentation in controlled and spontaneous fermentations, often results in more production of metabolites and better flavoured end product compared to controlled fermentation using single culture fermentation (Papadimitriou et al., 2015). When fermentation of a substrate involves two or more cell types, there is communication between the cells (Papadimitriou et al., 2015; Sieuwerts, De Bok, Hugenholtz, & van Hylckama Vlieg, 2008). These interactions could be nutritional (metabolite trading), physical or through the production of diffusible chemicals (Sieuwerts et al., 2008) and they are broadly said to be either antagonistic or synergic (De Vuyst & Neysens, 2005; Smid & Lacroix, 2013). They can be more specifically divided into competitive interactions (-/-), mutual interactions (+/+) commensal interactions (+/0), amensal interactions (-/0) and parasitic interactions (+/-) where + represents a positive effect and – a negative effect (Smid & Lacroix, 2013). The dynamics of the microbial ecosystem changes and shifts as fermentation progresses depending on nutrient transport and uptake and organisms' sensitivity to fermentation output (Fleet, 2011). These network of interactions are indicated by the growth and/or death of organisms present within the community (Fleet, 2011) and generally contribute to the flavour characteristics of final fermented product (Zupan et al., 2013).

1.12.2 Nutritional interaction

In cereal foods, the community of organisms is comprised mainly of LAB and yeast (Nyanzi & Jooste, 2012). Co-culture of yeast-LAB could be synergic or antagonistic depending on the available carbon, energy and nitrogen sources in the substrate (De Vuyst & Neysens, 2005), as well as the strains of organisms involved in the fermentation (Romano, Capece, & Jespersen, 2006). The absence of competition for carbon source is a major pre-requisite for a stable and successful association between LAB and yeasts (Romano et al., 2006). Cereals are very high sources of carbohydrates (**Table 1.4**), 55 – 70% of which is starch and approximately 3% of sugars (Koehler & Wieser, 2013).

Fermentable sugars of approximately 3% could mean limited availability of a carbon source for the microorganism consortium during cereal grain fermentation. However, this is managed by amylolytic activities that occur during fermentation. Carbohydrates are stored as starch in the endosperm of the cereal grain (Koehler & Wieser, 2013). Cereal grains have the amylolytic enzymes, amylase - α-amylase, β-amylase and glucoamylase, embedded in the endosperm (Gangadharan & Sivaramakrishnan, 2009; Oyewole & Agboola, 2011). The enzyme is known to be dormant or with limited activity in dry grains but gets activated as germination commences (Ernst, 1971) which is usually when moisture is introduced (Arendt & Zannini, 2013). Amylase hydrolyses the starch present in the endosperm into fermentable carbohydrate molecules that can be used as carbon and energy sources by the fermentation organisms. Even though yeast strains are known to be able to synthesize amylase, the form of amylase synthesized by most yeast strains, α-amylase, is unable to degrade raw starch on its own (van Zyl, Bloom, & Viktor, 2012). *Saccharomycopsis fibuligera* nevertheless is able to degrade raw starch due to the ability to synthesize glucoamylase along with α-amylase (Hostinova, 2002; van Zyl et al., 2012).

Table 1.4: Average nutritional composition of raw cereal grains

g/100g	**Wheat**	**Rye**	**Barley**	**Oats**	**Rice**	**Millet**
Moisture	12.6	13.6	12.1	13.1	13.0	12.0
Protein	11.3	09.4	11.1	10.8	07.7	10.5
Lipids	01.8	01.4	02.1	07.2	02.2	03.9
Carbohydrates	59.4	60.3	62.7	56.2	73.7	68.2
Fibre	13.2	13.1	09.7	09.8	02.2	03.8
Minerals	01.7	01.9	02.3	02.9	01.2	01.6
mg/kg						
Thiamine	04.6	03.7	04.3	06.7	04.1	04.3
Riboflavin	00.9	01.7	01.8	01.7	00.9	01.1
Nicotinamide	51.0	18.0	48.0	24.0	52.0	18.0
Pantothenic acid	12.0	15.0	06.8	07.1	17.0	14.0
Vitamin B6	02.7	02.3	05.6	09.6	02.8	05.2
Folic acid	00.9	01.4	00.7	00.3	00.2	00.4
Total tocopherols	41.0	04.0	23.0	18.0	19.0	40.0

Adapted from: Koehler, P., & Wieser, H. (2013). Chemistry of cereal grains. In M. Gobbetti & M. Ganzle (Eds.), Handbook on Sourdough Biotechnology (Vol. 4, pp. 11-45): Springer

S. fibuligera is primarily known for its amylolytic activities in indigenous food fermentation using rice and cassava (Chi et al., 2009). Amylolytic yeast cells are important for starchy substrate fermentation (Fleet, 2011). Most LAB strains do not have amylolytic activities and are therefore unable to hydrolyse starch (Fossi & Tavea, 2013). The few species that are able to undergo amylolysis, known as Amylolytic Lactic Acid Bacteria (ALAB), belong to the *Lactobacillus* genera and are associated with indigenous fermentation of starchy substrates in the tropics (Fossi & Tavea, 2013). Examples of these include *Lb plantarum, Lb fermentum* and *Lb manihotivorans* (Fossi & Tavea, 2013). Even though fermentable sugars are made available through amylolytic processes, competition or its absence depends on the strains of organisms that are co-cultured. The degree of fastidiousness of LAB present in the consortium may dictate the competition that may ensue within the community. Romano et al. (2006) reported that when sucrose is the main carbon source within a substrate, competition in a culture consisting of *S. cerevisiae, S. exiguus* and *Lb plantarum* favours the LAB. According to the authors, yeast strains are able to hydrolyse sucrose into glucose and fructose which are more readily fermented by *Lb plantarum* than sucrose resulting in a high yield of lactic acid. There is also a rapid depletion of sucrose, the preferred sugar for yeast since it can also be metabolized by *Lb plantarum* (Plumed-Ferrer et al., 2008). On the other hand, where maltose and glucose are the main carbon sources, there is less production of acetic and lactic acid by *Lb plantarum* in the co-culture with the mentioned yeast compared to if it was a mono-cell culture. This is because whilst *S. cerevisiae* strains are able to metabolise glucose, they may also consume maltose competitively, limiting the growth of *Lb plantarum* (Romano et al., 2006). Yeast cells are high in proteins, free amino acids and vitamins, especially B vitamins (Romano et al., 2006) which are then made available for LAB cell growth at the death or autolysis of yeast cells in adverse environmental conditions (Fleet, 2011). On the other hand, when there is shortage of supply of fermentable sugars, yeast cells may be able to further metabolize organic acids produced (Fleet, 2011). LAB though have preferences for mono and disaccharides, are also able to ferment more complex carbohydrate compounds such as xylose and arabinose, with the potential to reduce possible metabolic competition that may occur between yeast and LAB (De Vuyst & Neysens, 2005). LAB to LAB interactions can also be observed in a consortium containing more than one species of LAB. For example, Gobbetti and Di Cagno (2012) reported that in sourdough fermentation, *Lb sanfranciscensis* CB1 have a high chance of surviving when co-cultured with *Lb rossiae* A7 than when co-cultured with *Lb plantarum* DC400 or *Lb brevis* CR13. The authors postulated that aside from easily definable factors such as acidity, synthesis of antimicrobial compounds and nutrient competition, DC400 and CR13 may create undefinable stressful conditions that may hinder the survival of CB1 during sourdough co-cultivation.

1.12.3 Quorum sensing – Microbial communication

Cells are able to interact by exchanging molecular signals (Smid & Lacroix, 2013), that are diffusible within the medium (Rul & Monnet, 2015; Sieuwerts et al., 2008). Each cell is able to produce signalling molecules known as auto-inducers, which when detected by other cells, results in gene expression that give rise to a common behaviour by the cells (Waters & Bassler, 2005). This form of interaction depends on the population density of cells and is therefore described as quorum sensing (QS) (Rul & Monnet, 2015). Auto-inducers are used for intra- and inter- specie communication (Waters & Bassler, 2005). LAB being gram positive organisms use modified peptides as auto-inducers which are also called pheromones (Rul & Monnet, 2015). Microbial cells use QS for virulence, stress response and competence enhancement (Sieuwerts et al., 2008). QS has been associated with the cell motility, exopolysaccharide (EPS) production, biofilm formation and antimicrobial substances such as bacteriocin production in a complex culture (Sieuwerts et al., 2008; Smid & Lacroix, 2013). Metabolites are secreted as fermentation progresses and increased cell density gives rise to a corresponding increase in metabolite secretion. At certain threshold levels the metabolites, acting as auto-inducers, trigger some stress-induced physiological responses from cells (Mohammadi et al., 2012; Zupan et al., 2013). The response may vary from LAB controlling the expression of genes involved in bacteriocin production (Sturme, Francke, Siezen, de Vos, & Kleerebezem, 2007) to enzyme secretion or secretion of aromatic alcohols by yeast cells (Fleet, 2011; Rul & Monnet, 2015; Zupan et al., 2013). A review by Smid and Lacroix (2013) showed that some auto-inducers have dual functions. According to them, lantibiotic peptide nisin, which is a type of bacteriocin is able to function as a metabolite, whilst also acting as a signalling molecule during QS. It is important to state that effective signalling mechanism in fermented foods is hindered by factors such as viscosity, pH and temperature (Rul & Monnet, 2015). The authors explained that viscosity influences accumulation, diffusion and reception of the signals while other factors may hinder the stability of these signals. They further stated that fermented foods contain QS inhibitors, which are able to degrade signalling molecules. Anti-quorum sensing substances in food fermentation may however be beneficial if they prevent food spoilage. Generally, survival of organisms in a complex microbial consortium is dependent on how much they are able to tolerate by-products of fermentation. For example, in yeast-LAB co-culture, alcohol produced by yeast may be inhibitory to some LAB strains while yeast strains may also be sensitive to lactic and acetic acids produced by LAB (Fleet, 2011; Romano et al., 2006).

1.13 Metabolite profiling of fermented foods

Metabolomics is generally defined as the detailed analysis of metabolites in a biological specimen (Clich, 2015). It involves the identification and quantitative analysis of intracellular compounds,

which are produced and modified by living organisms, during metabolic processes (Mozzi, Ortiz, Bleckwedel, De Vuyst, & Pescuma, 2013). These metabolites often have low molecular weights (b1500 Da) and are not genetically encoded (Mozzi et al, 2013). Metabolite profiling - study of the production and modification of metabolites - of fermented cereal products has been widely reported with examples of endogenously and exogenously produced metabolites during microbial fermentation identified as peptides, amino acids, nucleic acids, carbohydrates, organic acids, alcohols, ketones, aldehydes, esters, vitamins, polyphenols, alkaloids, minerals, antibiotics and enzymes (Koistinen & Hanhineva, 2017; Mozzi et al., 2013; Koistinen, Mattila, Katina, Poutanen, Aura & Hanhineva, 2018; Deshpande, 2000; Divya et al., 2012; Pereira et al., 2013; Mienda et al., 2011; Subramaniyam & Vimala, 2012)

Koistinen et al., (2018) undertook metabolite profiling of bread and sourdough, using rye flour vs wheat flour. Sourdoughs were made using a strain each of *Lactobacillus brevis*, *Lactobacillus plantarum*, *Candida milleri* while straight doughs were prepared with a commercial baker's yeast. Due to consumer acceptability of the bread made from sourdough, microbial inoculum size and length of fermentation were higher and longer with rye flour than wheat flour. They consequently reported a significant difference in the metabolite profile between wheat vs rye, between whole-grain dough and doughs made from refined or processed wheat and rye flour, and between sourdoughs and straight doughs (made with baker's yeast), highlighting the implication of type of cereal, fermentation organisms and method of food processing in the final biochemical composition of the fermented cereal products.

Metabolites profiling however provides information on sensory properties, nutritional composition, functionality and nutraceutical quality of the fermented end product (Mozzi et al, 2013; Adebo et al., 2017).

1.14 The ecosystem in the human gut

The interior and exterior parts of the human body are colonized by an extensively diverse range of microbial cells consisting of fungi, protozoa, viruses and archaea but dominated by bacteria, and they are generally described as the human microbiota (Cénit, Matzaraki, Tigchelaar, & Zhernakova, 2014; Maier, Anderson, & Roy, 2014; Ravel et al., 2014). These cells are said to outnumber human cells by a factor of 10 (Cénit et al., 2014). The human gut houses the largest percentage of the microbiota (Ravel et al., 2014), with the stomach, small intestine and colon believed to consist of up to 10^2, 10^7 and 10^{12} bacterial cells/ml respectively (Vyas & Ranganathan, 2012).

Human genes have also been said to be 150 times outnumbered by the genome of the microbiota, popularly described as the human microbiome, (Cénit et al., 2014; D'Argenio & Salvatore, 2015) with the largest percentage found in the human gut, especially the colon (Ravel et al., 2014).

It is generally said that the GIT of human foetus is sterile and the development of human microbiota does not commence until after birth (D'Argenio & Salvatore, 2015). However, studies such as those carried out by Jiménez et al. (2008), Mshvildadze et al. (2010) and Aagaard et al. (2014) suggest that the gut of a healthy human foetus from healthy mothers may not be sterile after all, as authors were able to isolate some microorganisms from meconium of healthy neonates though with limited microbial diversity. Jiménez et al. (2008), undertook further investigation with the aim to clarify whether oral administration of a bacterial strain to pregnant mice may lead to its presence in foetal meconium in a randomized controlled trial. In contrast to the non-inoculated group where the investigated strain could not be detected, *E. fecium* strain previously isolated from breast milk of a healthy woman and orally administered to pregnant mice was isolated and PCR-detected from meconium of mice obtained by caesarean section one day before the predicted date of labour. Though the study was not a human trial, the authors theorize that some of the microbes orally administered to healthy pregnant women may permeate into the placenta allowing access to the foetus. A more diverse microbial colonization nevertheless begins to develop immediately birth (Derrien & van Hylckama Vlieg, 2015).

1.14.1 Development and Composition of the gut microbiome

The range and type of organisms comprising the gut microbiota is initially influenced by the method of delivery, whether vaginal or caesarean and subsequent feeding practices, breast or formula feeding (Cénit et al., 2014; Derrien & van Hylckama Vlieg, 2015). The initial gut microbiota of new-borns delivered through the vagina is believed to be similar to the mothers' vaginal microbiota while gut microbiota of caesarean born babies correlate with the mothers' skin microbiota (Dominguez-Bello et al., 2010). The bifidogenic effect of the breastmilk causes *Bifidobacteria* along with *Lactobacillus* spp to initially dominate the gut of exclusively breastfed babies (Conlon & Bird, 2014; Laparra & Sanz, 2010). The infant gut microbiota is rather unstable and continues to change until about 24 - 36 months, when the gut microbiota evolves to an adult-like pattern of diversity (Derrien & van Hylckama Vlieg, 2015; Palmer, Bik, DiGiulio, Relman, & Brown, 2007). There is however the assumption that the initial colonization in infancy may influence the composition of the gut microbiota through adulthood (Sekirov, Russell, Antunes, & Finlay, 2010).

The adult gut ecosystem is usually dominated by the phyla Bacteroidetes (e.g. *Parabacteroides distasonis*, *Bacteroides vulgatus*), Firmicutes (*Clostridia* and LAB), Proteobacteria (e.g. *Escherichia coli*) and Actinobacteria (*Colinsella* and *Bifidobacterium* spp) with the first two phyla being the most abundant, followed by Proteobacteria and Actinobacteria (Sun & Chang, 2014; Xu et al., 2007; Zhang et al., 2015). Some other bacterial cells in the gut belong to Verrucomicrobia, Cyanobacteria and Fusobacteria phyla (Sun & Chang, 2014; Kim, Choi & Yim, 2019). It is nonetheless important to note that the diversity of the human gut microbiota varies along the GIT from facultative anaerobes in the stomach to obligate anaerobes as the gut descends (Maier et al., 2014). There is no universal gut microbiome strain composition (Vyas & Ranganathan, 2012) as there is a notable degree of intra- and inter- individual variability in its specific composition (Hisada, Endoh, & Kuriki, 2015). The gut microbiota is dynamic and responds to physiological perturbations it encounters everyday (Sun & Chang, 2014). Factors such as diet, medication, age, and organism that are ingested significantly alter the inter-microbe and host-microbe relationships (De Filippo et al., 2010; Derrien & van Hylckama Vlieg, 2015; Laparra & Sanz, 2010; Sun & Chang, 2014). Organisms in the gut microbiota are often described as commensal organisms (DiBaise, Frank, & Mathur, 2012; Laparra & Sanz, 2010; Sekirov et al., 2010; Sun & Chang, 2014; Vyas & Ranganathan, 2012), but a mutually beneficial relationship between the microbiota and the human has been established, with the human gut hosting and consistently making nutrients available for the microbes, while the microbes provide health benefits to the host (Cénit et al., 2014).

1.14.2 Gut microbiome and health

Increasing evidence shows that human health is greatly influenced by the activities of the gut microbiome (Derrien & van Hylckama Vlieg, 2015). The gut microbiome has been said to be responsible for processes such as fermentation of indigestible nutrients ingested by the host, synthesis of nutritional factors such as B and K vitamins, protection against pathogens, detoxification, development of the robust systemic and intestinal immune function (Cénit et al., 2014; De Filippo et al., 2010; Sekirov et al., 2010; Sun & Chang, 2014). Though not officially described as an organ, the gut microbiome is completely relied upon by the human body for these metabolic functions which is the reason the gut microbiome was described as the ‘forgotten’ organ by Cénit et al. (2014).

Fermentation of indigestible carbohydrates by the gut microbiota produces short-chain fatty acids (SCFA) - acetate, butyrate and propionate, which are the main source of energy for colonocytes and the gut microbiota and they also help to maintain homeostasis in the gut (Sun & Chang, 2014).

Results from recent experimental studies have implied that the gut microorganism community may play some critical role in determining nutrient uptake and energy regulation in relation to obesity (Delzenne & Cani, 2011; DiBaise et al., 2012). Though these compounds are produced in the colon, a small percentage (Deroover, Boets, Tie, Vandermeulen, & Verbeke, 2017) is absorbed into the blood and carried to the liver by the hepatic portal vein where they are further metabolised (Sun & Chang, 2014). The gut microbiota is also able to produce conjugated linoleic acid, which has been suggested to have anti-inflammatory and metabolic pathway regulatory effects, by metabolising dietary linoleic acid (Delzenne & Cani, 2011). Butyrate may be able to provide the colonocytes anti-inflammatory functions (Conlon & Bird, 2014; Maier et al., 2014) and anti-carcinogenic effects by promoting cell differentiation and encouraging apoptosis of transformed colonocytes (Wong, De Souza, Kendall, Emam, & Jenkins, 2006). It has however been suggested that based on end products of metabolism and fermentation processes, the gut microbiota may be said to comprise of both beneficial and harmful organisms and their effects are not limited to the GIT, but also extend to other organs in the body (Bedani, Isay Seed, & Sivieri, 2015; Sun & Chang, 2014; Zhang et al., 2015).

1.14.3 Gut microbiome and the immune function

Over the years, there has been an increased understanding that the gut microbiome plays an essential role in the emergence, development and function of the host immune system (Belkaid & Hand, 2014). The gut intestinal mucosa houses immune cells and it is suggested that it may be the location for up to 80% of the immune cells in the human body (Furness, Kunze & Clerc, 1999; Sipos & Muzes, 2011). The epithelial cells, the highly permeable covering of the intestinal mucosa, is exposed to the activities of the gut microbiome (Fureness et al., 1999). Beneficial bacteria in the microbiome produce a barrier or competitive-exclusion effect, with mechanisms such as competition for nutrients and production of antimicrobial substances, to inhibit the attachment and possible invasion of pathogenic organisms into the epithelial cells (Bull & Plummer, 2014). The intestinal mucosa consists of specialised tissues known as gut-associated lymphoid tissue (GALT) and isolated lymphoid follicles (ILF) that play a vital role in the immune system of the human body (Furness et al., 1999). The GALT and ILF comprise of antigen sampling cells (M cells), lymphocytes and other immune-associated cells such as macrophages, eosinophils, mast cells, dendritic cells and neutrophils (Furness et al., 1999; Sipos & Muzes, 2011).

Though the immune cells are present in the foetus from about 11 weeks of gestation, the cells however do not develop until after birth (Olin et al., 2018). It is believed that the development of the immune cells at this time is triggered by the exposure of infants to microorganisms after birth

(Olin et al., 2018). Nevertheless, evidence from studies using germ-free (GF) vs microbial colonized gut mice also suggest that the human gut microbiome plays a significant role in the development of immune cells (Gensollen, Iyer, Kasper & Blumberg, 2016). It was discovered that the intestinal epithelial cells of the GF mice differ physiologically from mice with microbial colonized guts (Round & Mazmanian, 2009; Gensollen et al., 2016), and the absence of the gut microbiome had negative impact on the structural and functional development of the immune cells with evidence showing defects in the development of the primary and secondary lymphoid organs such as the GALT, spleen, and thymus, impaired development and maturation of the ILF, and decreased size of Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) (Li et al., 2018; Gensollen et al., 2016; Round & Mazmanian (2009). These as a consequence have been associated with a decreased number of intestinal T cells, B cells and intraepithelial lymphocytes which are responsible for the activation of immune responses when required (Gensollen et al., 2016; Furness et al., 1999; Mosconi et al, 2018; Baptista et al., 2013). Rakoff-Nahoum & Medzhitov (2008) and Gensollen et al., (2016) also suggest that not only does the gut microbiome significantly contribute to the host immune development, the composition and ecological successions of the microbiota before the age of 3, may also determine the efficiency of the immune function in adult life and the risk of development of diseases such as diabetes, GIT diseases and food allergies. Nevertheless, though the existence of the microbiome-host interaction for immune development is not ignored, the specific channel of this interaction is not well understood (Gensollen et al., 2016). It is believed however that the metabolites produced by the gut microbiota are able to interact with the pattern recognition receptors in the intestinal epithelial cells, the immune cells in the peripheral blood and the cells of the central nervous system (CNS) (Zhu et al., 2020). As a result, an alteration of the gut microbiome may cause some immunological responses by the mucosal layers and the intestinal lymphocytes with possible manifestations such as allergies (Zhu et al., 2020).

1.14.4 Perturbation of the gut microbiome

Wallace et al. (2011) and Roberfroid et al. (2010) suggest that members of the consortia with the potential to cause diseases are opportunistic pathogens in the absence of homeostasis. Homeostatic relationships are initiated and enhanced through intra- and inter species communications that occur in the gut microbiome (Swift, 2000; Sun & Chang, 2014). Studies suggest that alteration of these relationships resulting in the imbalance of the gut microbiome, i.e. dysbiosis, may be associated with the emergence of a number of diseases (Derrien & van Hylckama Vlieg, 2015; Ritchie & Romanuk, 2012; Zhang et al., 2015). Intestinal microbiota dysbiosis has been defined as a change in the intra- and inter species communications and dialogue between the host intestinal cells and enteric bacteria due to disruption of microbial diversity which is usually manifested as the

overgrowth of a given taxon, often the opportunistic pathogens (Xu et al., 2019). Antibiotics and food intake are known to rapidly alter the gut micrbiome (Myers, 2004; David et al., 2014). Antibiotics are generally administered with the aim to deplete pathogenic bacteria, however due to their wide spectra of activities, combined or single orally administered antibiotics may indiscriminately destroy beneficial organisms in the gut resulting in the changes in the gut microbiome (Zhang & Chen, 2019). For example, it has been suggested that a combination of cefoperazone, clindamycin, and vancomycin may be associated with the loss of Lachnospiraceae and Ruminococcaceae in the large intestine, leading to reduced transformation of primary bile acids to secondary biles acids and increased risk of *Clostridium difficile*, an opportunistic pathogen, infection (Zhang & Chen, 2019). Willmann et al., (2019) also reported a consistent decrease in the diversity at phylum level of the gut microbiome of haematological patients over the course of administration of ciprofloxacin or cotrimoxazole. Some bacterial strains isolated from the gut, particularly those belonging to *Lactobacillus* and *Bifidobacterium* spp, have been tested and acknowledged for their protective and beneficial immunoglobulin responses to the alteration of homeostasis that may occur in the gut microbiome (Laparra & Sanz, 2010). These organisms have also been known to be present in fermented food products Rezac, Kok, Heermann & Hutkins, (2018). The effects of diet on the gut microbiome is further discussed below. It is nevertheless important to mention that there are speculations other microbial strains in the gut that may confer health benefits may be available but are yet to be identified or may not be culturable (Keeney, Yurist-Doutsch, Arrieta, & Finlay, 2014; Sanders, 2008).

1.14.5 Gut microbiome and diseases

The gut microbiota has been implicated in various diseases known to man including GIT diseases, metabolic disorders, cancer, neurological and neurodegenerative disorders (Patterson et al., 2016; Kim, Choi & Yim, 2019; Zhao, 2013; Wolf & Lorenz, 2012). Obesity is a global epidemic that has been defined, according to the WHO, as an excessive accumulation of fat which increase the risk of development of metabolic diseases such as type 2 diabetes mellitus (T2D) and cardiovascular diseases, some kinds of cancer and non-alcoholic fatty liver diseases (WHO, 2020; Kim et al., 2019). Though obesity is said to develop as a result of excessive energy intake in relation to expenditure (WHO, 2020), the gut microbiome has been implicated as the environmental factor which contributes to the development of fat adiposity and obesity in the host (Wolf & Lorenz, 2012; Greiner & Bäckhed, 2011). The causative link between the gut microbiome and development of obesity was first recorded by Bäckhed et al., (2004) who in an intervention study, showed that colonization of GF mice with a conventional gut microbiota harvested from the cecum of conventionally raised 8 weeks old mouse resulted in increased accumulation of body fat by up to

60%, over 2 fold increase in hepatic triglycerides and reduced insulin sensitivity in comparison with GF mice, in spite of 29% less consumption of rodent chow diet (57% carbohydrates, 5% fat) for 14 days. Bäckhed, Manchester, Semenkovich, & Gordon (2007) further reported in a follow-on study that, in contrast to mice with a gut microbiota, germ-free (GF) mice were protected against obesity after consuming a high-fat, high sugar Western-style diet for 8 weeks.

Metabolites produced by the gut microbiome have therefore been associated with the development of obesity. These metabolites may be beneficial to the host, providing anti-inflammatory and anti-oxidative effects, producing gut barrier functions and being sources of micronutrients to the host (Zhao, 2013). Nonetheless, some metabolites such as SCFAs may modulate energy balance to the host whilst others such as bacterial endotoxins may promote pro-inflammatory immune responses (Cénit et al., 2014; De Filippo et al., 2010; Sekirov et al., 2010; Sun & Chang, 2014; Zhao, 2013; Kim et al., 2019; Wolf & Lorenz, 2012), both of which have been suggested to influence the host's metabolic processes (Zhao, 2013; Kim et al., 2019; Wolf & Lorenz, 2012). Lipopolysaccharide (LPS), an endotoxin produced by gram-negative bacteria induces pro-inflammatory responses i.e. increased production of cytokines, which is suggested to be linked to excessive lipid absorption and accumulation and insulin resistance (Fei & Zhao, 2013; Shen, Obin & Zhao, 2013; Zhao, 2013). Bäckhed et al., (2007) implied in their study that with the gut microbiota there is an increased calorie harvest from plant polysaccharides that are normally non-digestible by the human digestive enzymes through fermentation. This results in increased amount of glucose available for uptake in the gut and the increased production of SCFAs which are absorbed and transported to the liver where they are synthesised into triglycerides. The authors further suggested that the gut microbiota may be able to modulate the genes that affect deposition of fat in the adipocytes which as a consequence, increases fat storage.

Due to ethical restrictions, there are no research articles available to affirm this causative links between the gut microbiota and development of obesity in humans. Nonetheless, studies have postulated that changes in gut microbiota may contribute to changes in the host metabolism by suggesting that there was a significant difference in the gut microbiota profile of obese versus lean humans (Kim et al., 2019; Sanz, Santacruz & Gauffin 2010; Federico, Dallio, Di Sarno., Giorgio & Miele 2017) with difference majorly reported among the Firmicutes and Bacteroidetes (Wolf & Lorenz, 2012; Kim et al., 2019; Musso, Gambino & Cassader, 2010; Federico et al., 2017). While Baothman, Zamzami, Taher, Abubaker & Abu-Farha (2016) and Patterson et al., (2016) reported decreased microbial diversity in the gut of the obese, other researchers have also suggested a higher number of Firmicutes and lower amount of Bacteroidetes in obese humans in comparison with lean

humans (Kim et al., 2019; Sanz et al., 2010; Federico et al., 2017). This was reported by various studies that showed an increase in Firmicutes and a decrease in Bacteroidetes when conventional mice were fed with high fat diets as reported by Wolf & Lorenz (2012). In contrast, Schwiertz et al., (2010), Duncan et al., (2007) and Gostner et al., (2006) in their experimental studies using larger sample sizes in comparison with previously mentioned articles, reported no differences in the amount of *Bacteroides* spp and the percentage of Firmicutes in overweight and obese versus lean participants. Although it is proposed that an increase in the population of Firmicutes may significantly contribute to increased fat storage in the host since they are typically more involved in carbohydrate and lipid metabolism compared to Bacteroidetes (Kim et al., 2019), Schwiertz et al., (2010) explains that increased production of SCFA in obese people may be a more important driving factor of obesity, rather than an increased population of the Firmicutes and the ratio between them and the Bacteroidetes. While butyrate is mainly utilized as energy source by the colonocytes, propionate is largely transported from the colon to the liver and may be an important substrate for gluconeogenesis, liponeogenesis and protein synthesis while acetate may be metabolised into cholesterol in the peripheral tissues (den Besten et al., 2013; Schwiertz et al., 2010; McNabney & Henagan (2017), all of which may cause an increased energy harvest. Increased energy harvest and fat storage may mediate insulin resistance and consequently, the development of T2D (Musso et al., 2010). Still, dysbiosis of the gut microbiome has also been implicated in the development of T2D (Tilg & Moschen, 2014). Qin et al., (2012) and Karlsson et al., (2012) showed up to 3% gut microbial gene difference between healthy people and patients diagnosed with T2D among the Chinese and Scandinavian populations respectively. Both studies reported significant decreases in butyrate-producing organisms such as *Roseburia intestinalis* and *Faecalibacterium prausnitzii*. Butyrate is known to be majorly responsible for the maintenance of epithelial cell integrity in the gut (Patterson et al., 2016; Yan & Ajuwon 2017; Peng, Li, Green, Holzman & Lin, 2009). A decrease in the production of butyrate may be a risk factor for an increased intestinal permeability of molecules such as LPS and pathogenic organisms from the colon into the blood stream, a condition commonly known as 'leaky gut' (Patterson et al., 2016; De Kort, Keszthelyi, & Masclee 2011). According to De Kort et al., (2011) serum LPS appears to be higher in patients with T2D than in healthy participants with as associative link to 'leaky gut'. Administration of metformin has nonetheless been shown to enhance gut microbial function in T2D patients, by decreasing intestinal lipid absorption and LPS-triggered local inflammation (Forslund et al., 2015; Iulia-Suceveanu et al., 2019; Sharma & Tripathi 2019). Furthermore, the anti-inflammatory and anti-carcinogenic benefits derived from butyrate (McNabney & Henagan 2017; Conlon & Bird, 2014; Maier et al., 2014; Wong, De Souza, Kendall, Emam, & Jenkins, 2006), may be compromised by its decreased production in the colon. A reduced anti-inflammatory and anti-carcinogenic activities in the colon

are known to be risk factors for the development of cancer in the host McNabney & Henagan (2017),

Brain disorders have also been linked to the gut microbiome and this was extensively discussed in a review article by Zhu et al., (2020). The authors have associated this link with the dysfunction of the blood brain barrier (BBB), is a membrane which protects the CNS by separating the cerebrospinal fluids from the peripheral blood, leading to anxiety, depression, autism spectrum disorders (ASDs), Parkinson's disease, Alzheimer's disease and schizophrenia. It is believed that increased permeability of the BBB could allow the entrance of metabolites of the gut such as phosphatidylcholine, trimethylamine oxide (TMAO), L-carnitine, glutamate, bile acids, and lipids from the peripheral blood, into the CNS (Zhu et al., 2020). All of these substances have been implicated to result in an increased risk of brain dysfunction (Zhu et al., 2020). And it has also been implied that perturbation of the gut microbiome can lead to increased production of such metabolites. Zhu et al., (2020) reported that abundance of *Akkermansia* and *Parabacteroides* - associated with high fat low carbohydrate intake - may increase the host's susceptibility of having seizures due to an increased level of gamma aminobutyric acid and glutamate in the brains.

1.15 Fermented foods and the gut health

In order to restore gut health, the re-establishment of homeostatic relationships in the gut may be necessary (Vyas & Ranganathan, 2012). Vitamin/mineral supplementation, fecal microbiota transplantation (FMT) have been identified as techniques that may be employed for gut microbiota restoration (Sun & Chang, 2014). Habitual food intake amongst other factors is believed to provide the most significant and rapid changes the gut microbiota (Sun & Chang, 2014). As a result, the use of diet and dietary component to re-establish homeostatic relationships within the gut and to enhance gut health has reported (Rezac, Kok, Heermann & Hutkins, 2018; David et al., 2014). According to Dimidi, Cox, Rossi, & Whelan, (2019) and Kim, Jeong, Kim & Seo, (2019), studies have suggested that consumption of kefir has the potential re-establish homeostasis by encouraging the growth of beneficial organisms such as *Lactobacillus*, *Lactococcus* and *Bifidobacterium* in the gut. This has been supported by Yılmaz, Dolar, & Özpınar, (2019) in a randomised controlled trial (RCT), showing that daily intake of 800 mL/day kefir by patients with inflammatory bowel disease (IBD) for 4 weeks significantly increased the population of *Lactobacillus* in faecal samples compared with the control group, though with a small sample size. Nielsen et al., (2018) in another intervention, though also underpowered, reported that daily consumption of 75 grams of pasteurized sauerkraut (control group) or unpasteurized sauerkraut (experimental group) for 6 weeks led to significant positive changes in the gut microbiota of the participant after faecal

analysis nonetheless, the experimental group recorded a significantly higher population of faecal LAB compared to the control group. According to Dimidi et al., (2019), studies have also reported similar results in relation to the consumption of kimchi but not with the consumption of sourdough, though significant improvements in GIT and metabolic symptoms and biomarkers were reported.

1.16 Fermented foods and GIT diseases

There are indications that consumption of fermented foods may be useful in the prevention or management of GIT and metabolic diseases (Marco & Golomb, 2016; Wilburn & Ryan, 2016). Gille et al., (2018) in their review suggested that habitual yoghurt consumption may be associated with reduced risk of developing T2D. Fermented milk products, including kefir have been suggested to be beneficial to people who are lactose intolerant in spite of the fact that yoghurts and unconcentrated milks have relatively similar lactose content (Savaiano, 2014; Gille, Schmid, Walther & Vergères 2018; Dimidi et al., 2019). African women, believe consumption of some fermented cereal foods such as 'uji' and 'kanun-zaki' significantly improves production of milk by nursing mothers (Nyanzi & Jooste, 2012). These fermented cereal products have as a result been widely consumed by lactating African mothers over many generations (Nyanzi & Jooste, 2012). Maize meal, germinated or non-germinated sorghum grains, millet grains and sour milk make final fermented products that are traditionally used as weaning food in many African countries (Blandino et al., 2003; Nyanzi & Jooste, 2012; Simango, 1997). Uncooked fermented maize is also traditionally believed in Western Nigeria to be effective in the treatment of diarhoea (Adebolu, Olodun & Ihunweze, 2007). While koko, a sour drink made from supernatant solution produced during fermentation of pearl millet is traditionally used for the treatment of stomach pains and aches as well as a refreshing drink in Ghana (Lei & Jakobsen, 2004). However, the mechanisms through which these fermented foods display their functionality are continuously being investigated. Benefits to the GIT health, that may be derived from the consumption of fermented foods may be expressed through various mechanisms. One of such is the interaction of ingested live fermentation microorganisms with the host (Wilburn & Ryan, 2016). Such organisms are subsequently discussed in this chapter. The authors also postulate that these benefits may also be derived from intake the metabolites synthesized during the process of fermentation.

1.17 Health functions of fermentation organisms

The healthy functional properties of fermentation organisms are attributed to those classified as probiotic. Probiotics are "live microorganisms that, when administered in adequate amounts, proffer a health benefit on the host" (Hill et al., 2014). The minimum requirements needed for

fermentation organisms to obtain probiotic status as stated by Joint FAO/WHO Working Group (2007), Parvez, Malik, Ah Kang & Kim (2006) and Vandenplas, Huys, and Daube (2015) include:

- Assessment of strain identity (genus, species, strain level)
- *In vitro* tests to screen potential probiotics
- Safety assessment: requirements for proof that a probiotic strain is safe and without contamination in its delivery form
- *In vivo* studies for substantiation of the health effects in the target host

In-vitro tests to screen potential probiotics according to Joint FAO/WHO Working Group (2007), Kechagia et al. (2013), Parvez, Malik, Ah Kang & Kim (2006) and Begley, Hill, and Gahan (2006):

- Must remain viable throughout the shelf life of the vehicle of delivery and must be of a high cell count (minimum of 10^6 colony forming unit cfu/ml) in its vehicle at the time of administration.
- Resistance to gastric acidity
- Bile acid resistance
- Adherence to mucus and/or human epithelial cells and cell lines
- Antimicrobial activity against potentially pathogenic bacteria
- Ability to reduce pathogen adhesion to surfaces
- Bile salt hydrolase activity which has associated to improved microbial intestinal survival and persistence

Fermentation organisms that cannot survive the gastrointestinal tract (GIT) may be of health benefits to consumers (Ouwehand & Salminen, 1998; Salminen, Ouwehand, Benno, & Lee, 1999), even as dead cells (Gosálbez & Ramón, 2015). However, are not classed as probiotics even though they are acknowledged for their health benefits by the European Food Safety Authority, as it is with *Streptococcus salivarius* subsp *thermophilus* and *Lb delbrueckii* subsp *bulgaricus* (Sanders, 2008; Tetens, 2011). Nonetheless, Elli et al., (2006) reported recovering *Lb delbrueckii* subsp *bulgaricus* from the stool of 10 out of the 20 healthy people who consumed the 250g of a commercial yoghurt for 7 days. The authors therefore concluded that the possibility of isolating *Lb delbrueckii* subsp *bulgaricus* from fecal samples after ingestion was dependent not only on the strain of organism but also on the reliability of the analytic procedures adopted for the isolation and identification of the strain.

Some physiological benefits may require the organism to survive the GIT transition but not necessarily proliferate in the intestine. Organisms with these characteristics are also categorised as probiotic (Hill et al., 2014). Not all probiotic strains are ideal for use as a starter culture during food fermentation, but they may be suitable for co-culturing with starter cultures provided the sensory characteristics of the fermented food product are not compromised (Mohammadi et al., 2012). When they are used as starter culture features such as ability to ferment, viability, cost, sensory properties such as appearance and flavour, and genetic stability will be of significance (Hati et al., 2013). Probiotic organisms often involved in cereal fermentation are LAB – *Lactobacillus* spp., *Bifidobacterium* spp. and yeast – *S. cerevisiae* var *boulardii* (Parvez, Malik, Ah Kang, & Kim, 2006).

Probiotic activities range from supporting a healthy digestive tract to enhancing the immune function (Hill et al., 2014). Specific mechanisms by which probiotic organisms are able to produce these effects have not been clearly stated, nevertheless, general ways by which probiotics organisms induce health benefits to the host according to Power, O'Toole, Stanton, Ross, and Fitzgerald (2014) are:

- Production of antimicrobial compounds (e.g. bacteriocins);
- Reduction of luminal pH through the production of SCFA;
- Competition with pathogens for nutrients and prebiotics;
- Competitive exclusion of pathogens for adhesion to epithelial cells;
- Production of growth substrates (such as vitamins, SCFA and exopolysaccharide;
- Enhanced intestinal barrier function (such as increased mucus and β-defensin secretion);
- Modulation of specific and non-specific immune responses.

It is important to note that results from studies on the effect of probiotic organisms, including those isolated from fermented food product, on humans have been inconclusive which may be attributed to the fact that activities of probiotic strains cannot be generalised. Probiotic effects on the host are dependent on various factors which include organism strain, type and duration of infection, dosage and period of treatment (Cencic & Chingwaru, 2010). Other factors such as the vehicle for administration, host's diet and life style may also influence the effect of the probiotic strain (Derrien & van Hylckama Vlieg, 2015; Tachon, Lee, & Marco, 2014).

Since the increased awareness of possible health benefits of the microorganisms, there have been many published studies on the efficacy of wide range "probiotic" strains isolated from fermented

foods for the treatment, management and prevention of GIT diseases. Disease that have been investigated include Irritable Bowel Syndrome (IBS), (Didari, Mozaffari, Nikfar, & Abdollahi, 2015; Hoveyda et al., 2009), constipation (Dimidi, Christodoulides, Fragkos, Scott, & Whelan, 2014), Necrotizing Enterocolitis (NEC) (Yang et al., 2014), Ulcerative Colitis, Crohn's disease, Pouchitis (Mardini & Grigorian, 2014; Shen, Zuo, & Mao, 2014), Helicobacter pylori infection (Gong, Li, & Sun, 2015) and diarrhoea (Freedman et al., 2015; Szajewska & Kołodziej, 2015; Xie, Li, Wang, Li, & Chen, 2015). These studies provide evidence suggesting that probiotic strains, other than those that have originated from the gut, may be able to confer health benefits when consumed (Derrien & van Hylckama Vlieg, 2015; Ritchie & Romanuk, 2012).

Hill et al. (2014) in their report explained that the mechanism through which probiotics work can be grouped into three – widespread mechanisms, frequent specie level mechanisms and rare strain specific mechanisms (**Table 1.5**). Extensive research show that a broad taxonomy groups of probiotic organisms may be able to provide health benefits using the widespread mechanisms such as inhibiting the growth and adhesion of pathogenic organisms to the epithelial cells, production of antimicrobial metabolites and alteration of the gut ecosystem (Hill et al., 2014; Kumar et al., 2013; Reid et al., 2011; Laparra & Sanz, 2010). Hill et al. (2014) also stated that evidence to show mechanisms that have been grouped as strain specific are inconclusive.

Table 1.5: Probiotic effects and mechanisms

Rare: Strain specific effects	**Frequent: Species-level effects**	**Widespread: Among studied probiotics**
• Neurological • Immunological • Endocrinological • Production of specific bioactives	• Vitamin synthesis • Neutralization of carcinogens • Bile salt activity • Direct antagonism • Gut barrier reinforcement • Enzymatic activity	• Colonization resistance • Acid and Short Chain Fatty Acids (SCFAs) production • Regulation of intestinal transit • Normalization of perturbed microbiota • Increased turnover of enterocytes • Competitive exclusion of pathogens

Adapted from Hill et al., (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology, 11*(8), 506-514.

1.17.1 Probiotics and Diarrhoea

According to the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), diarrhoeal diseases are some of the most common diseases of the GIT with about two billion worldwide recorded cases every year (Farthing et al., 2013). Diarrhoea is a leading cause of death in children under 5, with about 2.2 million deaths occurring yearly, with 90% of the cases occurring in the developing countries (Farthing et al., 2013; World Health Organisation, 2014).

The WHO has defined diarrhoea as three or more loose or watery stools that take the shape of the container, in a 24-hour period (WHO, 1996). It results from the alteration of the movement of water and ions in the large intestine where the transport of water and electrolyte primarily takes place (Kiela & Ghishan, 2016; Hodges & Gill, 2010). Diarrhoea is often characterised by 2 conditions namely the transepithelial hypersecretion of fluid and defects in water absorption in the GIT (Pelagalli, Squillacioti, Mirabella & Meli, 2016). The main pathophysiologic mechanisms through which diarrhoea develops as reported by Sweetser (2012) include

Secretory mechanism: Defect in the electrolyte transport resulting in decreased water absorption
Osmotic mechanism: Malabsorption of substances giving rise to increased secretion of water from plasma into the intestinal lumen along the osmotic gradient
Altered motility: Compromised motility of the intestine or colon, which may be associated with a defect in the neuromuscular functions, may result in a defect in fluid absorption by increasing or decreasing the exposure of luminal content to intestinal absorptive surface
Inflammatory disease: May be as a result of mucosal invasion giving rise to exudative, secretory, or osmotic defects

These conditions may arise due to microbial enterotoxins, secretion of inflammatory mediators such as histamine, serotonin, prostaglandins, decreased expression or availability of transporters, which may be associated with incompetent functions of the epithelial barrier (Pelagalli et al., 2016). Diarrhoea may be also be induced by pathogenic infection or as a result of gut perturbation, following the use of antimicrobials as it is with the case of antibiotic associated diarrhoea (AAD) (Allen, Martinez, Gregorio, & Dans, 2010; Pimentel, 2003; Keeney et al., 2014).

Over 20 viral, bacterial and parasitic organisms have been associated with acute, less than 14 days, diarrhoea (WHO, 1996). Rotavirus has been described as the most common cause of severe (over 14 days) diarrhoea and diarrhoeal mortality in children under 5 years (Cunliffe 1998 as stated by Allen et al., 2010). Important diarrheagenic bacteria include*, Salmonella, Shigella, Yersinia,*

Campylobacter, and *Vibrio cholerae* with pathogenic *E. coli* being the common cause of travellers' diarrhoea (TD) (Allen et al., 2010). Oral rehydration salts (ORS) solution is the recommended routine treatment of acute diarrhoea (Guarino et al., 2008; WHO, 2005). However this is used only to prevent dehydration and to replace water and electrolytes that may be lost in the faeces (WHO, 2005) as against solidifying the stool, reducing stool loss or reducing the duration of the disease (Francavilla et al., 2012). The use of a zinc supplement was thereafter included as a guideline for the management of disease based on convincing evidence that administering 10 – 20 mg daily until cessation of diarrhoea may be associated to significant reduction in severity and duration of diarrhoea in children under 5 years (WHO, 2005). Nevertheless, accessibility to zinc supplements may be a challenge in rural environments where hospitals are remote. The efficacy of probiotic strains have been investigated for antibiotic associated diarrhoea (AAD), infectious diarrhoea (ID), TD and *Clostridium difficile* disease (CDD) (Ritchie & Romanuk, 2012).

Using foods containing probiotics may therefore be a more suitable adjunct for the treatment of this disease due to easy accessibility and affordability. Medical health providers in a few countries such as Vietnam administer probiotic solutions containing *Lb acidophilus* to children with acute diarrhoea as the initial treatment regimen (Kolader et al., 2013). Despite overwhelming evidence showing the efficacy of the use of probiotic strains for the treatment and management of diarrhoeal diseases (Farthing et al., 2013), the use of these organisms has not been included in the universal guidelines for the management of the disease. This has however been attributed to the fact that no standard has been put in place for dosage and method of preparation of these probiotics; coupled with the inconsistency of results obtained within various populations and with varied single strains (Dutta et al., 2011; Francavilla et al., 2012).

1.17.1.1 Probiotics and Acute diarrhoea

Francavilla et al. (2012), conducted a double-blind RCT to investigate the efficacy of *Lb reuteri* DSM 17938 for the treatment of diarrhoea caused by rotavirus and adenovirus in Southern Italy. Sixty-nine children between the ages of 6 and 36 months, hospitalised with acute diarrhoea with 2 or more consecutive watery stools during 24 hours (h) and duration of no more than 7 days, with clinical signs of mild to moderate dehydration completed the study. Children that were prescribed antibiotics, with bacterial infection and those that deviated from the research protocol were excluded from the study. Participants were randomly assigned to receive orally either *Lb reuteri* (4×10^8 cfu/day) or a placebo as an adjunct to ORS which replaced lost fluid. Overall results showed that *Lb reuteri* significantly reduced the duration of watery diarrhoea as compared with the placebo (2.1 ± 1.7 vs. 3.3 ± 2.1 days; $p < 0.03$). The effects of reduction in the frequency of diarrhoea and

normalised stool consistency were observed by the end of the first 24 hours but with significant effects in days 2 and 3 ($p = 0.03$ and 0.02 respectively), the authors however did not define normalised stool. The mechanism by which the probiotic provided its effect was not investigated but the authors proposed mechanisms such as the production of reuterin which is a broad-spectrum antimicrobial substance; competition for binding sites and substrates; enhancement of mucosal barrier and a decrease in intestinal permeability; and stimulation of intestinal immune responses. *Lb reuteri* may be able to induce colonization according to Connolly, Valeur, Engel, Carbajal, and Ladefoged (2003) as stated by Weizman, Asli, and Alsheikh (2005) since they are able to grow *in situ* on stomach and intestinal biopsies. The authors further mentioned that the organism may be able to induce immunomodulatory activities, including recruitment of T-helper cells in the small intestine. Excluding children with bacterial infection limits the efficacy of the effect of *Lb reuteri* DSM 17938 in this study to diarrheagenic viruses only which was the initial aim of the study. Studies have also shown that another strain of *Lb reuteri, Lb reuteri* DSM 17938, has the potential to significantly shorten the duration of rotavirus caused acute diarrhoea (Francavilla et al., 2012) but evidence suggests that the strain may be able to carry transferable resistance traits for tetracycline and lincomycin (Rosander, Connolly, & Roos, 2008). Antibiotic resistance traits in probiotics, with the possibility of being transferable, flags up safety concerns, especially with regard to potential food or gut pathogens (Gueimonde, Sánchez, Clara, & Margolles, 2013).

Results from a RCT by Aggarwal et al. (2014) investigating the efficacy of *Lb casei* GG (1×10^9 cfu/day) for the treatment of acute diarrhoea in children under 5 years in India indicated that irrespective of rotavirus negativity or positivity in stool tests, the duration of diarrhoea was significantly shorter in children in LGG group [60 (54-72) h vs. 78 (72-90) h; $p < 0.001$] and faster improvement in stool consistency in children receiving *Lactobacillus* GG than the control group [36 (30-36) h vs. 42 (36-48) h; $p < 0.001$]. There was also a significant reduction in average number of stools per day in LGG group ($p < 0.001$) compared to the control group. Stools were not tested for bacterial infection, even though stools from about 75% of the participants had negative rotavirus test results. There are other viral causes of diarrhoea including astrovirus, human caliciviruses (norovirus and sapovirus) and enteric adenoviruses (Allen et al., 2010), therefore results of this study cannot be extended to diarrhoea caused by bacteria or other parasitic organisms. All participants also received 20 mg of zinc supplements daily, which may be a confounding variable in the study.

On the other hand, a study by Basu, Chatterjee, Ganguly, and Chandra (2007) showed contrary results when similar variables were measured but using 10^6 cfu/day of *Lb rhamnosus* GG. However,

the authors repeated the study using higher dosages of the same strain, 10^{10} and 10^{12} cfu/day and recorded significant positive results in both dosages with insignificant differences in the results obtained from both dosages (Basu, Paul, Ganguly, Chatterjee, & Chandra, 2009). In both cases, zinc supplements were not administered to the participants but outcome can only be attributed to diarrhoea caused by rotavirus. Weizman et al. (2005), investigated the efficacy *Bifidobacterium lactis* BB-12 or *Lb reuteri* SD 2112 for the prevention of diarrhoea. Results showed that healthy term 4 to 10 months old non breastfed infants in the Beer-Sheva area of Israel fed with infant formula containing 10^7 cfu/g of *B. lactis* BB-12 n = 73 or *Lb reuteri* SD 2112 n = 68 for 12 weeks (mean daily ingested dose of 1.2 X 10^9 cfu/day), had significantly less febrile episodes, episodes and duration of diarrhoea compared to infants that were fed with the same infant formula but without the probiotics (60) ($p < 0.001$). Also, the *Lb reuteri* group, compared with BB-12 or controls, had significantly fewer days with fever ($p < 0.001$), less visits to the clinic ($p = 0.002$), less absences from the child care unit ($p = 0.015$) and fewer prescriptions of antibiotics ($p = 0.037$). Stools of diarrhoea cases tested positive for rotavirus for 34% to 41% and *Shigella*, *Salmonella*, and *Campylobacter* species for 9% to 11% of participants in all groups. The authors however did not investigate the causes of diarrhoea of those that tested negative to the mentioned organisms neither did they isolate results for bacterial infections.

A similar result was stated by Chen et al. (2010) when they investigated the effect of a product called Bio-three consisting of three probiotic organisms namely *Bacillus mesentericus, Enterococcus faecalis,* and *Clostridium butyricum*. The study indicated that administering $2.5*10^7$ cfu/kg/d of the probiotic product significantly reduced the duration of diarrhoea after the start of therapy was at by 26 h ($p = 0.003$) and length of hospital stay by over 24 h ($p = 0.009$) in the probiotic groups when compared to the placebo group. Participants n = 293, were children aged 3 months to 6 years that had been hospitalized with acute diarrhoea caused by rotavirus (31%), norovirus (15%), *Salmonella enterica* (15%), rota + noro (7%), *Campylobacter jejuni* (4%), adenovirus (2.5%) and no identified pathogen (25%). *S. boulardii* was also shown to significantly shorten the duration and normalisation of stool consistency and frequency of diarrhoea caused by *E. coli* (Htwe, Yee, Tin, & Vandenplas, 2008). A meta-analyses by Bernaola, Bada, Carreazo, and Rojas (2010) suggested that probiotic organisms may reduce duration of persistent ($\geq$ 14 days) diarrhoea in children (between ages 0 and 18 years) but the review had a limited number of studies and participants and may therefore have not given a reliable estimate of the efficacy of probiotics on the treatment of persistent diarrhoea in children.

1.17.1.2 Antibiotic-associated diarrhoea (AAD)

AAD varies among populations but it is believed that it affects between 5% and 39% of patients on antibiotics (Xie et al., 2015). The patient's age, type and spectrum of antibiotics are risk factors to the disease (Xie et al., 2015) with older patients that are over 65 years, at more risk to AAD due to weakened immune system (Xie et al., 2015). Broad-spectrum antibiotics such as clindamycin, β-lactams, 3rd generation cephalosporins and quinolones; and those with long term treatment tend to have greater effects than antibiotics with narrow-spectrum or short treatment regimen (McFarland, 2009; Xie et al., 2015). *Clostridium difficile* is the leading cause of nosocomial AAD worldwide, accounting for up to 39% of recorded cases (Awad, Johanesen, Carter, Rose, & Lyras, 2014; Xie et al., 2015), although it has also been reported among outpatients and residents of long-term care facilities (McFarland, 2009). Other pathogens associated with AAD include *Clostridium perfringens, Staphylococcus aureus, Candida* spp, *Klebsiella oxytoca*, and *Salmonella* spp (Barbut & Meynard, 2002). The routine treatment and management regimen of AAD depending on the severity include rehydration or discontinuation of the causative agent or its replacement with an antibiotic with a lesser risk of causing AAD (Barbut & Meynard, 2002). Since probiotics have been said to restore balance in the gut microbiome (Vyas & Ranganathan, 2012) studies have investigated the efficacy of probiotic *Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus,* and *Bacillus* spp, alone or in combination for the prevention, treatment and management of AAD and *C. difficile* diarrhoea (CDD), (Divya et al., 2012; Ritchie & Romanuk, 2012; Xie et al., 2015). Results have however been inconsistent which may be attributed to varied quality of methodology, age of participants, duration of intervention, type and dosage of probiotics. Allen et al. (2013), investigated the efficacy of an experimental capsule containing $6*10^{10}$ cfu/g of two strains of *Lb acidophilus, Bifidobacterium bifidum* CUL20, NCIMB 30153 and *Bifidobacterium lactis* CUL34, NCIMB 30172 against an identical placebo capsule in the prevention of AAD and CDD in older ($\geq$ 65 years) patients. A total of 2,941 participants, (1,470 experimental and 1,471 placebo) completed the study and dosage was one capsule/day for 21 days when possible, between antibiotic doses. Follow up continued until 8 weeks after recruitment. With no statistical differences in the characteristics of participants in both groups, study results indicated that the intervention failed to give the significant effect of AAD and CDD prevention compared to the placebo group. The authors however did not state which antibiotics were administered to the participants.

The results from the mentioned study is a contrast to other RCTs by Hickson et al. (2007), Selinger et al. (2013) and Kamhuber et al. (2009) which though had same study objectives, used smaller sample sizes and different experimental probiotic strains - 100 g of fermented milk containing *Lb*

casei DN-114 001 (*Lb casei imunitass*) ($1.0*10^8$ cfu/ml), *Streptococcus thermophilus* ($1.0*10^8$ cfu/ml) and *Lb bulgaricus* ($1.0*10^7$ cfu/ml); a sachet of VSL#3 containing $4.50*10^{10}$ cfu/g of *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lb acidophilus, Lb plantarum, Lb paracasei, Lb delbrueckii* subsp *bulgaricus, Streptococcus thermophiles*; and probiotic milk drink containing $6.5*10^9$ *Lb casei* SHIROTA respectively. The least number of participants among these studies was 113 and intervention covered the period of antibiotics and a minimum of three days with a follow up of between 20 and 28 days after the intervention. All studies focussed on participants on the elderly as the minimum mean age was 57 years but none of the studies mentioned stated which antibiotics were administered. Various meta-analyses show results that favour the efficacy of probiotics but with some degree of heterogeneity in the strain of probiotics, population of participants and quality of RCTs used (Xie et al., 2015). Nevertheless a large meta-analysis by Hempel et al. (2012) with 11,811 participants from 63 RCTs summarizes that pooled evidence suggests probiotics may be effective for the treatment of AAD, (relative risk (RR), 0.58; 95% CI, 0.50 to 0.68; $P < 0.001$; heterogeneity $I^2 = 54\%$; [risk difference, −0.07; 95% CI, −0.10 to −0.05] with most studies using *Lactobacillus* based interventions either alone or in combination with other genera.

In conclusion, among the organisms that have been investigated for their efficacy in the prevention, treatment and management of diarrhoea, the genera most associated with cereal fermentation which is the focus of this study is *Lb* with species including *Lb acidophilus, Lb plantarum/paraplantarum, Lb delbrueckii* subsp *bulgaricus, Lb casei, Lb reuteri, Lb fermentum, Lb brevis,* and *Lb rhamnosus* (Franz et al., 2014).

1.18 Prebiotics

Prebiotics are also components of food, including fermented foods which may influence the gut microbiota (Rezac et. al., 2018). The human gut microbiota is able to ferment a wide range of substrate that are derivatives of the diet or endogenous secretions (Bedani et al., 2015). These include different forms of dietary carbohydrates, proteins, fats, products from lysed cells, sloughed epithelium cells and mucin (Bedani et al., 2015). Dietary carbohydrates that have not been digested in the upper gastrointestinal tracts are nevertheless the major fermentable substrates for the gut microbiota (Gibson & Roberfroid, 1995). These types of carbohydrates are generally referred to as dietary fibre. Dietary fibre (DF) has been clearly defined by the American Association of Cereal Chemist (AACC) as “the remnants of edible part of plants and analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the human large intestine” (Camire et al., 2001). AACC further explains that DF

"includes polysaccharides, oligosaccharides, lignin and associated plant substances" (Camire et al., 2001). Consumption of DF encourages microbial growth, hence significantly altering the composition of the gut microbiota (Simpson & Campbell, 2015). Sudden increased intake of DF may yield non-beneficial results, dysbiotic conditions to the host (Binns, 2013; Zhang et al., 2015). In addition, a wide range of organisms in the gut, especially those belonging to the genera *Bacteroides, Ruminococcus, Eubacterium, Bifidobacterium* and *Lactobacillus* are able to metabolise non-digestible carbohydrates (Binns, 2013) and some organism species belonging to these genera such as *Bacteroides fragilis,* given the right conditions, have the potential to effect some harmful changes in the human gut (Gibson & Roberfroid, 1995; Zhang et al., 2015; Roberfroid et al., 2010). Therefore DF would be prebiotic if it selectively supported that growth of only the beneficial organisms in the gut (Bindels, Delzenne, Cani, & Walter, 2015; Gibson et al., 2010; Roberfroid et al., 2010; Slavin, 2013). The concept of prebiotics was first introduced by Gibson and Roberfroid (1995) and initially defined by authors as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activities of one or a limited number of bacteria in the colon, and this improving host health". The definition was most recently revised by ISAPP (2008) as stated by Roberfroid et al. (2010) to "a dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health". The conditions by which as colonic food can be described as being a prebiotic according to Gibson et al. (2010) include:

- It must be neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract
- It must be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated
- It must be able to alter the colonic flora in favour of a healthier composition

Following the inception of the concept of prebiotics, studies were able to establish that only inulin-type fructans (ITF) e.g. fructooligosaccharides, inulin and oligofructose; galactooligosaccharides and lactulose as the non-digestible carbohydrates that met the stated requirements to be called prebiotics (Binns, 2013; Gibson et al., 2010; Roberfroid et al., 2010). This has been associated with the fact that *Lactobacillus* and *Bifidobacterium* were the target organisms for test of prebiotic potentials (Gibson et al., 2010; Hutkins et al., 2016; Roberfroid et al., 2010). EPS formed during food fermentation have also been noted for their prebiotic properties (Ripari 2019; Zhou, Cui & Qu, 2018).

The main mechanism by which prebiotics exhibit health advantages to the host is through the production of metabolites such as lactate and short chain fatty acids (SCFAs) - acetate, butyrate and propionate (Slavin, 2013). SCFAs apart from being energy sources in the colon, also possess antimicrobial and pH lowering functions (Bindels et al., 2015; Roberfroid et al., 2010; Slavin, 2013). Bindels et al. (2015), mentioned that the target organisms of interest, *Bifidobacterium* and *Lactobacillus* mainly produce butyrate and propionate from established prebiotics, but are also able to produce all butyrate, propionate as well as acetate from other dietary fibre such as resistant starch. The authors however pointed out that no DF is able to selectively grow one or two bacterial organisms, neither is there any DF that can be fermented by all the organisms in the human gut. Other non-digestible carbohydrates such as resistant starches, arabinoxylans and resistant dextrins are hence being investigated as candidates for prebiotics (Binns, 2013; Krumbeck, Maldonado-Gomez, Ramer-Tait, & Hutkins, 2016; Roberfroid et al., 2010). In addition, suggestions have been made for a review of the definition of the term 'prebiotics' and the 'selectivity' restriction in order to accommodate recent findings in prebiotic and probiotic research (Bindels et al., 2015; Hutkins et al., 2016; Krumbeck et al., 2016; Rastall & Gibson, 2015). Rastall and Gibson (2015), suggested that even though other non-digestible substance, aside ITFs, galactooligosaccharides and lactulose, may have prebiotics potentials, the importance of selective growth or metabolic activities of beneficial organisms only, for positive health impact should not be overlooked. Therefore, prebiotics should be defined based on the overall metabolic and physiological benefits exerted on the host and not about characteristic selectivity (Bindels et al., 2015; Hutkins et al., 2016).

1.19 The complex matrix of fermented foods

Outcomes of studies about the nutritional benefits of fermented foods have been consistent (Hotz & Gibson, 2007). Fermentation has been reported to increase the quantities of some food nutrients (Hotz & Gibson, 2007). For instance, fermentation of milk has been reported contain more B and K vitamins, compared to unfermented milk (Hugenholtz, 2013) and fermented fruits, vegetables and tea (kombucha) have increased level of anti-oxidants, polyphenols, folate and other B vitamins when compared with non-fermented ones (Dufresne & Farnworth, 2000; Hugenholtz, 2013). On the other hand, in the attempt to identify and specify mechanisms through which fermented foods may be functional, outcomes of studies that have investigated the efficacy of specific components of fermented food products on specific diseases or symptoms or biomarkers of GIT diseases (Melini, Melini, Luziatelli, Ficca & Ruzzi, 2019; Rezac et al., 2018) have been inconsistent. (Dimidi, Cox, Rossi, & Whelan, 2019; Marco & Golomb, 2016). Nevertheless, most studies have focussed on the impact of the fermentation organisms (Marco & Golomb, 2016). Many of such studies have undertaken *in vitro* investigation of the probiotic potential of organisms isolated from

fermented foods (Franz et al., 2014; Nyanzi & Jooste, 2012; Sanni et al., 2013; Todorov & Holzapfel, 2014). However, the Joint FAO/WHO Working Group (2007) state that although results from *in vitro* tests may be indicative, they are not enough to describe a strain as probiotic. The group encourages carrying out human trials for result authentication. *In vivo* studies have suggested the fermented foods may provide some forms of health benefits to the consumers, but results have been inconsistent probably owing to varied research methodologies or due to the specificity of the strain used in relation to the symptom being investigated (Aoki et al., 2014; Giovannini et al., 2007; Mo, Zhu, & Nout, 2012; Wenus et al., 2008). As noted by Nielsen et al., (2018), sterile fermented food products may have positive impact on health, but there is minimal information on the possible effects of the end products of the fermentation (Cencic & Chingwaru, 2010; Chelule et al., 2010; Divya et al., 2012; Tripathi & Giri, 2014). Fermentation organisms, aside from having probiotic potentials, may able to produce exopolysaccharides during the metabolic process (Donot, Fontana, Baccou, & Schorr-Galindo, 2012; Onilude, Sanni, Ogunbanwo, Fadahunsi, & Afolabi, 2002). Exopolysaccharides produced during fermentation may be advantageous to the consumer of the fermented food product due to their possible antitumour, antioxidant or prebiotic functions (Liu et al., 2010). Detailed evaluation and efficacy of contents of fermented food products may require the targeted identification of certain bioactive compounds such as phytochemicals and phenol compounds which may not be present in unfermented foods (Wilburn & Ryan, 2016). Nonetheless, the type and quantity of metabolites and fermentation organisms in a fermented food product are multifactorial as previously discussed. It is important to note that *in vivo* investigation of the effectiveness of bioactive compounds, may not yield consistent and valid results due to the possibility of damage by the harsh environment in the GIT, causing such compounds to be less bioactive or totally inactive (Wilburn & Ryan, 2016). Few clinical trials have investigated the effect of the complex food matrix on the chosen symptom or biomarker (Rezac et. al., 2018). As previously noted, fermented food products may be able to re-establish homeostasis to the gut microbiome and due to the previously noted link between the gut microbiome and health, may by extension enhance the GI and metabolic functions in the host. Nevertheless, limiting the investigation of plausible mechanisms to a specific component of the fermented food product, may result in the losing the plausible observation of collaborative and connected mechanisms that may yield the health benefit, as exemplified by Bove, Russo, Capozzi, Gallone, Spano & Fiocco (2013 who reported the complex food matrix may contribute to the probiotic actions of some fermentation organisms.

1.20 Research gap & justification of study

According to Wilburn & Ryan (2018) questions such as safety of fermentation under different conditions, amounts required to confer health benefits and the degree of accessibility of the beneficial component to the body are yet to be answered. Where studies have suggested the functionality of fermented foods, authors unable to provide information about what component of the fermented food matrix may have provided the effects (Hill et al., 2014). Understanding the possible process through which fermented foods modulate or manifest their health benefits to the host might help to identify the components of the food matrices that may singly or collaboratively provide the therapeutic or preventive health effect. This may nevertheless involve investigating the activities and potentials of multiple components of the fermented food product. There are *in vitro* and *in vivo* studies have investigated the activities of fermented dairy products (Mäkeläinen et al., 2009; Rezac et al., 2018; Savaiano, 2014; Gille et al., 2018) but there is dearth of studies that have investigated the activities of multiple components of fermented cereal that may have beneficial effects on the GIT upon ingestion with the potential of obtaining the 'functional food' label. Therefore, the thesis aims to explore possible mechanisms through which components of fermented corn may be able to enhance the health of its consumer.

1.21 Simulated digestion models

The gold standard for investigating the human digestive process is an *in vivo* study design involving human participants (Minekus et al., 2014; Wickham, Faulks, & Mills, 2009). This method is however expensive, requiring extensive time and labour intensive (Minekus et al., 2014). It involves the assessment of samples of digestion output from all sections of the GIT which can induce some stringent ethical restrictions and technical difficulties (Wickham et al., 2009). The use of simulated human digestion techniques (*in vitro*) on the other hand are cheaper, quicker and with limited ethical or technical restrictions (Minekus et al., 2014). These techniques aim at mimicking the processes and conditions of the human GIT (Wickham et al., 2009). They include, just like the human GIT, the mouth, stomach, small intestine and sometimes the colon phases of the digestion process, taking factors such as the presence of enzymes, pH, salt and digestion time into consideration (Minekus et al., 2014). Results from the use of simulated digestion are nonetheless less accurate than *in vivo* methods due to limitations such as inability to incorporate physiological aspects (such as age and gender and health status) psychological and the psychophysiological complex of food and drink ingestion (Hur, Lim, Decker, & McClements, 2011; Minekus et al., 2014). For example, the type and amount of enzymes produced depend on the type and amount of nutrient source has been consumed, a complex which a simulated digestion may not be able to mirror (Boisen & Eggum, 1991). There have been a few studies to compare the correlation of *in*

vivo and *in vitro* digestion but they have majorly investigated digestion of drugs (Dahan & Hoffman, 2007; Reymond & Sucker, 1988). There is therefore inadequate information about the correlation or lack of outcomes of digestion of food substances using simulated digestive systems and *in vivo* human studies (Hur et al., 2011).

The static model, also known as the biochemical model, is most commonly used during *in vitro* digestion (Minekus et al., 2014; Wickham et al., 2009). Studies have used diverse techniques of the static model and have produced considerably varied number of steps, type and volume of enzymes, digestion times, food flow and mechanical stresses (Hur et al., 2011) which has given rise to a standard protocol being developed by a group of scientists in 2014 (Minekus et al., 2014). According to the authors, this standard protocol was designed following the consolidation of all protocols for simulated digestion to arrive at simple reproducible steps that create "a set of conditions that are close to human physiological situations…" with the exception of the elderly and infant. Minekus et al. (2014), however mentioned that, like every other static model, that standardised protocol, may not be an exact mirror of human digestion as it involves some degree of control and there are no provisions for adjustment for factors such as food texture, bolus size and "other complex interactions between the food and the individual". The standard protocol in addition does not include the colon phase of digestion which may be a disadvantage if effect of digested food on the gut microbiota is being investigated. The dynamic model, is the other model used during *in vitro* digestion. This multistage continuous model is described to be a more holistic and realistic representation of the human GIT and compared to the static model as it is able to adjust for factors such as food texture and peristaltic movement and digestion times (Wickham et al., 2009). Static models are ideal, cheap and practicable methods for multiple experiments whilst dynamic models are expensive and time consuming unable to perform multiple experiments at the same time (Alminger et al., 2014). The research question will nevertheless inform what model is more appropriate.

1.22 Aim of study

The aim of this study was to investigate the potentials for 'ogi' to be a functional food in the management of gastrointestinal diseases by investigating the mechanisms through which it may be able to confer the health benefits when consumed. In order to achieve this, the following will be investigated

- Probiotic potential of 'ogi' during the spontaneous and controlled methods of fermentation
- Production of SCFAs by fermentation organisms during spontaneous and controlled fermentation

- Potential of ‘ogi’ to modulate or influence the immune response

Prior to carrying out experimental studies to investigate the functions mentioned above, it was important to explore and review the analysis of the effectiveness of fermented foods, especially fermented cereals in enhancing gut health. Therefore, an independent chapter which contained a meta-analysis aimed to investigate whether fermented foods may significantly reduce the duration of diarrhoea, was undertaken.

1.23 Hypotheses of study

- ‘Ogi’ will have probiotic potential
- Fermentation organisms in ‘Ogi’ will produce SCFAs during spontaneous and controlled fermentation
- ‘Ogi’ will have the potential to modulate or influence the immune response in the colon

Chapter 2

The efficacy of fermented foods in the treatment of diarrhoea – Meta Analysis

2.1 Introduction

Fermentation, an ancient food processing technique, is a biochemical process which involves conversion of complex organic substrates carbohydrates into smaller substances such as organic acids, by enzymes produced by living cells for energy (Marshall & Mejia, 2011). This ancient food processing technique is however still being used within households and industries as preservation method, prolonging shelf life of foods and beverages (Franz et al., 2014; Mokoena, Mutanda, & Olaniran, 2016; Ray, Ghosh, Singh, & Mondal, 2016) and to enhance sensory properties of food products (Franz et al., 2014; Karovicova, 2007). For instance, proteomic analysis of 'kefir', a fermented milk product that originated from Caucasus Mountains and is popularly consumed in Europe, suggests that the beverage came into existence over three thousand years ago (Yang et al., 2014). Aside from dairy and cereal substrates, meat, fish, fruits and vegetables are also processed using fermentation techniques (Mokoena et al., 2016; Rivera-Espinoza & Gallardo-Navarro, 2010). End products of these fermentable substrate vary between regions. Over the years, food fermentation has contributed to the economic development of household and countries (Marshall & Mejia, 2011). Though fermented foods are sources of nutrients to the consumer (Hugenholtz, 2013), there are also suggestions that they also have the potential to provide therapeutic effects particularly in the management or prevention of some gastrointestinal diseases (Marsh, Hill, Ross, & Cotter, 2014). The increased awareness of these potential health benefits has resulted in higher consumption of fermented foods and beverages over the last few decades (Corbo, Bevilacqua, Petruzzi, Casanova, & Sinigaglia, 2014; Marsh et al., 2014; Ray et al., 2016). Though not clearly understood, there are various speculations of possible ways fermented foods and beverages may be able to proffer health benefits to the consumer. Components of the fermented product matrix, including fermentation organisms, fermentation output such as organic compounds and exopolysaccharides produced during the process could collectively or individually produce varied effects on the consumer of the fermented food product (Hill et al., 2014).

2.1.1 Fermentation organisms

Lactic acid bacteria (LAB) and yeast strains are the organisms most commonly used in the food and beverage fermentation (Ali, 2010; Chelule, Mokoena, & Gqaleni, 2010; Tamang, Watanabe, & Holzapfel, 2016), particularly dairy and cereal fermentation (Nyanzi & Jooste, 2012; Widyastuti & Febrisiantosa, 2014).

LAB are generally regarded as safe and have not yet been reported to be harmful when consumed in foods or beverages (Song, Ibrahim, & Hayek, 2012). The LAB genera that are popular in food fermentation include *Lactobacillus, Leuconostoc, Lactococcus* and *Streptococcus* (Das & Goyal, 2012). Some species of these organisms may be able to confer health benefits through mechanisms such as enhancement of gut immune response, competition with pathogenic organisms for GIT or production of antibacterial substances (Beausoleil et al., 2007; Song et al., 2012; Hill et al., 2014). Such species may be described as probiotic if they are able to survive the harsh environment of the gastrointestinal tract (GIT) in order to reach the colon in substantial quantity, where they may be able to enhance gut function (Donot, Fontana, Baccou, & Schorr-Galindo, 2012; Franz et al., 2014; Liu et al., 2010). There are however species of fermentation organisms which may not be probiotic as they are unable to survive in the GIT, but may still be able to provide health benefits even as dead cells by stimulating the production of helper T cells in the large intestine (Gosálbez & Ramón, 2015; Park & Jeong, 2015; Vinderola et al., 2005) as stated by Adams (2010), who reported that dead LAB cells may be able to enhance immune response in the gastrointestinal tract in an in-vivo study using rats subjects.

2.1.2 Organic acids in fermented food

Lactobacilli that are able to reach the human gut undergo fermentation of non-digestible carbohydrate releasing organic acids as by-products, hence lowering pH and exhibiting antimicrobial effects against non-acidophilic pathogens in the gut (Chifiriuc, Cioaca, & Lazar, 2011; Rivera-Espinoza & Gallardo-Navarro, 2010). Lactic acid is an important organic acid produced in the gut by Lactobacilli along with other LAB present (Morrison & Preston, 2016). Lactate could be further metabolised by other organisms into butyrate (Morrison & Preston, 2016; Rautiola, 2013), a short chain fatty acid (SCFA) which has been reported to give anti-inflammatory and anti-carcinogenic effects in the human gut, whilst also boosting the gut immune function (Nicholson et al., 2012). Increased gut population of Lactobacilli may therefore result in the increased production of butyrate and consequentially, an enhanced gut immune function.

2.1.3 Exopolysaccharides in fermented foods

LAB during fermentation are able to produce extracellular polysaccharides, commonly known as exopolysaccharides (EPS) (Leroy & De Vuyst, 2004; Ruas-Madiedo et al., 2007; Tieking, Korakli, Ehrmann, Gänzle, & Vogel, 2003). EPS is a compound composed of sugars and sugar derivatives (Sanlibaba & Çakmak, 2016), formed by the bacterial cells from simple or complex carbon substrates (Nwodo, Green, & Okoh, 2012). EPS are formed by bacterial cells as a coping mechanism in harsh environment, enhancing colonization of habitat by the EPS producing

organism (Salazar, Gueimonde, de los Reyes-Gavilán, & Ruas-Madiedo, 2016). EPS produced influences the rheological and textural properties of fermented foods as seen in the viscosity of fermented milk. EPS produced by Lactobacilli organisms which include α-D-glucans, inulin-type fructans, oligosaccharides, (Sanlibaba & Çakmak, 2016), have been reported to act as prebiotics in the human gut (Das, Baruah, & Goyal, 2014; Hutkins et al., 2016; Roberfroid, 2007; Whelan, 2013), selectively stimulating the growth of beneficial organisms in the colon (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004; Gibson & Roberfroid, 1995). Furthermore, enhanced production of SCFAs in the human gut has also been associated with EPS with the explanation that it serves as substrate to organisms, such as LAB and Bifidobacteria, which are involved in the production of SCFAs in the gut (O'Callaghan & van Sinderen, 2016).

2.1.4 Research gap

Randomised controlled trials (RCTs) and other clinical studies have investigated the effect of fermented foods on diarrhoea amongst other gastrointestinal diseases or symptoms. Studies have mostly concentrated on the effect of organisms used as started culture or added on to the fermented product, testing for probiotic effects of these organisms and using heated treated matching fermented product as the comparator (de Vrese, Kristen, Rautenberg, Laue, & Schrezenmeir, 2011; Merenstein, Foster, & D'Amico, 2009; Pitkälä, Strandberg, Finne-Soveri, & Ouwehand, 2007). Nevertheless, LAB are able to produce heat-stable bacteriocins as well as heat-labile antimicrobial proteins which also have antimicrobial characteristics (Grosu-Tudor, Stancu, Pelinescu, & Zamfir, 2014) and may be able be effective in the management of diarrhoea. There are however some other studies that have compared the efficacy of fermented food products vs non fermented similar product but findings have not been consistent (Beausoleil et al., 2007; Lei, Friis, & Michaelsen, 2006), which could have resulted from variations in sample size, the fermented substrate or method of food fermentation employed. As far as we know, the few systematic reviews and meta analyses that have been done have investigated the efficacy of fermented foods in the prevention and treatment of diarrhoea jointly, using dairy products as the intervention (Agostoni et al., 2007; Patro-Golab, Shamir, & Szajewska, 2015; Patro-Gołąb, Shamir, & Szajewska, 2015; Szajewska, Skórka, & Pieścik-Lech, 2015). This study therefore aims to systematically review and analyse where feasible, evidence available on the efficacy fermented products, including dairy and cereal products, focussing on the treatment of diarrhoea.

2.2 Methods

2.2.1 Inclusion and exclusion criteria

Randomised control trials (RCTs), clinical trials, controlled clinical trials or quasi-randomized trials were considered eligible for inclusion in this analysis. There was no age restriction for subjects. Studies must have included human subjects with acute/chronic diarrhoea and must have had duration of diarrhoea as an outcome measure. Participants on antibiotics were excluded. Intervention had to be consumption of any fermented food product only at any dosage scheme and duration of delivery with control being placebo, consumption of unfermented or ultra-heat treated or pasteurized fermented food product similar to the intervention or no intervention. Studies with no control, unspecified control and non-heat treated fermented controls were excluded. The outcome measure for this review was duration of diarrhoea.

2.2.2 Electronic Search

Preliminary search was done to ensure that there was no duplication of this review. Electronic search of COCHRANE CENTRAL (http://www.cochranelibrary.com/about/central-landing-page.html), OVID MEDLINE (https://www.ovid.com/) and PUBMED (https://www.ncbi.nlm.nih.gov/pubmed/) databases was done in May 2017 and March 2020. There was no language restriction and publication date was left open to avoid limited number of search output. Search terms were "Fermented foods AND diarrhoea"; "Fermented foods AND gastrointestinal diseases", "Fermented foods AND ferment$ food$.mp AND Diarrhea, Infantile/ or Diarrhea/ or diarrhea.mp". Relevant review articles from reference list of chosen studies were also searched.

Articles that were irrelevant following an initial screening based on title, abstract and keywords were excluded. Full text of potentially relevant studies were then obtained for further screening. Consensus was reached by 2 reviewers on the final chosen articles. Risk of bias was assessed using the Cochrane Collaboration tool which includes the following criteria: adequacy of random sequence generation; allocation concealment; blinding of participants; personnel, and outcome assessors; incomplete outcome data and selective reporting. In all cases, an answer of "+", "-" and "?" indicated low risk, high risk and unclear risk respectively. There was no formal assessment of publication bias using funnel plot due to small number (< 10) of selected articles (Sterne et al., 2011).

Information extracted from each article include: author; year of publication; baseline characteristics of the participants (age, setting, country of origin) sample size; type of intervention and comparator;

dosage of intervention; duration of the intervention; definition of diarrhoea; outcome measures and results. Where feasible, meta-analysis was undertaken and the data were entered into the Review Manager (RevMan) (Computer program, Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) for analysis. Mean difference between the experimental and control groups was selected to be the effect size of the continuous outcome with 95% confidence intervals. Duration of diarrhoea was the only common outcome variable in all selected articles, hence was the only outcome variable analysed. Where there were more than 1 intervention group, similar experimental groups were combined to create a single pair-wise comparison (Higgins, 2011). A conversion of result of outcome measure, shown as median and range to mean and standard deviation was achieved as recommended by Hozo, Djulbegovic, and Hozo (2005), in order to pool data together. It is however important to note that conversions such as this could result in overestimation of mean and standard deviation (Patro-Gołąb et al., 2015), therefore, interpretation of pooled results of this meta-analysis should be with caution.

2.3 Results

Six RCTs were identified to have met the inclusion criteria (**Figure 2.1**). Characteristics of these articles and assessment of risk of bias can be found in **Tables 2.1** and **2.2** respectively. Data from 6 (Agarwal & Bhasin, 2002; Bhatnagar, Singh, Sazawal, Saxena, & Bhan, 1998; Boudraa et al., 2001; Isolauri, Rautanen, Juntunen, Sillanaukee, & Koivula, 1991; Pedone, Bernabeu, Postaire, Bouley, & Reinert, 1999; Pashapour & Iou, 2006) out of the 7 articles having a total of 612 participants were entered into RevMan for meta-analysis. Agarwal and Bhasin (2002) undertook their study in 2 settings, the community and the hospital. Data from these settings were separately entered into RevMan. Though the 8th article (Yartey, Nkrumah, Hori, Harrison, & Armar, 1995) met the inclusion criteria, the study investigated if there was any significant difference in the outcome of managing diarrhoea using fermented maize and ORS which is the WHO guideline for the management of diarrhoea which was quite contrary to what this review aimed to achieve.

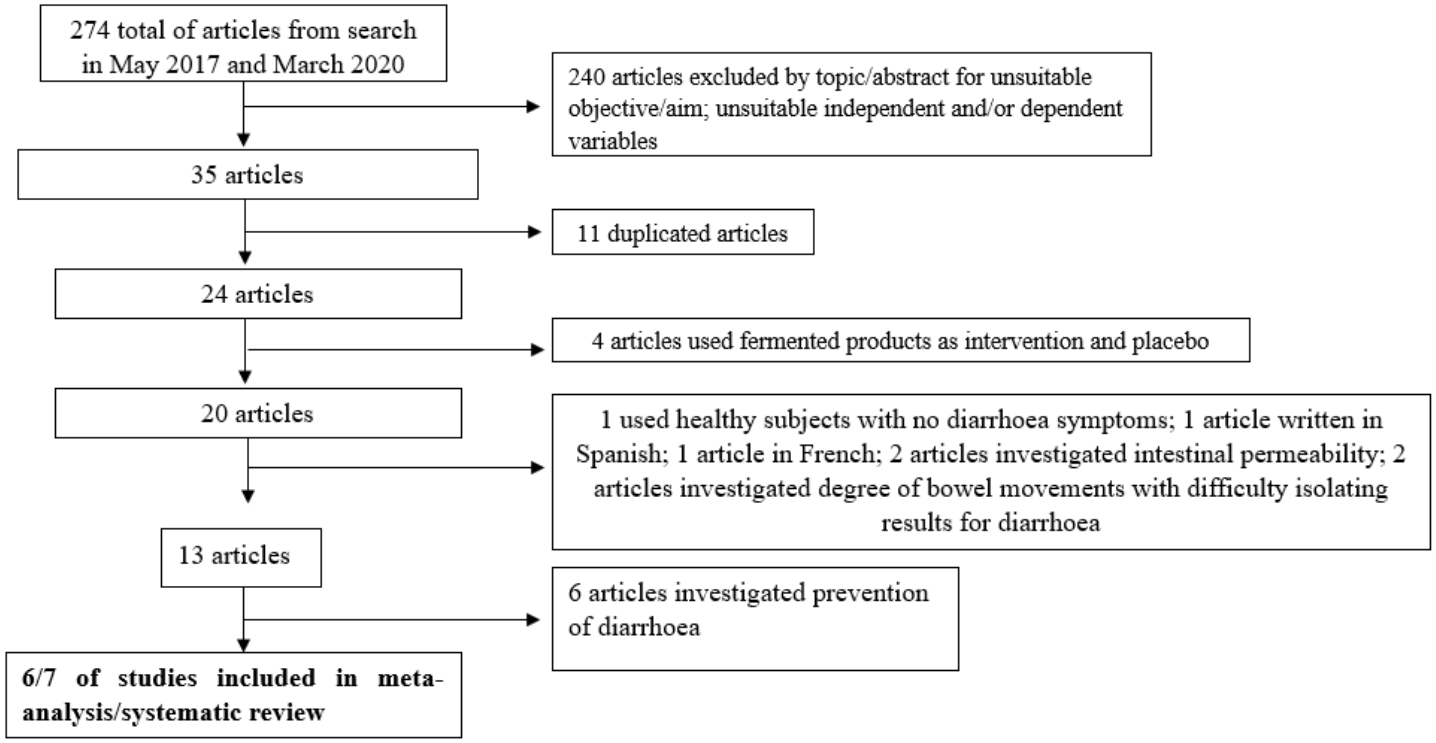


Figure 2.1 shows the PRISMA flow chart for literature search.

Table 2.1: Characteristics of included articles

Title, Authors, Country	Population	Blinding	Intervention	Comparator	Duration of intervention	Definition of Diarrhoea	Results
Effect of feeding yogurt versus milk in children with acute diarrhoea and carbohydrate malabsorption (2001) Boudraa G, Benbouabdellah M, Hachelaf W, Boisset M, Desjeux JF, Touhami M. Oran, Algeria 9	112 well-nourished children, aged 3 to 24 months, admitted to Cabral Clinic, Oran with acute watery diarrhoea of less than 5 days	Double blind	Y group Infant formula fermented with *Lactobacillus bulgaricus* (10^8) and *Streptococcus thermophilus* (10^8). N = 56 Attrition = 9 ITT	M group Infant formula N = 56 Attrition = 10 ITT	180 ml / kg*day was offered to each participant. Actual intake was 71 $\pm$ 39 ml /kg*day for breastfed children and 113 $\pm$ 65 for non-breastfed children. Length of intervention not stated (180 hr?)	More than 3 watery stools during the previous 24 hours	• No case of bacterial diarrhoea. Rotavirus found in 54% of subjects. • Duration of diarrhoea was significantly reduced in group Y (44.1 hr; 95% CI 36 – 54) compared with group M (61.7 hr; 95% CI 52 - 71), $p < 0.05$ • Number of stools per day significantly reduced in group Y (12; 95% CI 9 - 15) compared with group M (17; 95% CI 14 – 20), $p < 0.05$. • Only in patients with reducing sugars (RS) did yogurt significantly reduce the duration of diarrhoea and number of stools per day. • Diarrhoea duration in patients in with RS was reduced in group Y (34; 95% CI 23 – 44) compared to group M (77; 95% CI 66 – 88) $p < 0.01$) • Number of diarrhoeal stools duration in patients in with RS was reduced in group Y (12; 95% CI 6 - 18) compared to group M (22; 95% CI 16 - 27) $p < 0.01$)
The effect of supplementation with milk fermented by Lactobacillus casei (strain DN-114 001) on acute diarrhoea in children attending day care centres (1999) Pedone CA,	287 (265 analysed; no ITT) children aged between 6 to 36 months, in whom dairy product had been introduced for more than a month before the beginning of the study, attending 1 of 12 selected day care 5 days a week and having both lunch and a 4 pm snack	Double blind	a) Standard yoghurt (Y) containing 10^7 cfu/ml of *L. bulgaricus* and *S. thermophilus* N = 92 b) milk fermented with *L. bulgaricus* and *S. thermophilus* with the addition of	Jellied milk (JM) free of LAB N = 87	125 g / day for children between 6 and 18 months for 6 months 250 g/day for children over 18 months for 6 months	Diarrhoea defined as 3 or more loose or watery stools per 24 hours. Acute diarrhoea defined as an episode that lasts	• Incidence of diarrhoea not s.d. between groups (YC 23.3%, Y 28.3% and JM 26.4% $p = 0.745$) • YC had significantly lower severity (duration) of diarrhoea attacks over the 6 months of intervention 4.3 (2.7) days compared with Y - 5.3 (2.5) days and JM – 8.0 (5.3) days, $p = 0.009$ • YC and Y gave a significantly shorter duration of 'one episode of diarrhoea' (3.9 [2.4] and 4.8 [2.3]

Bernabeu AO, Postaire ER, Bouley CF, Reinert P Val de Marne in Paris 10			*L. casei* ($3.2*10^3$ cfu/ml) (YC) N = 86			less than 2 weeks An episode of diarrhoea is the total period of stool perturbation, including the acute phase and the return to normal	days respectively) when compared with JM (6.9 [3.0] days) p = 0.002 • Time required for stools to return to a normal consistency after acute diarrhoea was 1.2 (1.0) days in YC group, 2.0 (1.6) days in Y group and 2.6 (2.4) days in JM group, p = 0.055
Clinical trial of fermented maize-based oral rehydration solution in the management of acute diarrhoea in children (1995) Yartey, J. Nkrumah, F. Hori, H. Harrison, K. Armar, D. Accra 12	108 children aged between 4 and 27 months presenting with acute diarrhoea of no more than 7 days duration, with mild to moderately severe dehydration	Not stated	Fermented maize N = 36 Unfermented maize N = 35	WHO/UNICEF ORS N = 37	Unlimited quantities aiming to achieve full hydration within 24 hours Fluid intake was similar between fermented maize and WHO/UNICEF ORS groups but significantly lower in unfermented maize group (p < 0.01)	Diarrhoea not defined by authors	• Mean duration of diarrhoea after admission of 17.8 (16.8 – 18.8), 20.6 (19.7 – 21.5) and 21.1 (20.0 – 21.1) for fermented maize, unfermented maize and WHO/UNICEF ORS groups respectively, were not s.d. • Mean number of stools within 24 hours of admission of 4.2(2.3), 4.3 (2.7) and 4.0 (2.2) for fermented maize, unfermented maize and WHO/UNICEF ORS groups respectively, were not s.d. • Mean no of children vomiting during rehydration of 6 (17.1%), 4 (11.4%) and 4 (10.8%) for fermented maize, unfermented maize and WHO/UNICEF ORS groups respectively, were not s.d. •
Efficacy of milk versus yogurt offered as part of a mixed diet in acute noncholera diarrhea among malnourished children Bhatnagar, S.	Malnourished boys aged 4 to 48 months with diarrhea for 96 hours or less and passage of five or more liquid stools in the preceding 24 hours were enrolled	Double	120 ml/kg/24 hr of yoghurt made from a combination of *Streptococcus thermophilus* and *Lactobacillus Bulgaricus* N = 47	120 ml/kg/24 hr of milk formula N = 49	Minimum of 3 days or till recovery whichever was later.	five or more liquid stools in 24 hours	• The median (range) duration of diarrhea (hours) was 45 [4, 183] for MF and 52 [7, 173] for YF, (p = 0.94), n.s.

Kiran DS., Sunil S., Shailendra KS., & Maharaj KB., (1998) India							
Feasibility studies to control acute diarrhoea in children by feeding fermented milk preparations Actimel and Indian Dahi (2002) Agarwal, KN And Bhasin, SK Delhi	75 children (6 months to 5 y) with diarrhoea were admitted for the study to the University College of Medical Sciences and Guru Teg Bahadur Hospital. Simultaneously, the study was also carried out on 75 children of the same age with diarrhoea in a slum area named (community study) in one of its dispensaries run by the State Government of Delhi.	Double	Group 1: Fermented milk containing 10^8 *Lactobacillus casei* DN-114001 per gram (Actimel by Danone, France). Community n= 25 Hospital n = 23 Group 2: Dahi, a type of yoghurt used in India containing 10^8 *Lactococcus lactis, Lactococcus lactis cremoris* and *Leuconostac mesenteroids cremoris* per gram Community n= 25 Hospital n = 27	Ultra-heat-treated fermented milk (no bacteria) Community n= 25 Hospital n = 25	100 ml, 2 times daily for children between 6 and 12 months and 3 times for other children	three loose stools per day without blood/ mucus	• Mean duration of diarrhoea (days) were 1.5 (SD = 0.48) and 1.9 (SD = 0.8) for Group 1 (Actimel) at the hospital and the community respectively • Mean duration of diarrhoea (days) were 1.8 (SD = 0.61) and 2.2 (SD = 1.0) for Group 2 (Dahi) at the hospital and the community respectively • Mean duration of diarrhoea (days) were 2.1 (SD = 0.72) and 2.4 (SD = 0.9) for Group 3 (control) at the hospital and the community respectively • P value were < 0.01 and < 0.05 between groups at the hospital and community respectively
A human Lactobacillus strain (Lactobacillus casei sp strain GG) promotes recovery from acute diarrhoea in children (1991)	4 and 45 months admitted at Tampere University Hospital or Satakunta Central Hospital for acute diarrhea of less than 7 days' duration and with more than three watery stools	Not stated by the authors	Group 1: *L.casei* sp strain GG (Lactobacillus GG)-fermented milk product, (10^{10-11} cfu) N = 24 Group 2: Lactobacillus	Group 3: Fermented and pasteurized yoghurt with insignificant amount of LAB N = 24	Group 1 and placebo: 125 g of milk, 2ce daily for 5 days Group 2, 1 dose of powder containing 10^{10-11} cfu twice daily for 5 days	More than three watery stools during 24 hours. Inferred, not clearly stated.	• In Groups 1 and 2, duration of diarrhoea was reduced by mean (95% CI), 1.0 (0.4, 1.6) days • Considering rotavirus-positive cases only, the mean (SD) duration of the diarrhoea was 1.4 (0.8) days in group 1 (n= 22), 1.4 (0.9) days in group 2 (n = 17), and 2.7 (1.0) days in group 3 (n = 19); F = 12.46, P < .001

Isolauri E1, Juntunen M, Rautanen T, Sillanaukee P, Koivula T. Finland	during the previous 24 hours. All patients were given ORT of at least two times the fluid deficit n = 71		GG given as a freeze dried powder N = 23		1 month follow up		• The effect of Lactobacillus GG on watery diarrhoea became apparent after the first day of treatment
Evaluation of yogurt effect on acute diarrhoea in 6-24-month-old hospitalized infants (2006) Pashapour, N., Iou, G. S. Iran	6-24 months with moderate dehydration and acute non-bloody, non-mucoid diarrhoea for fewer than four days admitted in Urmia Imam Hospital n = 80	Not blinded	At least 15 ml/kg/day of pasteurized cow milk yogurt (fat 2.5%, sugar 3%, lactic acid 1%, water 74%, *Lactobacillus bulgaris* 50,000/ml, *Streptococcus thermophilus* 50,000/ml and pH=4.7) orally plus routine hospital treatment	Timely routine hospital treatment. Hospital treatment for moderate dehydration due to possible non-bacterial gastroenteritis in the infant period is a compound of intravenous serum therapy, oral rehydration solution (ORS) and mother's milk in breast-feeding infants and complementary food according to their month of age	From admission to discharge	Increase in the frequency, fluidity and volume of feces	• Mean duration of hospitalization (mean duration of diarrhoea) (days) was 2.7±0.91 vs 3.1±0.74 days for case vs control groups, respectively; p = 0.035 • Mean weight gain was 435± 89.20 vs 383±98.9 g for case vs control groups, respectively; p = 0.049 • Mean reduction in diarrhoea frequency was 4.30±1.74 vs 3.60±1.23 times for case and control groups, respectively; p = 0.017

Table 2.2: Risk of bias assessment using the Cochrane risk assessment tool

Author	Random sequence generation	Allocation Concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
Boudraa et al (2001)	⊕	⊖	⊖	⊖	⊕	⊕	⊖
Pedone et al (1999)	⊕	⊕	⊕	⊕	?	⊕	⊕
Yartey et al (1995)	⊕	?	?	?	⊕	⊕	⊖
Bhatnagar et al (1998)	⊕	?	?	?	⊕	⊕	⊖
Agarwal & Bhasin (2002)	⊕	⊕	⊕	⊕	⊕	⊕	⊖
Isolauri et al (1991)	⊕	⊖	⊖	⊖	?	⊖	⊖
Pashapour & Iou (2006)	⊕	?	⊖	⊖	⊕	⊕	⊖

Table shows the summary of risk of bias based on authors' judgement using the Cochrane Collaboration tool. In all cases, an answer of "+", "-" or "?" indicates low risk, high risk and unclear risk respectively

The pooled results from the articles, using random effect models, showed that mean duration of diarrhoea was significantly less in the group administered fermented food products in comparison with the control group; mean = -0.70 days; 95% CI (-1.06, -0.34). Subgroup analysis was undertaken by separating those that have used non-fermented foods including no intervention and heat-treated fermented foods as control and mean duration of diarrhoea was significantly lower in the group administered fermented foods in both cases [-0.75 days; 95% CI (-1.39, -0.10) and -0.74 days; 95% CI (-1.24, -0.24) respectively] (**Figure 2.2**). Results were similar when analyses were done using fixed effect models.

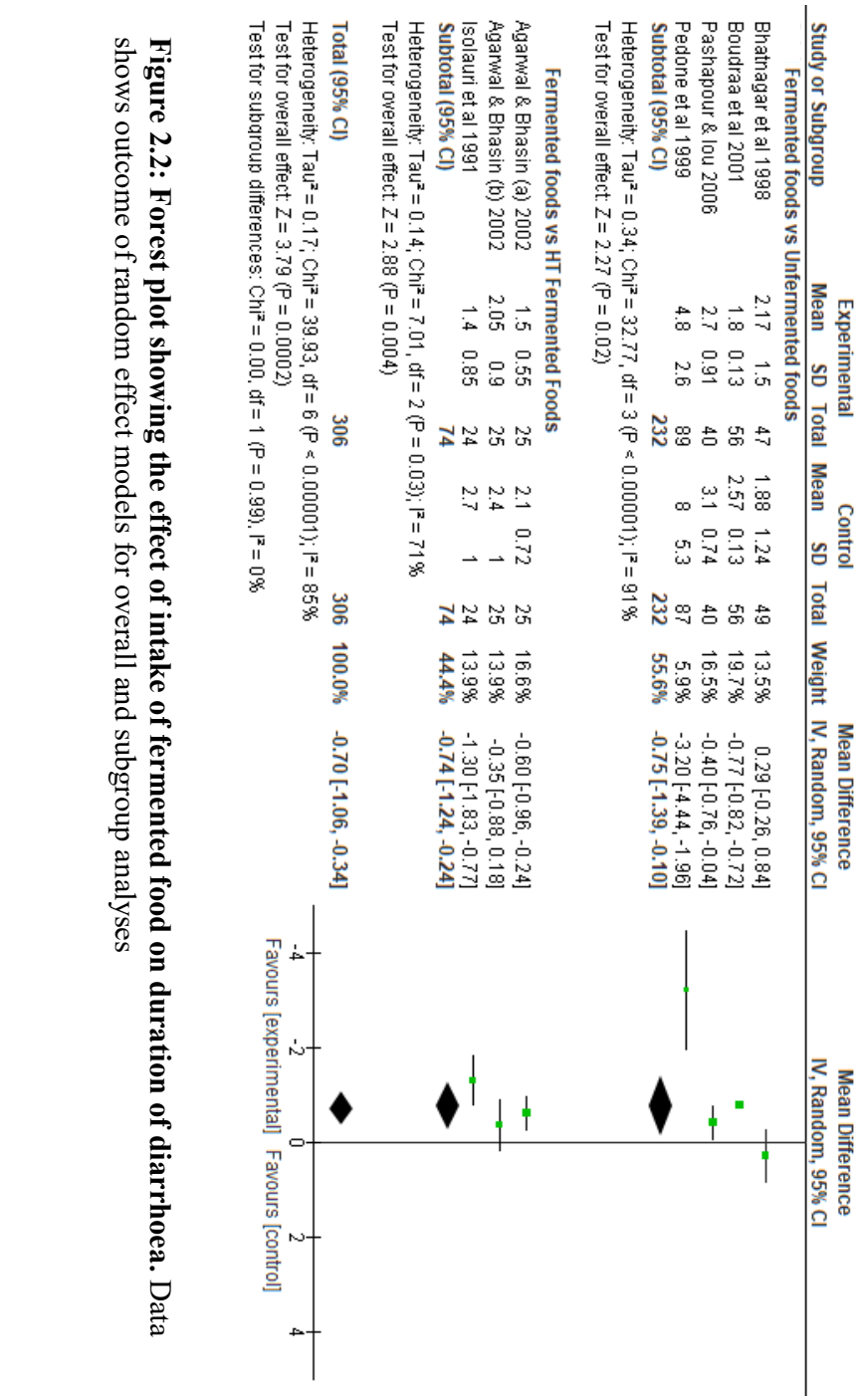


Figure 2.2: Forest plot showing the effect of intake of fermented food on duration of diarrhoea. Data shows outcome of random effect models for overall and subgroup analyses

2.4 Discussion

This review aimed to pool together evidence from studies that have investigated the efficacy of fermented food in the treatment of diarrhoea, considering dairy and non-dairy fermented products. Data from six out of the seven articles that met the inclusion criteria were pooled and results following meta-analysis of the data suggest that intake of fermented foods during an incidence of diarrhoea could reduce the duration of the disease in comparison to unfermented foods and pre-heated fermented foods. Furthermore, results from the 6th article, Yartey et al. (1995), suggests that fermented maize has similar effect in the treatment of diarrhoea as WHO recommendation – ORS. This suggests that fermented corn may be an effective alternative when ORS is not easily available especially in locations where corn is a staple meal.

The significant difference observed in the mean duration of diarrhoea between the experimental group administered with fermented foods vs the control group administered with unfermented groups could be associated with various components of the fermented foods which includes the fermentation organisms and products of fermentation process. However, the difference in the therapeutic effect observed in fermented foods using heat treated fermented food as the comparator may be associated with the fermentation organisms present in the fermented product.

Species of LAB including *Lb fermentum, Lb. acidophilus, Lb. rhamnosus, Lb. plantarum*, *Lb. casei*, have been investigated in *in vitro* and *in vivo* studies and have been reported to have probiotic properties (Bao et al., 2010; Millette, Luquet, Ruiz, & Lacroix, 2008; Pisano et al., 2014; Rivera-Espinoza & Gallardo-Navarro, 2010; Watson & Preedy, 2015; Zheng et al., 2013; Hosseini, Hussain, & Britz, 2015). Very often, strains tested in these studies have had origins from fermented foods products or human fecal samples (Rivera-Espinoza & Gallardo-Navarro, 2010). Meta-analysis of clinical studies, by Manzanares, Lemieux, Langlois, and Wischmeyer (2016) suggested that administering *Lb. plantarum*, either alone or in combination with other probiotics significantly reduced infections in critically ill patients. Furthermore, an *in-vivo* study Zheng et al. (2013) reported that some selected strains of *Lb acidophilus*, *Lb plantarum* and *Lb kefiri*, isolated from Tibetan kefir grains, may be able to help reduce serum lipid in humans. The authors also mentioned that the tested strains of these organisms were able to adhere to Caco-2 epithelial cells as much as or significantly better than *Lb. rhamnosus* GG (LGG), a strain known to adhere to human gut epithelial cells, in the *in-vitro* aspect of the study, indicating that the three investigated strains may be able to adhere to or colonize human intestinal tract. Adherence of probiotic organisms to the human intestinal walls decreases the chances of adherence for pathogenic organisms hence, reducing the risk of diseases. According to the meta-analysis by Ritchie and Romanuk (2012), *Lb*

rhamnosus GG (LGG) and *Lb casei* are LAB organisms which have most commonly been investigated for the management of gastrointestinal diseases ranging including diarrhoea. The authors however reported that these probiotic LAB had the potentials for preventive and therapeutic management of infectious diarrhoea but not for travellers' diarrhoea. There were variations in factors such as duration of administration, quantity administered varied and age of subject, across the studies included in the meta-analysis which may have caused the variations in the outcomes of both types of diarrhoea. Furthermore, the specificity of the health benefits that may be derived from organisms with probiotic properties may have contributed to inconsistencies that may have been observed between studies, as strains of the same species may not proffer the same health benefit to their host (Campana, van Hemert, & Baffone, 2017; Ramos, Thorsen, Schwan, & Jespersen, 2013; Rivera-Espinoza & Gallardo-Navarro, 2010). Ritchie and Romanuk (2012) reported a more significant probiotic effect in combined species of Lactobacilli compared to studies that used only one species but higher doses of probiotic organisms did not significantly enhance the therapeutic effect of probiotics organisms against gastrointestinal diseases. They therefore suggested that dosage size of metabolites formed in fermented foods on the other hand may be crucial to the effectiveness of these foods in enhancing gastrointestinal health.

In the current review, a significant reduction in duration of diarrhoea was observed in the group that was administered a fermented food vs control and during subgroup analyses. Nevertheless, *in vivo* studies that have investigated the effect of cell free fermented foods vs unfermented foods were unavailable. Such *in vivo* studies may be able to give insights on the effect of the combined components of the complex matrix of fermented food on health. Though the results of this current study may indicate that the fermentation organisms may help improve gut health during an episode of diarrhoea, it also supports the possibility that the complex matrix of the fermented food, which includes the organisms, end-products and by-products of fermentation may be of greater significance, than the isolation of each component.

Majority of the studies found during the search investigated the efficacy of fermented foods in the prevention rather than treatment of diarrhoea and other related gastrointestinal diseases (Beausoleil et al., 2007; de Vrese et al., 2011; Fox, Ahuja, Robertson, Ball, & Eri, 2015; Wenus et al., 2008). Furthermore, there is limited number of studies that have investigated treatment of diarrhoea using fermented foods other than dairy products (Fox et al., 2015; Patro-Golab et al., 2015; Patro-Gołąb et al., 2015; Rivera-Espinoza & Gallardo-Navarro, 2010). Two articles that have used fermented cereal as the intervention for the treatment of diarrhoea (Lei et al., 2006; Yartey et al., 1995) were

found during the search however trial by Lei et al. (2006) was excluded as it did not meet the inclusion criteria.

2.5 Limitation of meta-analysis

Due to limited number of studies that have investigated the effectiveness of fermented foods, especially foods non-dairy fermented foods, in the treatment of diarrhoea, only 6 articles were included in the current review. On the other hand, many more studies have investigated the effectiveness of fermentation organisms particularly those that have tested for probiotic potentials (Allen, Martinez, Gregorio, & Dans, 2010; Marsh et al., 2014; Millette et al., 2008; Mokoena et al., 2016) in the prevention of diarrhoea. Secondly, a significant degree of statistical heterogeneity was observed but not surprising as there were marked diversity in the types of participants, population size, duration of diarrhoea, duration and dosage of intervention between studies. Thirdly, only 2 studies included in the review (Agarwal & Bhasin, 2002; Pedone et al., 1999) showed low risk of bias using the Cochrane assessment tool. The quality of trials included in a meta-analysis will a great impact on the validity of the results (Greco, Zangrillo, Biondi-Zoccai, & Landoni, 2013).

2.6 Conclusion and future research

Fermented food products are widely available and consumed around the world. Probiotic potential of the organisms involved in the fermentation process have been documented. Health benefits of some of the end-products and by products of fermentation have also been documented. Fermented foods are traditionally consumed in some African and Asian communities, not only for the nutritional benefits but also for possible therapeutic effects they are perceived to have in the treatment of gastrointestinal diseases such as diarrhoea. There is however limited number of studies to evidence this perception. The current review has attempted to do this using limited number of articles. Results indicate that fermented foods may be helpful in the treatment of diarrhoea, maybe to the same degree as ORS recommended by the WHO. Given the small number of trials and methodological limitations of the included studies, the evidence in this review should be viewed with caution. More good quality trials are required to investigate the complex matrix of fermented food products, other than dairy foods, in the management, particularly treatment of gastrointestinal diseases such as diarrhoea.

Chapter 3

Methodology

This chapter aims explores the principles and justification for all experiments undertaken in the study. Detailed experiments are further described in each experimental study. **Table 3.1** provides the summary of where in the thesis discussed methodology has been utilised.

Table 3.1: Cross reference of methodology against relevant chapter of the thesis

Methodology	**Related Chapter**
Microbial Analysis Organism Identification Fermentation pH & Titration Antimicrobial Test Simulated Digestion Statistics	Chapter 4
SCFA Analysis using GC-FID Statistics	Chapter 5
Nitric Oxide analysis Statistics	Chapter 6

3.1 Microbial analysis

3.1.1 Pure culture

In order to determine the physiological, biochemical and metabolic characteristics as well as identify organisms present in a mixed culture sample, it is essential to isolate pure cultures of these organisms and cultivating them on an appropriate growth media for optimal growth (Richards et al., 2014). It is often assumed that a visible colony is a mass of a single cell that has undergone multiplication (Refai, 1979), and as such, a colony is believed to consist of a single type of organism. It is however possible to have more than one type of organism in a colony, which is usually characterised by different colour pigmentations or varied edges around the colony (Benathen, 1990). Therefore subculturing a single colony more than once may be ideal to isolate a pure culture from a mixed culture sample. Environmental factors significantly influence the culturability of microorganisms (Lagier et al., 2015; Richards et al., 2014). Organisms which have specific nutritional requirements for optimal growth may not thrive on general-purpose media, unless they are enriched (Lagier et al., 2015). Selective media are used to grow such types of

organisms, while also inhibiting the growth of others, depending on the degree of selectivity (Todar, 2011).

Food products are often fermented by mixed culture composed of lactic acid bacteria (LAB), moulds and yeast (Battcock, 1998; Holzapfel, 2002; M. K. Tripathi & Giri, 2014). de man, rogosa and sharpe (MRS) agar was developed for selective growth of lactobacilli (Corry, Curtis, & Baird, 2011a). Found not to be selective (Diaz et al., 2013) except when supplemented with an inhibitor such as sorbic acid and lowering the pH to about 5.7, MRS agar or broth is generally used as an elective medium for the cultivation of LAB particularly those belonging genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Reuter, 1985; Schillinger & Holzapfel, 2011). Rose bengal chloramphenicol (RBC) agar is a growth media used to selectively cultivate yeast and moulds from food products, with chloramphenicol (10% w/v) as its selective agent, inhibiting the growth of bacteria (Corry, Curtis, & Baird, 2011b). RBC is only available to use as an agar; Yeast Extract Peptone Dextrose (YPD) broth is an alternative media that can be used to cultivate yeast is. In the current study, MRS agar and broth were used to isolate and culture LAB while RBC agar supplemented with chloramphenicol was used to isolate yeasts from the fermented test sample. YPD broth was also used to culture yeast for further experiments.

Table 3.2: Composition of MRS Agar, RBC Agar and YPD Broth

MRS Agar (Oxoid)	**RBC Agar (Oxoid)**	**YPD Broth (Sigma)**
Peptone 10.0 g/L 'Lab-Lemco' powder 8.0 g/L Yeast extract 4.0 g/L Glucose 20.0 g/L Sorbitan mono-oleate 1 ml Dipotassium hydrogen phosphate 2.0 g/L Sodium acetate $3H_2O$ 5.0 g/L Triammonium citrate 2.0 g/L Magnesium sulphate $7H_2O$ 0.2 g/L Manganese sulphate $4H_2O$ 0.05 g/L Agar 10.0 g/L	Mycological peptone 5.0 g/L Glucose 10.0 g/ L Dipotassium phosphate 1.0 g/L Magnesium sulphate 0.5 g/L Rose-Bengal 0.05g/L Agar 15.5 g/L	Bacteriological peptone 20 g/L Yeast extract 10 g/L Glucose 20 g/L

3.1.2 Organism storage

There are 3 main categories of preservation methods of microorganisms: continuous growth, drying or freezing (Kumar, Kashyap, Singh, & Srivastava, 2013). Continuous growth is a short term preservation method which requires periodic transfer of organisms into fresh media (Kumar et al., 2013). Continuous periodic transfer to fresh plates put the organisms at the risk of changes in phenotypic and genotypic characteristics (Kumar et al., 2013; Prakash, Nimonkar, & Shouche, 2013; Sandle, 2015). The frequency of subculture should be dependent on the organism and the temperature of storage, for example, *Escherichia coli* requires a monthly transfer ("Maintenance and Preservation", 2008). However, the general recommendation is for organisms to be transferred unto fresh media every week and stored in the refrigerator, temperature 0 – 5^{O}C, to slow down rate of metabolic activities or the organisms (Prakash et al., 2013). Metabolic activities may also be reduced if organisms are not cultured in media that support optimal growth but cultured in media that will provide minimum required amount of nutrients ("Maintenance and Preservation", 2008). Nevertheless a maximum of 5 subcultures is recommended in order to minimize the risk of phenotypic variations, genetic drift, and contamination (Kumar et al., 2013; Prakash et al., 2013; Sandle, 2015).

Freezing is a common storage technique for microorganisms that provides a more extended preservation time compared to refrigeration. Temperature lower than 0 ^{O}C slows down metabolic rate in organisms more effectively than refrigeration. Enzymatic activities in cells continue at – 25 ^{O}C but physiochemical changes are frozen at temperature lower than – 40 ^{O}C (Bakhach, 2009). Therefore, freezing preservation of organisms at temperature that is higher than – 40 ^{O}C may only be long term if 15% (v/v) glycerol is added growth medium before storage ("Maintenance and Preservation," 2008). Storage of cells at freezing temperature could result in osmotic stress, dehydration of the cells and rupturing of the cells during thawing (Bakhach, 2009; Kumar et al., 2013; Prakash et al., 2013; Smith & Ryan, 2012). Cryoprotectants such as glycerol are able to minimize these negative effects of ice crystals (El-kest & Marth, 1992). Preservation of microorganisms in cryogenic temperatures, −80 ^{O}C, (dry ice) or −196 ^{O}C, (liquid nitrogen), is considered the most reliable method of long term cells preservation (Prakash et al., 2013). If stored in cryogenic temperatures, bacteria and fungi cells can be revived and restored, preserving the viability and genomic integrity of the cells (Bakhach, 2009; Kumar et al., 2013; Prakash et al., 2013). However, there still is the risk of osmotic stress, dehydration of the cells and rupturing of the cells during thawing (Bakhach, 2009; Kumar et al., 2013; Prakash et al., 2013; Smith & Ryan, 2012). Hence, storage temperature, method and frequency of cooling, and appropriate type of

cryoprotectants need to be considered for effective cryopreservation of microorganisms (Kumar et al., 2013; Prakash et al., 2013; Smith & Ryan, 2012).

In this study, long term storage was done using multi-coloured Microbank™, Pro LAB, which uses cryovials containing 25 beads that have been treated with cryopreservative solution (composition not stated by manufacturer). Each vial was inoculated with a pure culture of isolated organism and stored following manufacturer's instructions. A bead was removed from the vial as required and directly placed onto an appropriate growth media to revive the organisms. Unused beads were thereafter refrozen. Repeated freeze-thaw cycles increases the risk of compromising the genetic integrity of the organisms (Smith & Ryan, 2012). However, this risk was controlled by having multiple vials for each pure culture. Short term storage of organisms was done on appropriate agar and broth at $5^{O}C$. A maximum of 5 subcultures on fresh growth media was done while experiments were being performed after which short term storage containers were autoclaved at $121^{O}C$, 30 minutes, 103kPa, following to the health and safety policies in the microbiology laboratory within the Biological Sciences Department, to kill the organisms and as recommended by the Healthcare Infection Control Practices Advisory Committee for Centers for Disease Control and Prevention (CDC) (2008)

3.1.3 Serial dilution

Quantitative estimation of the number of viable organisms present in a food sample involves serial dilution and plate counting. Serial dilution reduces the concentration of the microbial load by a known dilution factor in order to prevent overcrowding (Ben-David & Davidson, 2014). The number of visible colonies that grows on the solid medium is used to estimate the number of viable cells (Sutton, 2011) present in a known volume or weight of the food sample being tested. Dilution factor is often a function of the population size (Duggan & Gurll, 1965). Ten-fold dilution also called logarithmic dilution is a large scale dilution often used in serial dilution in microbiological analysis (Refai, 1979). Ten-fold dilution reduces the concentration of microbial cells to one which helps make counting easier on the agar plate

The accuracy of quantitative estimation of microbial load in cultured sample is dependent on environmental factors such as the growth media, incubation time and temperature, dilution factor (Franklin, Garland, Bolster, & Mills, 2001) and characteristics of the cells (Oliver, 2005, 2010). Mixed organisms in test samples such as traditional fermented foods may have varied requirements for optimal growth which is a risk factor for counting and sampling errors (Ben-David & Davidson, 2014). In addition, viable but non culturable organisms (VBNC), though alive, are unable to grow

on bacteriological media (Oliver, 2010) which may be present in these fermented foods will be missed during plate counting leading to increased risk of inaccurate estimation of the microbial load. Serial dilution often causes a significant change in the population of the organisms - the concentration of organisms and diversity of their population - particularly organisms that have low concentration in the sample which may result in sampling error (Ben-David & Davidson, 2014). It is therefore logical that the least dilution will be most representative of the microbial load of the sample, however, it is also important that counting error due to overcrowding or merges colonies is avoided. It is nevertheless imperative that dilution factor most closely representing the population of organism in the test sample is used for increased accuracy of quantitative estimation. As a result, Breed and Dotterrer (1916) (as cited in Sutton, 2011) suggest a lower limit of 50 colonies and upper limit of 200 colonies may be ideal for best results of quantitative estimation. Tomasiewicz, Hotchkiss, Reinbold, Read JR, and Hartman (1980) argues that appropriate number of countable colonies may vary based on factors such as type of sample that is being analysed. The authors further recommend in triplicate, a countable range of 25-250 colonies using raw milk as their test sample. Putting many variables into consideration, The United States Pharmacopeia (2011) recommends for best result, a range of 20 – 300 colonies, with a minimum of 6 colonies on a plate being acceptable. The USP recommendation was followed in the current study.

3.2 Organism identification

3.2.1 Gram staining

Gram staining is a primary and rapid diagnostic test for the identification of microorganisms, providing information about the morphological characteristics of the cells based on the organism's cell wall (Beveridge, 2001; Matheson, 1999; Steinbach & Shetty, 2001) by making them visible under the light microscope. Viruses do not have cell walls hence cannot be visualised by gram staining. The test differentiates bacterial cells into 2 main categories ``Gram-positive,'' or ``Gram-negative'' depending on the thickness of the cell walls (Beveridge, 2001; Steinbach & Shetty, 2001). Bacterial cell wall consists of a polymer known as peptidoglycan, which are long strands of glycans cross-linked by stretchable peptides, (Huang, Mukhopadhyay, Wen, Gitai, & Wingreen, 2008). Peptidoglycan gives the bacterial cell its characteristic shape (Silhavy, Kahne, & Walker, 2010). The amount of peptidoglycan contained in the cell wall determines what colour will be retained by the cell during gram stain test. The cell wall of gram negative bacteria is thinner, containing significantly less amount and layers peptidoglycan, compared gram-positive bacteria which have up to 100 nm thickness and layers of the compound (Huang et al., 2008; Silhavy et al., 2010). Thin layer of peptidoglycan in gram negative bacteria cell wall causes it be decolourised,

losing the violet colour initially obtained from crystal violet stain, and picking up the red colour from safranin stain (**Figure 3.1 and 3.2**).

Gram staining is a simple but erroneous test prone to providing false results. Excessive thickness of smears of bacterial cells have been shown to cause incomplete decolourization, causing gram negative bacteria to appear as gram positive (Chandler, 2013; Claus, 1992). In addition, excessive heat fixation has been shown to not only result in a change in the morphological characteristics of the cells (Chandler, 2013; Claus, 1992), but to also cause gram positive bacteria to stain as gram negative (Steinbach & Shetty, 2001). In this study, standard procedure published by Public Health England (2014) was followed to enhance accuracy and reliability of outcomes. Overnight cultures were gram stained as it has also been shown that cell age is able to influence colour retention characteristics of bacterial cells (Chandler, 2013; Claus, 1992). Furthermore, due to inherent inconsistency in the ability of some cells to gram stain there is the risk of the technical error of misinterpretation of results (Chandler, 2013).

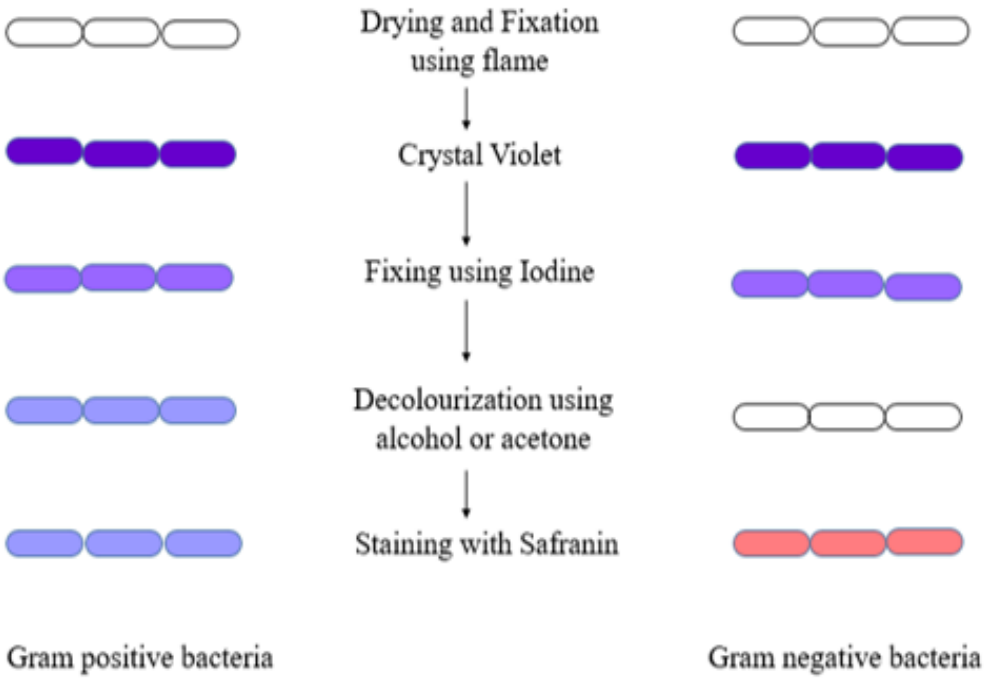


Figure 3.1. Response of Gram-positive and Gram-negative bacteria to gram staining

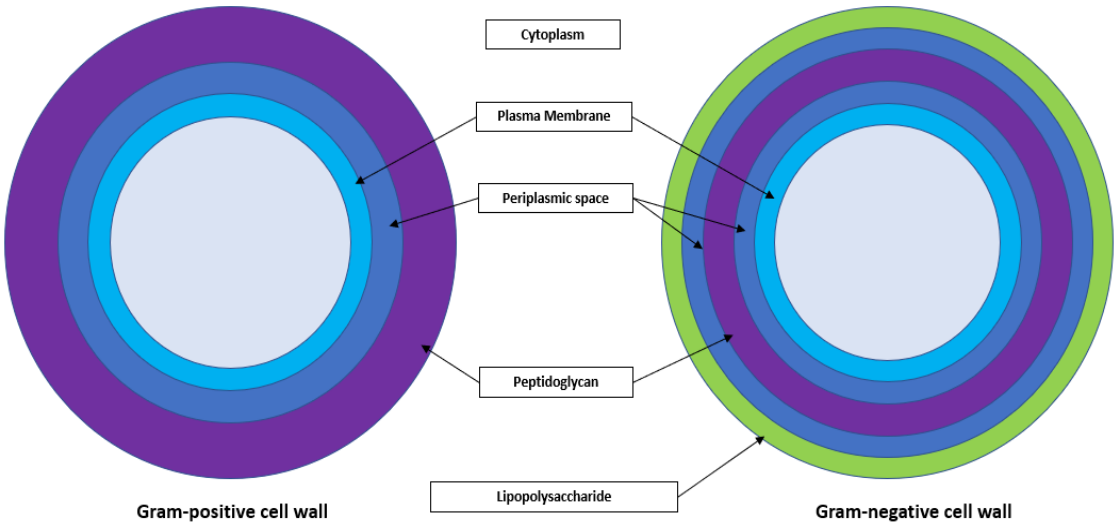


Figure 3.2 Cell wall of Gram-positive and Gram-negative bacteria

3.2.2 Molecular identification

Techniques traditionally used in the identification of microorganisms relied mainly on the morphological, growth variables and biochemical characteristics of the organisms (Bisen, Debnath, & Prasad, 2012). These phenotypic test are necessary as they provide information about basic morphological and biochemical characteristics, they are nevertheless easily influenced by environmental factors surrounding the growth of the organisms, hence compromising the precision and reproducibility of the methods and reliability of the results (Klijn, 1996). API® (Biomerieux©, Italia SpA), is a commercial kit used to identify organisms based on their metabolism of various carbohydrates (Anderson, Vredeveld, Brake, Buchanan, & Lewis, 1983; Carpana, Marocchi, & Gelmini, 1995; Crist, Johnson, & Burke, 1996). API® 50 CH (Biomerieux, Italia SpA), is commonly used for the identification of Lactobacilli by investigating how the organisms are able to metabolise 49 carbohydrates in 48 hours (Biomerieux, n.d.; Diaz et al., 2013; Ozgun & Vural, 2011). Inaccuracies and inconsistencies of results obtained from the kit has however been reported (Diaz et al., 2013; Donelli, Vuotto, & Mastromarino, 2013). Bosshard et al. (2006) reported that out of 107 isolates, using API® 20 NE, 57 (54%) were assigned to species in excellent, very good, good, and acceptable species identifications and 7 (7%) to genus level, and 42 (39%) could not be discriminated at any taxonomic level. The authors further stated in 14 isolates identified to the species level, not a single species but a mixed taxon was reported. It can therefore be inferred that phenotypical and biochemical characteristics are not precise enough to identify the organisms based on species and strain characteristics such as antibiotic susceptibility and enzymatic profiles (Bisen

et al., 2012). In the last 20 years, molecular approaches for identification of organisms up to strain level, utilizing stable genomic properties have been developed (Donelli et al., 2013; Land et al., 2015). Due to limitations of the commercial identification kits and the requirement to identify food fermentation organisms to strain level by Joint FAO/WHO Working Group (2007), the use of genotypical methods for the identification of these organisms is necessary.

There are 2 categories of genotypic microbial identification methods; 1) the pattern-based techniques where a series of fragments of the organisms's chromosomal DNA is reproduced and separated by size to generate a profile; 2) the sequence-based techniques where the sequence of a specific stretch of the DNA often asscociated with a specific gene (Emerson, Agulto, Liu, & Liu, 2008). In both techniques, a database containing information of many organisms is generated. Information gathered from test organisms is compared with database in search for similarity. In the pattern-based technique, a match of test organisms profile on the database will mean that organisms are related usually are species and strain levels. Whilst in sequence-based technique, the degree of similarity between sequence of test organism and that of the an organism on the database can be used to determine the degree of relation between the both organisms to species level.

DNA-DNA hybridization technique, Amplified ribosomal DNA restriction analysis (ARDRA) and 16S and 23S rDNA sequencing, are techniques used in the identification of microorganisms to species level (Donelli et al., 2013). 16S and 23S rRNA is commonly used in the identification of food fermentation organisms. Evolution in the 16S rRNA molecule and lesser extent, of 23S rRNAs is slow, so the molecules are therefore conserved throughout all bacterial speciess (Donelli et al., 2013; Prakash et al., 2013), hence ideal to for the identification of organisms, even those that have been in existence for a very long time. This process begins with extraction of DNA from the organism. The Polymerase chain reaction (PCR) then amplifies the gene sequencing 16S rRNA from the DNA using primers. Amplified output is then assessed and studied for taxonomic similarities and relationships between organisms (Cruaud et al., 2014). The major limitation of 16S rRNA sequencing as previously mentioned, is its inability to differentiate organisms to strain level (Cruaud et al., 2014; Donelli et al., 2013; Emerson et al., 2008; Prakash et al., 2013). However, in the current study, identification of organism to species level was sufficient since probiotic characteristics were only being investigated *in-vitro* and not *in-vivo*.

In this study, DNA extraction was done using DNeasy Blood & Tissue Kits which was purchased from QIAGEN®. This kit uses the solid phase extraction method of DNA extraction (Elkins, 2012).

This kit was chosen due to easy accessiblity as it is often used for teaching within the department. Extracted DNA was then submitted to Eurofins Scientific© for 16S rRNA sequencing.

3.3 Spontaneous and controlled fermentation; sterilization of corn

Fermented food products are processed either spontaneously, allowing the organisms existing naturally on and in the food, water and environment to ferment the food; or controlled, when known quantities of known live organisms are inoculated in the sterile food product as starter culture (Giraffa, 2004). The end product of spontaneous fermentation is unpredictable, hence the need for controlled fermentation in research, where fermentation output can be predicted, with possibility of being repeatable and reproducible. In order to imitate spontaneous fermentation in a controlled setting, all organisms responsible for spontaneous fermentation of the food product need to be isolated, quantified and identified. It might not be possible to achieve this as the dynamics of the fermentation organisms - population and type of organisms - changes as fermentation progresses, in response to changes in the composition of the food (Bautista-Gallego et al., 2014). In addition, sterilization of food sample in preparation for controlled fermentation may cause an alteration the composition structure of the food product, depending on the method of sterilization employed.

Food sterilization can done be by thermal or non-thermal processing (McAllister, Rode, Cheng, & Forsberg, 1991). Irradiation, pulsed electric fields (PEF) and high pressure processing (HPP) (Jan, Sood, Sofi, & Norzom, 2017; Min & Zhang, 2005) are examples of non-thermal food sterilization. Although non-thermal sterilization has been described to cause a lower deterioration in the quality and loss of nutrients compared to the conventional heat treatment (Escobedo-Avellaneda et al., 2011; Ramesh, 2003), equipment used are more expensive (Muredzi, 2012). Furthermore, not all foods types can be effectively sterilized using all non-thermal techniques (Gupta & Abu-Ghannam, 2012). Apart from HPP, other methods are still in the development stage hence a combination of methods may be required to achieve sterility (Gupta & Abu-Ghannam, 2012; Jan et al., 2017). Thermal processes could be done using moist or dry heat (Ramesh, 2003). The temperature and length of time for sterilization is often dependent on factors such as the type of food being sterilized, the type of organisms expected to be found in the food and the method of heating to be employed (Hartel & Heldman, 2012). Steam sterilization can be done using a pressure cooker or an autoclave which functions by allowing the flow high temperature steam with high pressure (Dion & Parker, 2013). McAllister et al. (1991) reported though effective, autoclaving ground corn, 120^{O}C, 20 minutes, 103kPa, caused a significant decrease in starch content by up to 11% compared to untreated corn powder. This according to the authors may have been due to Maillard reaction that might have accorded between the sugar and amino acid present in the food product. The author

concluded that in comparison with autoclaving, chemical sterilization 12% (v/v) ethylene oxide, 105 minute and gamma irradiation, 1,500 krads, 50 h had digestive properties that closely resembled the untreated ground corn. In the current study, due to low cost and accessibility, corn was sterilized by autoclaving 121^{O}C, 15 minutes, 103kPa (1.03 psi), which is sufficient to kill all microorganisms and spores (Ray & Bhunia, 2013) with no visible change in the colour.

3.4 pH and Titration

Organic acids produced during fermentation result in a drop in substrate pH (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). Therefore fermentation process can be followed by checking pH (potential hydrogen) and organism population at intervals. pH is defined as concentration of free hydrogen or hydronium ions present in a solution (Buck et al., 2002; Van Slyke & Zacharias, 1914) and values range from 0 to 14 (Buck et al., 2002). The pH value gives information of the ratio of hydrogen ion and hydroxide ion in a solution. hence a higher concentration of hydrogen ion is an indication that the solution is acidic (pH < 7) and a higher concentration of hydroxide ion means that the solution is alkaline (pH >7) while equal concentration of hydrogen and hydroxide ions, pH 7, indicates that the solution is neutral (Kohlmann, 2003). pH can be measured using colour indicators, undergoing colour changes when exposed to different pH values. (Kohlmann, 2003). For example, a simple the litmus test, utilises colour indicators to test for acidity or alkalinity of a solution (Brownlee, 2004). pH stripes using colour indicators may also be used to estimate the degree of alkalinity or acidity of a solution (Foster & Gruntfest, 1937). pH stripes are available in limited pH ranges which therefore decreases the accuracy of the pH (Kohlmann, 2003). The use of a measuring electrode is described as the most accurate, repeatable and precise method of measuring pH (Kohlmann, 2003; Toledo, 2007). The pH meter uses electrodes, measuring electrode and a reference electrode to determine the pH of a solution (Riddle, 2011). The reference electrode provides that standard for the pH to be measured against. Most pH meters used in laboratories have both electrode combined as a pH electrode for convenience (Kohlmann, 2003; Riddle, 2011; Toledo, 2007). For accuracy, prior to use, the reference electrode needs to be calibrated with standard solutions that have predetermined pH (Cheng & Zhu, 2005; Kohlmann, 2003; Riddle, 2011). The temperature of the solution can also influence the accuracy of the pH meter (Kohlmann, 2003; Riddle, 2011; Toledo, 2007). It is recommended that temperature of sample be within $\pm$ 5^{O}C of temperature of buffer during calibration for accuracy of measurement (Riddle, 2011). In this study, calibrations and pH measurements were done when buffers and tests were at 25^{O}C. Mettler Toledo's pH meter was used in the current study and a recommendation of daily calibration for accurate measurements was made by the manufacturer (Toledo, 2007).

Titratable acidity (TA) is determined by the amount of strong base required to be added to the solution in order to achieve an endpoint (Siggaard-Andersen, 1966) which is either a specific pH (potentiometric titration) or a change in colour (colorimetric titration) depending on the method being used (Friedrich, 2001; Siggaard-Andersen, 1966). This process is known as titration (Friedrich, 2001). Potentiometric titration is recommended if the colour of the test substance would interfere with change in colour should the colorimetric titration be used (Friedrich, 2001). In the current study, colorimetric titration was used to determine TA as fermentation progressed, using Phenolphthalein 10% (w/v) as indicator. Supernatant solution of fermented corn was colourless, hence there was no interference with colour change during titration.

pH and TA are determined by the amount of free hydrogen ions and the amount of total hydrogen ions respectively present in a solution (Sadler & Murphy, 2010), nevertheless, there is no direct relationship between both measures. As stated by Baldwin (1919), 0.1 N acetic acid has ten times the titratable acidity of 0.1 N hydrochloric acid, yet 0.1 N hydrochloric acid has about 22.4 times the hydrogen ion concentration of 0.1 N acetic acid. More significantly, while pH maybe significant to the survival of microorganisms in the test substrate (fermenting corn in this study), titratable acidity better has more influence than pH on the flavour of the food (Sadler & Murphy, 2010).

3.5 Antimicrobial test

Antibacterial agents function by either being bacteriostatic, inhibiting the growth of the test organisms or by being bactericidal, which do not only inhibit the growth of the test organisms but also activate pathways that lead to cell death (Coyle, 2005). Test for detection of antimicrobial is generally classed into 3 which include bioautographic, diffusion, and dilution methods (Valgas, Souza, Smânia, & Smânia Jr, 2007). Though described as a qualitative technique that is only able to suggest the presence or absence of antimicrobial activities (Valgas et al., 2007), the diffusion method is a common method employed to investigate the antibacterial activities of fermented foods and fermentation organisms (Halder, Mandal, Chatterjee, Pal, & Mandal, 2017; Kazemipoor, Radzi, Begum, & Yaze, 2012; Othman et al., 2011; Prabhurajeshwar & Chandrakanth, 2017; Samot & Badet, 2013; Yesillik, Yildirim, Dikici, Yildiz, & Yesillik, 2011). This may be because there are multiple mechanisms of antimicrobial actions for fermentation organisms and fermented foods. Such mechanisms include production of bacteriocins by the organisms, lowered pH in fermented foods, competition for nutrients especially is pathogenic organisms are fastidious (Halder et al., 2017; Kazemipoor et al., 2012) and further tests might be required to investigate the precise mechanism for antimicrobial actions.

The types of diffusion methods popularly used include spot diffusion (Hagi & Hoshino, 2009; Samot & Badet, 2013), agar disk-diffusion, agar well diffusion (Balouiri, Sadiki, & Ibnsouda, 2016). Spot diffusion involves using spot inoculation of the fermentation organism or fermented food on the overlaid plate inoculated with the test organism which is then inoculated (Samot & Badet, 2013). In the agar disk-diffusion method, paper discs (about 6 mm in diameter), which has been soaked in the broth containing a known concentration of the fermentation organism or fermented food is placed on the overlaid agar surface containing the test organism (Balouiri et al., 2016). The well diffusion technique involves aseptically boring wells up to 6 mm wide, in overlaid agar with the test organisms, filling the holes with up to 100 µl of the antimicrobial agent and incubating at appropriate temperature (Halder et al., 2017; Pundir, Rana, Kashyap, & Kaur, 2013). In all cases, presence of inhibition zone around the spot is an indication of antimicrobial activity (Hagi & Hoshino, 2009). The antagonistic activity of the antimicrobial agent can then be estimated based in the inhibition zone diameter (Balouiri et al., 2016). In this current study, the agar well diffusion technique was used as it is sufficient to investigate the antimicrobial activities (Valgas et al., 2007) of fermentation organisms against strains of *E. coli* and *S. epidermidis*.

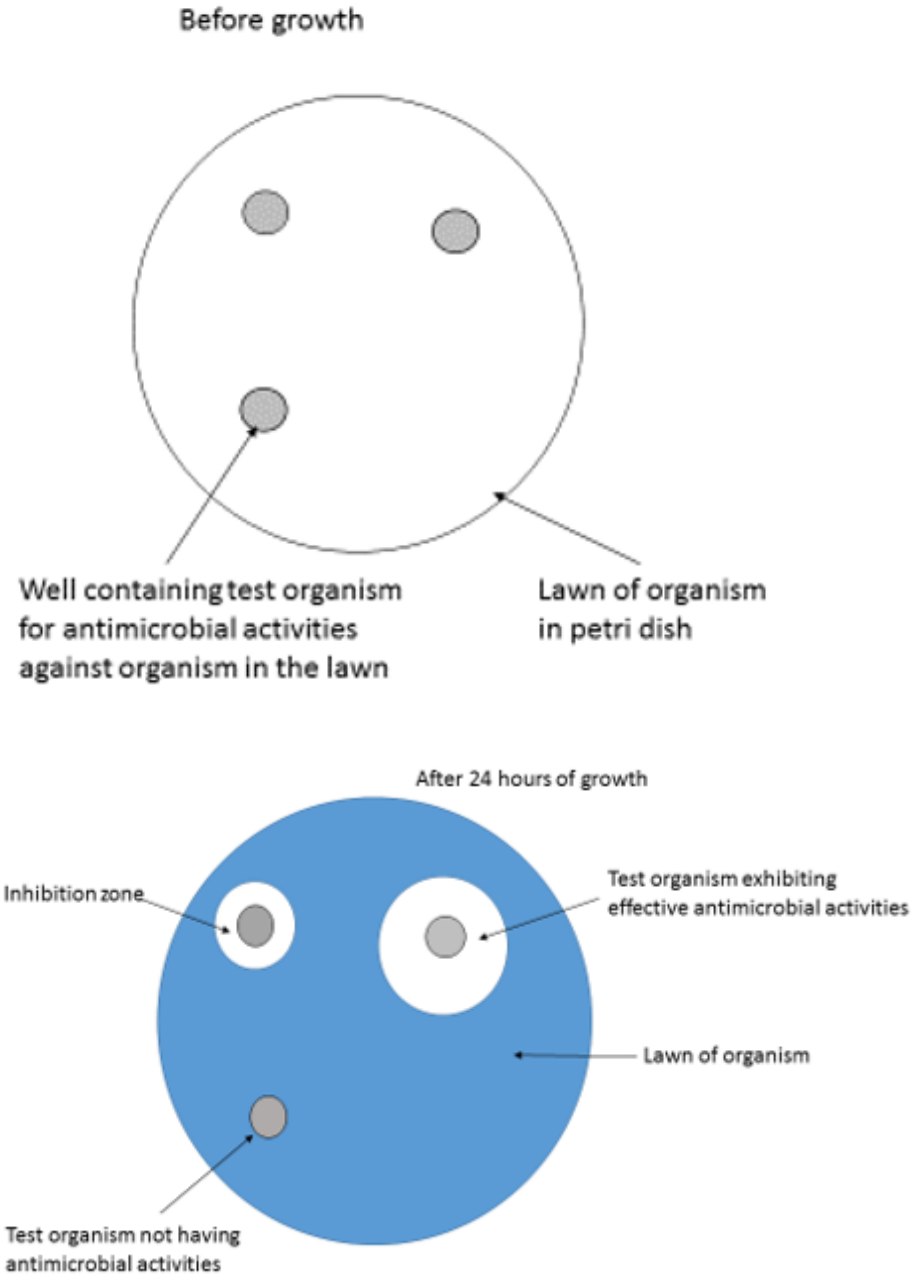


Figure 3.3: Antimicrobial screening using well diffusion technique

3.6 Short Chain Fatty Acid (SCFA) analysis using Gas Chromatography - Flame ionization detection (GC-FID)

Heterofermentative LAB are able to produce SCFA - also known as volatile fatty acids (Fernández, Dinsdale, Guwy, & Premier, 2016), such as acetic and propionic acids during fermentation of sugars (Hayek & Ibrahim, 2013; Prückler et al., 2015). SCFAs including butyric acid, are also produced in the gut during the fermentation of indigestible carbohydrates by the gut microbiome

(Deroover, Boets, Tie, Vandermeulen, & Verbeke, 2017; Primec, Mičetić-Turk, & Langerholc, 2017), an outcome that may be possible when complex carbohydrate rich foods are anaerobically fermented by microbiome of fermented foods.

Determination and quantification of individual SCFA can be done using separation techniques (Fernández et al., 2016). Though analyses of SCFAs have been done using High Performance Liquid Chromatography (HPLC) (Primec et al., 2017), Gas-Chromatography (GC) appears to be the most popular instrument used for the detection and quantification of SCFAs (Primec et al., 2017), which may be due to the high volatility and polarity characteristics of the compounds. A GC system is made up of four major parts namely - carrier gas source, sample introduction system, column, and detector (Wang & Paré, 1997) (**Figure 3.4**). The system's principle rests on a carrier gas, Nitrogen, Helium or Hydrogen, which serves as a moving or mobile phase with which sample compounds are transported to the column at the stationary phase, where they are separated by differential interaction, based on the affinity of the sample compounds to the column (Primec et al., 2017). Therefore, the most significant part of the GC is the column, where the separation of compounds take place (Al-Bukhaiti, Noman, Qasim, & Al-Farga, 2017; Rahman et al., 2015). Packed and capillary columns are the 2 types of columns used in the GC. However, based on factors such as resolution power, separation time and material it is made with, capillary column is often described as more effective for GC (Rahman et al., 2015). Flame ionization detection (FID) is the most common detector used in GC particularly in food analysis (Al-Bukhaiti et al., 2017). FID detectors are sensitive to organic or carbon containing compounds (Primec et al., 2017) and respond to molecules that have been ionized using flames that have commonly been generated using hydrogen and air (Al-Bukhaiti et al., 2017). The ions formed generate amplified current between two electrodes in the detector and in varied measures proportional to the quantity of the compound in the sample (Al-Bukhaiti et al., 2017; Primec et al., 2017). The major advantages of FID detectors the degree of accuracy, wide range of linearity and the option of temperature regulation (Al-Bukhaiti et al., 2017; Primec et al., 2017). In GC-FID, the time taken to reach the detector known as the detection time and the detector signal known as chromatography peaks, are used to for quantitative evaluation of compounds (Primec et al., 2017). Nevertheless, the system is unable able to provide qualitative information i.e. identification of the detected compounds (Cadwallader, 2007). In the current study, quantitative analyses of SCFA in fermented corn, a food sample, was done using the GC-FID. Compounds detected were further qualitatively evaluated by comparing chromatography peaks from known pure SCFA with peaks derived during analyses of fermented foods.

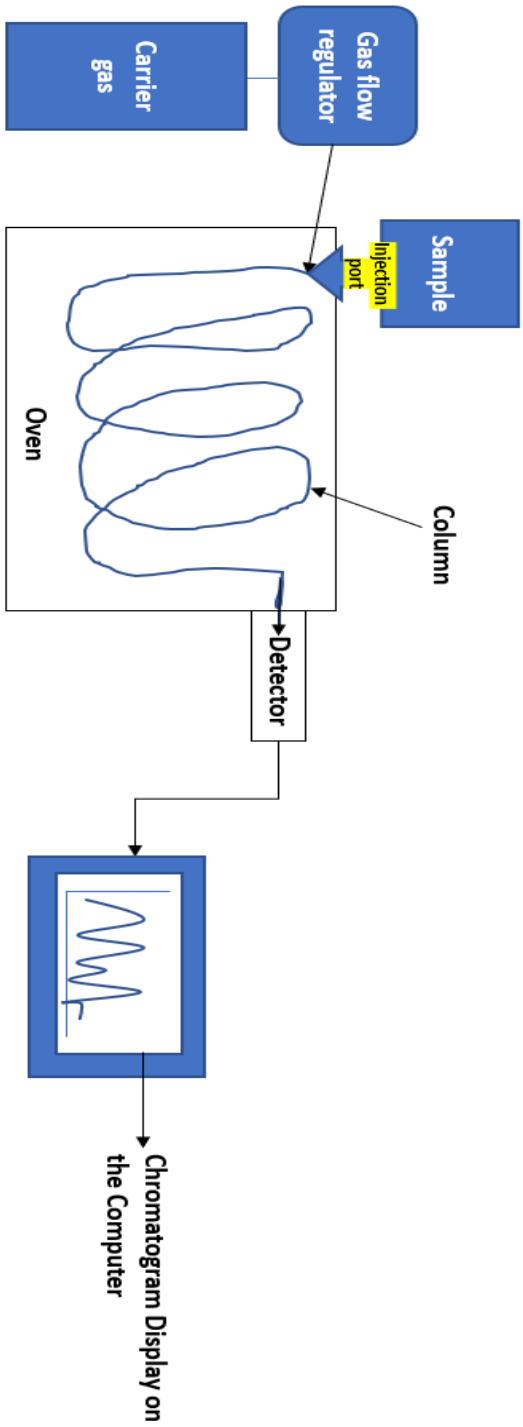


Figure 3.4 Schematic Diagram of a Gas Chromatograph

3.7 Digestion

A fermentation organism may have some probiotic potential if it has the ability to survive the hostile environment in the digestive tract in order to arrive at the colon where it is expected to confer health benefits to the host (Joint FAO/WHO Working Group 2007). The accurate method to measure this would be to take sample specimen at varied points in human gut during transit time to investigate the presence and population of the organism of interest (Minekus et al., 2014;

Wickham, Faulks, & Mills, 2009). However, apart from cost implications, the invasive nature of this type of experiment may result in strict ethical limitations and technical problems. Instead, studies have evaluated human faecal sample to investigate the survival the organisms of interest (Elli et al., 2006; Oozeer et al., 2006; Soares dos Santos Pozza, da Silva Miglioranza, Garcia, Garcia, & Pozza, 2011). The accuracy of this method may nevertheless be compromised if the presence and population of such organism is not predetermined in the volunteer's faecal sample before oral administration. *In vitro* test using simulated human gastrointestinal tract (GIT) on the other hand is less ethically restrictive, cheaper and quicker (Minekus et al., 2014). The simulated GIT is designed to mimic as much as possible, the physical conditions in the mouth, stomach and intestine with respect to agitation, enzymes, pH, salt and digestion time (Minekus et al., 2014). This technique nevertheless is not accurate as it does not accommodate physiological factors including age, gender and state of health (Minekus et al., 2014) and psychophysiological aspect of food intake, such as the measure of enzymatic responses to nutrients ingested (Hur, Lim, Decker, & McClements, 2011; Minekus et al., 2014). Therefore, neither *in vivo* methods - faecal analyses nor in vitro tests involving simulated GIT is able to give an accurate representation of actual events in the human gastrointestinal tract. Unfortunately, there is dearth of information on the correlation between digestive output using *in vivo* or *in vitro* methods (Hur et al., 2011). Therefore, the aim of a study will determine which of the 2 methods will be most suitable.

The static method of in vitro digestion is a simple technique which includes the oral, gastric and small intestinal phases of digestion (**Figure 3.5**), but with the process being largely immobile, in a static vessel (Alegría, Garcia-Llatas, & Cilla, 2015). This model does not mirror the mobile process of the human digested tract neither does it include the large intestine phase of digestion (Alegría et al., 2015; Minekus et al., 2014), but it is cheap (Minekus et al., 2014). The process is however said to be sufficient for quantitative assessment of the release of functional ingredients and nutrients in food (Kong & Singh, 2008) and has been successfully employed to investigate the survival of potential probiotic organisms in harsh environment of the human gut (Succi et al., 2017; Vemuri et al., 2018). In this study, digestion of fermented food to investigate the survival of fermentation organisms through the digestive tract was done using the standard static in vitro digestion protocol that was designed by Minekus et al. (2014). According to the authors, this standard protocol was designed following the consolidation of all published protocols for simulated digestion to arrive at these simple reproducible steps that create conditions that may be close to physiological situations

in humans generally except infant and elderly whose digestive system is undeveloped and prone to development of diseases respectively.

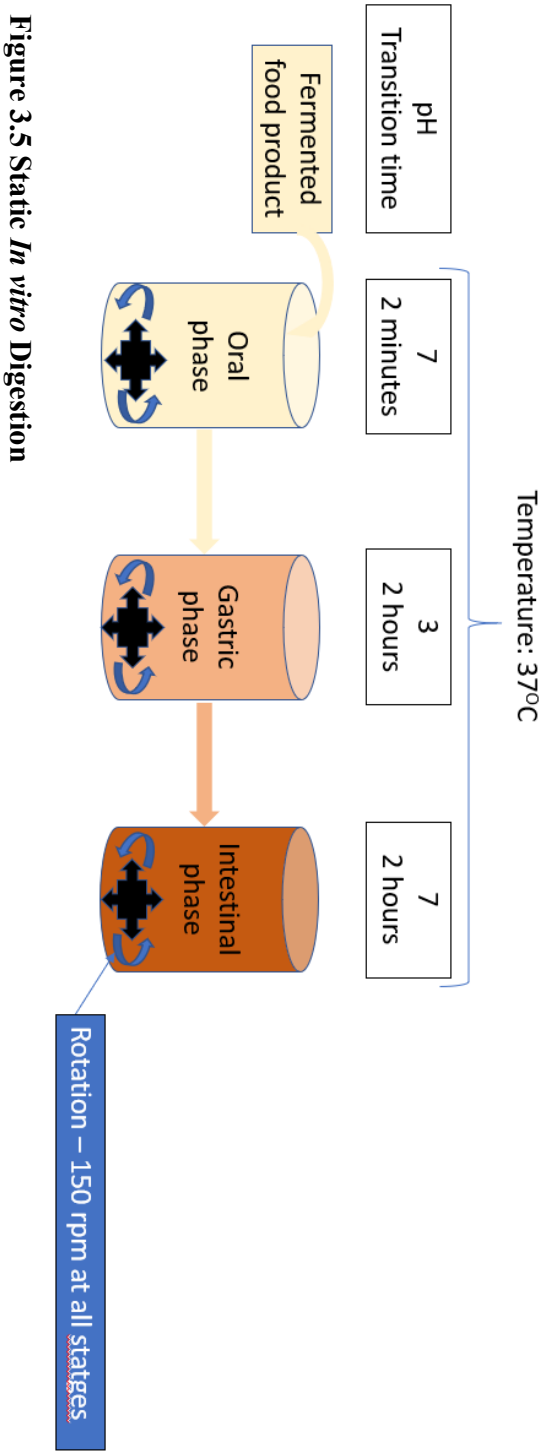


Figure 3.5 Static *In vitro* Digestion

3.8 Nitric oxide response

Nitric Oxide (NO) is a free radical that controls a wide range of signalling pathways in the human body (Pereira, Ferreira, Rocha, Barbosa, & Laranjinha, 2013) and plays a significant role in the human immune function (Tripathi, Tripathi, Kashyap, & Singh, 2007). It is synthesised from the amino acid L-arginine by a group of enzymes known as Nitric Oxide Synthase (NOS) through the L-arginine–NO pathway (Tripathi et al., 2007). Isoforms of NOS responsible for the synthesis of NO include endothelial NO synthase (eNOS), neuronal NO synthase (nNOS) and inducible NOS (iNOS) (Ibiza & Serrador, 2008; Tripathi et al., 2007). eNOS and nNOS are constitutively or constantly expressed within the body while iNOS is expressed by immune cells only when activated (Ibiza & Serrador, 2008; Tripathi et al., 2007). This activation occurs when calmodulin (calcium modulated protein) binds with calcium ions, in response to inflammation or infection (Hosking, 2009; Tripathi et al., 2007). nNOS is not only found in the nerve cells, but also in testes and skeletal muscle while eNOS are found in the Golgi apparatus of the endothelial cells regulating the vascular reactivity in the periphery and brain (Bogdan, 2001; Bredt, 1999; Deckel, 2001; Hosking, 2009; Tripathi et al., 2007). Production of NO is an important feature of cells of the immune system including dendritic cells, natural killer (NK) cells, mast cells and phagocytic cells including monocytes, macrophages, microglia, Kupffer cells, eosinophils and neutrophils, and other cells such as endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts, keratinocytes, chondrocytes, hepatocytes, mesangial cells and Schwann cells, which are also involved in immune responses in the body (Hosking, 2009; Tripathi et al., 2007).

NO is short-lived, having a shelf life of up to 20 seconds in aqueous and oxygen-containing solutions (Ibiza & Serrador, 2008; Moncada, 1991; Tripathi et al., 2007). It is therefore readily oxidized to nitrite (NO^{2-}) and nitrate (NO^{3-}) in the human cells (Weitzberg, Hezel, & Lundberg, 2010). Activities of NOS and NO in food can only be investigated by measuring NO^{2-} and NO^{3-} (dos Santos Baião, Conte-Junior, Paschoalin, & Alvares, 2016; Tsikas, 2005; Weitzberg et al., 2010; Yang et al., 1997). NO has been analysed using colorimetric and ultraviolet spectrophotometric methods, fluorometric assays, chemiluminescence (CL), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), gas chromatography (GC), and gas chromatography–mass spectrometry (GC–MS) (Tsikas, 2005). Nevertheless the authors argued that mass spectrometry-based approaches were most reliable for quantitative analysis of nitrite and nitrate (Tsikas, 2005). Tsikas et al. (1997) reported that Griess assay (colorimetric method) showed lower nitrate basal levels than GC–MS. Becker et al. (2000) on the other hand reported no significant difference in the quantities of nitrite and nitrate detected from blood cells from healthy volunteers using GC-MS and Colorimetric determination using Griess assay. This variation may

be due to the varied sources of body fluid analysed in both studies. Griess assay is the oldest and most frequently used method for the quantification of nitrite and nitrate (Tsikas, 2005) probably due to the fact that it is well-established, cheap and quick to use, with sample preparation not required before analysis. In the current study, quantitative analysis of nitrite and nitrate was done using a commercial nitric oxide assay kit purchased from Thermo Fisher Scientific. Using this kit, nitrite and nitrate are detected as a coloured azo dye product of the Griess reaction (**Figure 3.6**).

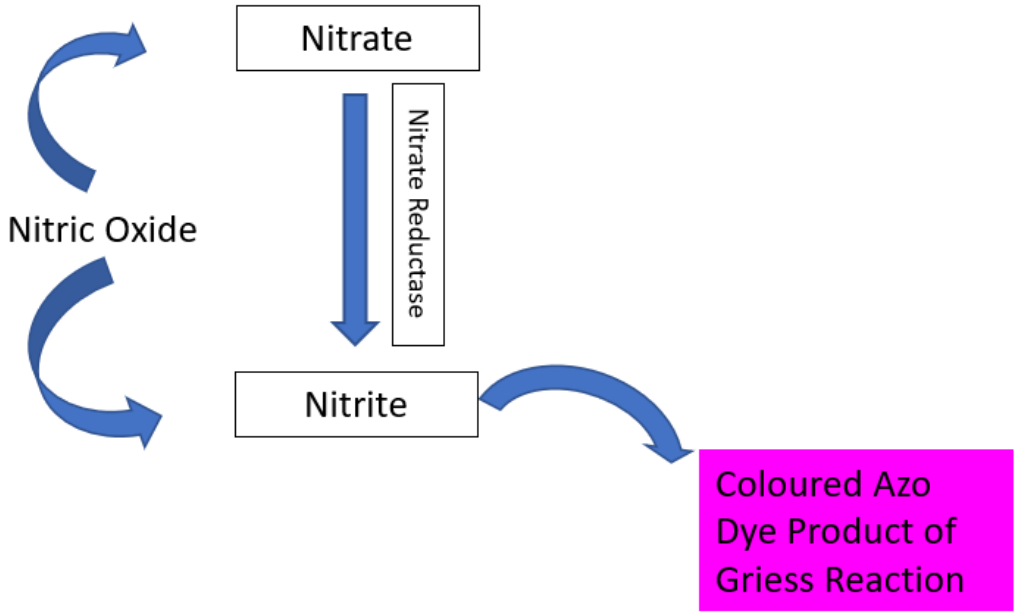


Figure 3.6 Detection of Nitric Oxide using Griess Assay

3.9 Statistical Analyses

Descriptive statistics were completed for all experiments. Where applicable, further statistical analyses were done using IBM SPSS Statistics version 24. Results were tested for normality to inform the decision of parametric or non-parametric tests. Statistical presentation of results was done using graphs and table with indication of standard error of mean (SE). Due to the possible sampling variation that may be observed when multiple samples are collected from the fermented food products that were analysed in the current study, the use of SE seemed to be an appropriate method of expressing variability from the mean of the population sample. The SE has been simply described by Altman & Bland (2005) and Lee, In & Lee (2015) as the standard deviation (SD) of the sampling distribution. SE is not only a more accurate measure of the precision of the sample mean, but it also a function of sample size and more effectively quantifies uncertainties in the estimated sample mean (Barde & Barde, 2012; Biau, 2011).

Chapter 4

Spontaneous vs Controlled Fermentation of Corn in the production of 'Ogi'

4.1 Introduction

Corn, also known as maize, is one of the world's most consumed cereal especially in Africa (Shiferaw, Prasanna, Hellin, & Bänziger, 2011). USA is said to be the largest producer of in the world with South Africa and Nigeria being the largest producing countries in Africa (Badu-Apraku & Fakorede, 2017). Corn is a good source of carbohydrate, but it also contains proteins, oils and B vitamins except B12 and Vitamin C (Ranum, Peña-Rosas, & Garcia-Casal, 2014). There are about 50 varieties of corn but the most common ones are the white and yellow corn kernels (Ranum et al., 2014). Corn can be processed by boiling or roasting of whole grains and by milling into flour to make meals such as porridge and bread (Nuss & Tanumihardjo, 2010). It can also be processed by fermentation (Ezekiel et al., 2018). Fermented corn meals is widely eaten in African countries, especially West African countries (Elmore et al., 2014; Ezekiel et al., 2018).

Traditionally, 'ogi', is spontaneously fermented, with corn kernels submerged in water and indigenous microorganisms present in the kernels, water and environment allowed to ferment the corn (Katongole, 2008; Nyanzi & Jooste, 2012) however outcome is majorly unpredictable (Katongole, 2008). Lactic acid bacteria (LAB) and yeast have been identified as the dominant organisms often isolated from spontaneously fermented corn (Leroy & De Vuyst, 2004). So, few studies have explored starter culture comprising of LAB and/or yeast that may be ideal for the controlled fermentation of corn in the production of 'ogi' and sourdough, and suitability was determined mainly based rate of acidification during fermentation (Edema & Sanni, 2008; Nwachukwu, Achi, & Ijeoma, 2010; Ojokoh, 2009).

4.1.1 Justification of study

The functional potentials of fermented cereal foods have been documented (Capozzi et al., 2017; Ekpa et al., 2018; Hasan, Sultan, & Mar-E-Um, 2014). There are suggestions that these fermented cereal foods may be probiotic based on the fermentation organisms, prebiotic based on the fibre content of whole cereal grains or symbiotic (based on fermentation organisms and fibre in whole grain) (Capozzi et al., 2017; Salmerón, 2017; Ukwuru & Ohaegbu, 2018). There are yet some inconsistencies in the effect outcomes of the studies that have investigated probiotic potential of fermented cereal in the treatment of diarrhoea (Lei, Friis, & Michaelsen, 2006; Lorri & Svanberg, 1994) possibly due to varied study designed such as varied to frequency and quantity of intake. Nevertheless, the risk that unpredictable fermentation organisms may result in inconsistent outcomes when using spontaneously fermentation cereal as an intervention cannot be overlooked.

Therefore, simulating the stages of spontaneous fermentation in controlled fermentation might afford the opportunity to effectively investigate these functional potentials using reproducible and repeatable methods, with the aim to provide reliable and consistent results.

The microbial diversity of fermented corn has been extensively investigated (Adebayo-tayo & Onilude, 2008; Banwo, Sanni, Tan, & Tian, 2012; Folarin Anthony Oguntoyinbo & Narbad, 2012; Oguntoyinbo, Tourlomousis, Gasson, & Narbad, 2011; Oyedeji, Ogunbanwo, & Onilude, 2013; Rahmawati, Dewanti-Hariyadi, Hariyadi, Fardiaz, & Richana, 2013) and some have examined changes in microbial diversity during spontaneous fermentation at intervals during the process of fermentation (Bello, Bello, Amoo, & Atoyebi, 2018; Nwokoro & Chukwu, 2012; Okeke et al., 2015; Omemu, 2011; Oyedeji et al., 2013; Wakil & Daodu, 2011). As far as we know, only one study (Edema & Sanni, 2008) has compared the outcomes of spontaneously fermented sourdough vs controlled fermented sourdough, looking at acidification, diacetyl production and antimicrobial activities, using isolates from spontaneous fermentation of corn as starter culture. This current study aimed to compare fermentation outcomes between spontaneously fermented (SF) 'ogi' vs controlled fermented (CF) 'ogi'.

4.1.2 Objectives of study

The objectives of this study (in this chapter) were:

- To isolate and identify the microorganisms in SF milled corn and use as single and mixed culture starters in CF of milled corn;
- To compare the growth of identified organisms in mixed culture CF vs single-strain CF vs SF;
- To compare the antimicrobial characteristics of the fermentation organisms against strains of *Escherichia coli*.
- To test using *in vitro* static digestion model, the survival of fermentation organisms in the upper digestive tract using a static digestion model

4.2 Methods and Materials

4.2.1 Purchase of Corn

White corn kernels were purchased from a market in Ogun State Nigeria and subsequently milled in the same market using a knife mill (Fritsch Industriestr. 8 0-55743, Idar-oberstein, Germany) (Edema & Sanni, 2008), into the grain size of very fine silt, about 3.9 μm according to Wentworth (1922). Milled corn was then posted to the University of Chester via DHL in July 2017 for analysis. Period of growth and harvest of the corn kernels are unknown.

4.2.2 Spontaneous fermentation of corn

Spontaneous fermentation was done following a modified version of the process described by Ojokoh (2009). Spontaneous fermentation of corn traditionally involves processing stages namely soaking, wet-milling, sieving and soaring (Adebayo-tayo & Onilude, 2008; Edema & Sanni, 2008; Omemu, 2011). Sieving is often done to achieve a smooth texture of 'ogi' after cooking. However, fermentation of dry milled corn or skipping the sieving step is also done by people who are not put off by the coarse textured outcome after cooking (Akingbala, Onochie, Adeyemi, & Oguntimein, 1987). Furthermore, 'ogi' made from whole corn contains higher fibre content that the sifted type (Akingbala et al., 1987) as a result, dry milled whole corn was used in the current study. The ratio of corn to water in the current study was 1:8 in order to have a sufficient amount of supernatant solution for all biochemical analyses. Fifty grams of milled corn was soaked in 400 ml of distilled water and allowed to spontaneously ferment at 30 $\pm$ 2 ^{o}C over 5 days. The chosen temperature is similar to the average room temperature in South-Western Nigeria (Salau, 2016), where 'ogi' is popular produced and consumed (Afolayan, Ayeni, & Ruppitsch, 2017). After 10 seconds of agitation by stirring using sterile rod to ensure uniform mixing, 50 ml of fermenting corn was aseptically harvested at 0 hours of fermentation and at 24-hour intervals until the end of fermentation for microbial and biochemical analyses. Twenty-five milliliters of supernatant solution was used to measure titratable acidity (International Organisation for Standard (ISO), 1998) and pH. Twenty-five milliliters from the 50 ml harvest was temporarily stored at -20^{O}C for later use.

4.2.3 Microbial and biochemical analyses

4.2.3.1 Serial dilution

The established protocol of serial dilution described by (Refai, 1979) was followed. One milliliter of fermented corn was added to 9 ml of sterile ringer solution (Sigma). A 10-fold dilution series was made and appropriate dilutions were plated on MRS agar (Oxoid) for lactic acid bacteria (LAB) growth and Rose Bengal (RB) with 10% Chloramphenicol agar (Oxoid) for yeast growth (Chapter 3). One hundred microliters of appropriate dilutions were aseptically spread on MRS and RB agar plates in triplicates and incubated aerobically at 30^{O}C for 48 hours (Omemu, 2011). Visible single colonies were counted and recorded.

4.2.3.2 Preparation of pure culture

Colonies were selected following protocol described by Omemu, (2011). Up to 10 single colonies each on MRS and RB agar plates from each plate incubated at 0 hours, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours of fermentation were either randomly selected or all selected if plates

contained less than 10 colonies for subculture. Randomisation is described as the golden method that may be employed for unbiased selection during research (Kahan, Rehal, & Cro, 2015; Silvestri et al., 2017; Suresh, 2011). Subcultured colonies were incubated at 30^{O}C overnight. Subculture was done the second time by transferring single colonies from first subculture in order to ascertain purity.

4.2.3.3 Gram stain

Twenty four hours growth of pure culture for all isolated organisms were gram stained using the standard protocol that was published by Public Health England (2014). Thin smear was made on the glass slide and fixed by applying gentle heat. Slide was thereafter flooded with 0.5% crystal violet and left for 30 seconds. Crystal violet was gently rinsed off using tap water and slide was lightly shaken to get rid of excess water. Slide was then flooded with (1%) Lugol's iodine allowed to remain for 30 seconds and gently rinsed off using tap water. Slide was thereafter gently shaken to get rid of excess water. Smear was decolourised with acetone until colour ceased to (usually up to 10 seconds) run out of the smear. Slide was gently rinsed using tap water and slide was gently shaken to get rid of excess water. Slide was flooded the slide 0.1% counterstain safranin and left for 30 seconds to 1min. Slide was gently rinsed using tap water and slide was gently shaken to get rid of excess water. Slide was left to dry completely before dropping immersion oil for observation under the microscope

4.2.3.4 Catalase test

Isolated pure cultures of LAB were tested for catalase status using 3% H_2O_2 (hydrogen peroxide), following a modified version of the procedure described by Public Health England (2018). Four to five drops of H_2O_2 solution were pipetted on a glass slide. A colony from overnight pure culture plate was aseptically picked with a sterile loop and mixed with the hydrogen peroxide solution on glass slide. Observation of immediate bubble formation (effervescence) was an indication that organism was catalase positive. No observed effervescence was an indication that organism was catalase negative.

4.2.3.5 Storage

Overnight cultures of isolates were subcultured into microbank™ vials containing 25 beads following the manufacturer's instruction, for storage at -80^{O}C. A bead was aseptically picked from the vial as required. Vials were labelled based on day organism was cultured from fermenting corn, for example 4.10 – meant tenth colony isolated from 96 hours (Day 4) of fermentation. Frozen cultures were subcultured twice before further analyses.

4.2.3.6 pH and titration

With LAB and yeast being the dominant organisms associated with the spontaneous fermentation of corn (Leroy & De Vuyst, 2004), a decrease in pH resulting from production of lactic acid, and other organic acids depending on the type of LAB, is evidence of a progressive fermentation process (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Decimo et al., 2017). In the current study, the pH of fermenting corn was taken using a METTLER TOLEDO pH meter which was calibrated using freshly made standard buffer solutions at pH 4.0 and 7.0 at room temperature. Buffer solutions provided by the technical staff for by all researcher using the laboratory. The pH was determined by direct insertion of the electrode at room temperature.

Titration is an established method of quantifying total acid concentration in food (Sadler & Murphy, 2010). In the current study, titration was done following protocol for the determination of titratable acidity for fruits and vegetables published by ISO (1998). Twenty-five milliliters of the clear supernatant solution of the fermented corn was diluted with distilled water to 250 ml mark of a volumetric flask. Four drops of phenolphthalein (Sigma), 10 g/l solution in 95 % (v/v) ethanol solution, was added to 50 ml aliquot of fermented corn solution and titrated against 0.1M of NaOH. Titration was done in triplicate. Titratable Acidity (TA), following the protocol was calculated as:

$$TA = \frac{250 \times V1 \times C \times 100}{V \times V0}$$

Where V1 = Volume of NaOH solution used in ml

V0 = Volume of test portion in ml

V = Volume of test sample in ml

C = Molarity of NaOH

4.2.3.7 DNA extraction and sequencing

DNA extraction for all isolates was done using DNeasy Blood & Tissue Kits which was purchased from QIAGEN®. Procedures for DNA extraction as stated by the manufacturers in DNeasy® Blood & Tissue Handbook (Qiagen, 2006) for bacteria cells and QIAGEN Supplementary Protocol for yeast cells were followed with no modifications. DNA of all pure organisms were eluted into sterile Eppendorf tubes and submitted at room temperature to Eurofins Scientific© for 16S and 23S rRNA sequencing. Sequence match with organisms on database were sent via email by the organisation.

4.2.4 Controlled fermentation

Controlled fermentation was doing following method described by Ojokoh (2009). Sterile corn and sterile distilled water were required for controlled fermentation. Fifty gram aliquots of milled corn and duran bottles containing 400 ml of distilled water were sterilized by autoclaving at 121^{O}C, 1.03 psi for 15 mins. Aliquots of sterile corn were stored on the bench at room temperature until used.

4.2.4.1 Preparation of fermentation organisms

As mentioned above, identified LAB and yeasts isolated from spontaneously fermented corn along with 2 organisms previously isolated from spontaneously fermented millet and identified using BioMérieux 50 CH API® strips as *Lb. plantarum* and *Lb. brevis*, were reactivated from cryopreservation by subculturing twice in MRS broth and YPD broth respectively (Chapter 3). Three milliliters of overnight culture of each identified organism was aseptically harvested into sterile 5 ml tubes and centrifuged at 3000 rpm for 10 mins (Jonganurakkun et al., 2008). Supernatant solution was then aseptically discarded using a sterile pipette. Organism pellets were washed by suspension, using 2 ml of sterile ringer solution. Pellets suspension was mixed by gentle inversion until completely mixed and centrifuged at 3000 rpm for 10 mins (Jonganurakkun et al., 2008) and supernatant solution was aseptically discarded using sterile pipette. Washing was done twice before inoculation into sterile corn.

4.2.4.2 Inoculation of sterile corn with fermentation organisms

All fermentation organisms isolated from SF corn were inoculated into sterile corn as single and mixed cultures. Pellets were re-suspended in 2 ml of sterile ringer solution and mixed together by gentle inversion until completely mixed before inoculation into sterile corn. Fifty gram of sterile corn was aseptically transferred into a sterile beaker and mixed with 400 ml sterile distilled water for fermentation. Suspensions of each identified organisms prepared as previously described were aseptically added to labelled sterile beakers containing submerged sterile corn separately as single strain starter culture for fermentation. Entire suspension of each organism was utilized. For mixed culture controlled fermentation, all identified organisms, prepared as previously described were aseptically inoculated into a sterile beakers containing submerged sterile corn. Controlled fermentation was done at 30^{O}C for 5 days

4.2.4.3 Analyses during controlled fermentation

At 0 hours, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours of controlled fermentation, fermenting corn was analysed for microbial and biochemical analyses. Fermenting corn was agitated for 10 seconds by stirring using a sterile rod to ensure uniform mixing after which 1 ml

was aseptically taken and added to 9 ml of sterile ringer solution using sterile pipette, to investigate microbial growth. Serial dilution and plate counting were done as previously described. Fifty milliliters of fermenting corn was also aseptically harvested for biochemical analyses out of which 25 ml of clear supernatant solution was used to measure titratable acidity following procedure previously described. pH of fermenting corn was also taken at these times using a METTLER TOLEDO pH meter. Twenty-five milliliters from the 50 ml harvest was temporarily stored at -20^{O}C for later use.

4.2.5 Digestion of corn

In vitro digestion of both fermented corns (SF and CF) and sterile unfermented corn was undertaken using a standardised static digestion model as described by Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carriere, et al. (2014). The protocol was divided into the oral, gastric and intestinal phases. Simulated digestion fluids were also prepared according to the stated protocol. Constituent of the digestion fluids can be found in the **Table 4.1**.

Table 4.1: **Composition of simulated digestive fluids**

	Simulated Salivary Fluid (SSF) pH 7		**Simulated Gastric Fluid (SGF) pH 3**		**Simulated Intestinal Fluid (SIF) pH 7**	
Constituent	Vol required in ml/100 ml	Conc in SSF (mmol L^{-1})	Vol required in ml/100 ml	Conc in SGF (mmol L^{-1})	Vol required in ml/100 ml	Conc in SIF (mmol L^{-1})
0.5 mol L^{-1} KCl	3.02	15.1	1.38	6.9	1.36	6.8
0.5 mol L^{-1} KH_2PO_4	0.74	3.7	0.18	0.9	0.16	0.8
1 mol L^{-1} $NaHCO_3$	1.36	13.6	2.5	25	8.5	85
2 mol L^{-1} NaCl	Not required	Not required	2.36	47.2	1.92	38.4
0.15 mol L^{-1} $MgCl_2(H_2O)_6$	0.1	0.15	0.08	0.1	0.22	0.33
0.5 mol L^{-1} $(NH_4)_2CO_3$	0.012	0.06	0.1	0.5	Not required	Not required

All fluids were prepared ahead and store in the refrigerator at 4^{O}C. Digestion fluids were utilized after they were brought to 37^{O}C using a water bath.

4.2.5.1 Oral phase

Five milliliters each of fermented corn and suspension of sterile unfermented corn was mixed with 3.5 ml of Simulated Salivary Fluid (SSF) electrolyte stock solution. Half a millilitre of salivary α-amylase solution of 1500 U mL^{-1} was made up in the SSF electrolyte stock solution. Twenty-five microliters of 0.3 M CaCl2 and 975 µl of distilled water was added to the corn mixture and mixed

together for digestion. Oral phase digestion was done in a shaking incubator, shaking at 150 rpm for 2 minutes at $37^{O}C$

Salivary α-amylase solution of 1500 U mL^{-1} was made up using 3 tubes of α-Amylase from human saliva (Type IX-A, 1000–3000 U mg^{-1} protein, Sigma-Aldrich®), containing 500 U each. Whole content of enzyme powder in the 3 tubes was dissolved in 1 ml of SSF. Solution was then mixed together by gentle inversion until totally dissolved. Half a millilitre of the enzyme solution was added to each corn for oral phase of digestion.

4.2.5.2 Gastric phase

The following was added to the output from mouth digestion: 7.5 ml of Simulated Gastric Fluid (SGF) electrolyte stock solution; 1.6 ml porcine pepsin stock solution of 25,000 U/ml made up in SGF electrolyte stock solution; 5 µL of 0.3 M $CaCl_2$, 0.2 mL of 1 M HCl to reach pH 3.0 and 0.695 mL of distilled water. Content was digested in an incubator with integrated shaker (for sufficient mixing), shaking at 150 rpm, for 2 hours at $37^{O}C$. One millilitre was taken and added to 9 ml of sterile ringer solution using sterile pipette, to investigate survival of microorganisms after the gastric phase. Serial dilution, plate counting and culture purification was done as previously described. DNA of pure isolated organisms were extracted as previously described and submitted to Eurofins Scientific© for 16S and 23S rRNA sequencing

To make up the porcine pepsin solution, porcine pepsin activity (units/mg) was estimated from porcine gastric mucosa lyophilized powder (Sigma-Aldrich®) containing 3,200-4,500 units/mg protein. Porcine pepsin activity was estimated as the mean value of unit range provided - 3,850 units/mg protein. Quantity of enzyme required was estimated by dividing the final U/ml (25,000) by 3,850 units/mg protein. Therefore, 13 mg of porcine was dissolved in 2 ml of SGF. Solution was mixed together by gentle inversion until totally dissolved. The required portion (1.6 ml) was added to the output from oral phase for gastric digestion.

4.2.5.3 Intestinal phase

Output from gastric phase of digestion was mixed with 11 ml of Simulated Intestinal Fluid (SIF) electrolyte stock solution including 5.0 ml of a pancreatin solution 800 U/ml made up in SIF electrolyte stock solution, 160 mM of bile from bile extract porcine (Sigma-Aldrich®), 40 ml of 0.3 M $CaCl_2$, 0.15 ml of 1 M NaOH to reach pH 7.0 and 1.31 ml of distilled water. The quantity of pancreatin solution was estimated from pancreatin from porcine pancreas, 8 × USP specifications (Sigma-Aldrich®) following the protocol described by (Minekus, Alminger, Alvito, Ballance,

Bohn, Bourlieu, Carrière, et al., 2014). Dissolution of pancreatin was difficult. Although the protocol mentioned dissolution in 1 mM HCl, comparison of enzymatic activity of pancreatin was investigated when dissolved in 1 mM HCl vs 70% Ethanol with PBS (50/50) vs DMSO/PBS (50/50) in concentration of 1 mg/ml. In all 3 solvents, dissolution was facilitated by 10 minutes of magnetic stirring, sonication for 10 minutes @ 37^{O}C. The powder was not completely dissolved therefore suspension was centrifuged at 4000 rpm for 3 mins for separation. Ten milliliters of 46 mM TRIS/HCl buffer, containing 11.5 mM $CaCl_2$ for pH adjustment to 8.1 was prepared and 3 aliquots of 2.6 ml was pipetted into quartz cuvettes. Test substrate was prepared with p-Toluene-Sulfonyl-L-arginine methyl ester (TAME), to 10 mM using sterile water and 0.3 ml was added into each quartz cuvettes containing TRIS/HCl and gently mixed together by inversion. Each cuvette was placed in a spectrophotometer and with wavelength set at 247 nm, absorbance was recorded. Enzymatic activity in each diluent was tested by adding 100 µl of supernatant solution from dissolved enzyme in each quartz cuvettes. Absorbance at every 50 seconds was recorded for 10 minutes. A graph of absorbance against time was draw and slope of the straight line part of the curve was determined. Slopes (unit absorbance/minute) for 1 mM HCl, 70% Ethanol with PBS (50/50), DMSO/PBS (50/50) were approximately 0.1 (0.100, 0.098, 0.108 respectively) therefore dilution for pancreatin was done using 1 mM HCl

Pancreatin was dissolved in 1 mM HCl (pH 3) with final concentrations being 0.5 mg/ml, 0.75 mg/ml and 1 mg/ml and dissolution was facilitated as described above. Absorbance and slope were determined as described above. Enzymatic activity at each concentration was determined using the following equation

$$Units/mg = \frac{[(\Delta A247\ Test - \Delta A247\ Blank) * 1000 * 3]}{(540 * X)}$$

Δ247 = slope of the initial linear portion of the Δ curve, [unit absorbance/minute] for the Test (with enzyme) and Blank
540 = molar extinction coefficient (L/(mol × cm) of TAME at 247 nm
3 = Volume (in millilitres) of reaction mix
X: quantity of trypsin in the final reaction mixture (quartz cuvette) [mg]

Mean of the 3 values of enzymatic activity (based on 3 concentration of dilutions) – 5.007 U/mg, was used to determine quantity of pancreatin required for digestion. Therefore, 800 mg of porcine pancreas dissolved in 5 ml of SIF was required for a 5 ml pancreatin solution containing 800 U/ml.

Quantity of bile extract required was determined using the predetermined concentration of bile salts in bile extract porcine by Tullberg et al. (2016) – 2.6 mM. Therefore, 61.5 mg of bile extract was added to 5 mls of previously prepared pancreatin solution in order to achieve a final bile salt concentration of 160 mM.

Content was digested in an incubator with integrated shaker (for sufficient mixing), shaking at 150 rpm, for 2 hours at 37^{O}C. One milliliter was taken and added to 9 ml of sterile ringer solution using sterile pipette, to investigate survival of LAB after the intestinal phase. Serial dilution, plate counting and culture purification was done as previously described. DNA of pure isolated LAB were extracted as previously described and submitted to Eurofins Scientific© for 16S and 23S rRNA sequencing. Digestion output was immediately and temporarily stored in – 20^{O}C until further analyses

4.2.6 Antimicrobial characteristics against *E. coli*

Pure culture of each isolated organism and supernatant solutions of both types of fermented corn were tested for antimicrobial characteristics against non-pathogenic diarrhoea causing organisms which were available in the microbiology laboratory, Biological Science Department, University of Chester - two strains of *E. coli*; *E. coli* B and *E. coli* K12. Adapted protocol for agar well diffusion method for antibacterial and antifungal activity as described by Pundir, Rana, Kashyap, and Kaur (2013) was used.

4.2.6.1 Preparation of test organisms

Test organisms were inoculated separately into media they are typically grown in the lab - sterile nutrient broth and incubated at 30^{O}C for 24 hours. To ensure sufficient microbial growth, Optical density of overnight culture was determined at wavelength of 600 nm. Minimum absorbance of 0.132, which is equivalent to 0.5 Mc Farland standard was taken as enough growth for the test (Pundir et al., 2013). Three and a half milliliters of sterile soft nutrient agar, containing 0.6 % of agar was inoculated with 100 µl of test organism, mixed together and overlaid on an already prepared sterile Nutrient Agar (containing 12 % agar) plate to make a lawn. Plates were thereafter allowed to dry and solidify. Wells with about 6 mm diameter were aseptically bored on the overlaid agar inoculated with the test organisms.

4.2.6.2 Screening of isolated bacterial cultures for antimicrobial activity

To test for antimicrobial properties, bored wells in overlaid plates containing test organism were filled with 100 µl of broth of overnight pure culture of isolated organisms and sterile supernatant solutions of both types of fermented corn. Plates were incubated at $30^{O}C$ for 24 hours. Inhibition zone around the wells was interpreted as indication for antimicrobial and antagonistic activities. Width of inhibition zones were measured and recorded. All tests were done in triplicate.

4.2.7 Statistical analysis

Microbial population data obtained was not normally distributed, and was therefore subjected to non-parametric tests. Mann Whitney U test was carried out to compare microbial growth during spontaneous vs controlled fermentation. Friedman ANOVA test was done to compare the growth of organisms over time under varied fermentation conditions. Wilcoxon test was done to compare microbial population during digestion process.

4.3 Results

4.3.1 Microbial population during spontaneous fermentation

There were no visible LAB and yeast cells at 0 hour of fermentation. This is contrary to studies such ones by Nwokoro and Chukwu (2012), Kigigha, Izah, and Okowa (2016), Wakil and Daodu (2011), Omemu (2011) and Bello et al. (2018) who recorded LAB and yeast growth at 0 hour of spontaneous fermentation. According to the authors, corn purchase and spontaneous fermentation was done in Nigeria. However, in the current study, though corn was purchased in Nigeria, spontaneous fermentation was done in the UK. A notable change in the environmental factors such as temperature may have influenced rate and pattern of microbial growth resulting in the mentioned contrary results (Dong, Jiang, Yu, Liu, & Zhang, 2010). There was no obvious growth of yeast cells for 48 hours, even at the lowest dilution factor (10^{-1}) until at 72 hours, indicative of increased population of culturable yeast cells at this stage of fermentation. Yeast cells continued to increase until 120 hours of fermentation with a population of $1.1 \times 10^{4} \pm 1.9 \times 10^{3}$ cfu/ml. On the other hand, there was visible microbial growth on MRS agar at 24 hours of fermentation. Maximum LAB population ($6.7 \times 10^{10} \pm 1.3 \times 10^{10}$ cfu/ml) was at 48 hours of fermentation, and LAB started to decrease at 72 hours of fermentation. At 120 hours of fermentation, population of culturable LAB was $3.7 \times 10^{9} \pm 6.7 \times 10^{8}$ cfu/ml (**Figure 4.1**). A LAB and yeast growth of up to 10^{14} and 10^{13} respectively in 48 hours of spontaneous until 72 hours and yeast has been reported by Wakil and Daodu (2011). On the other hand, Omemu (2011) reported a maximum growth of 10^{8} and 10^{7} for LAB and yeast after 48 hours, Nwokoro and Chukwu (2012) reported 10^{8} and 10^{5} as maximum growth of LAB and yeast in 3 days of fermentation. Bello et al. (2018) also reported a maximum

LAB of 10^8 in 96 hours of fermentation did not report any yeast growth after 48 hours. Microbial growth in spontaneous fermentation is very fluid and easily influenced by many variables giving it some degree of unpredictability especially with LAB being known to be fastidious about growth conditions (Hayek & Ibrahim, 2013; Mohammadi, Sohrabvandi, & Mortazavian, 2012). This often results in notable variations in the reported growth amongst studies.

Further effect of change in environment may have been observed in the number of isolates that did not grow after the first subculture. Over 120 hours of spontaneous fermentation, 49 single colonies (10 from 24 hours; 8 from 48 hours; 10 from 72 hours; 11 from 96 hours and 10 from 120 hours) and 12 single colonies were randomly isolated from the MRS and RB plates respectively. Six out of the 49 isolates from MRS agar did not grow after the first subculture from mixed culture plates. All 12 yeast isolates survived the second subculture for purification. All isolates were gram positive and all 43 LAB isolates were catalase negative organisms. View of gram stained LAB under 100x oil immersion microscope showed that isolated LAB had rod and cocci shapes (**Table 4.2**).

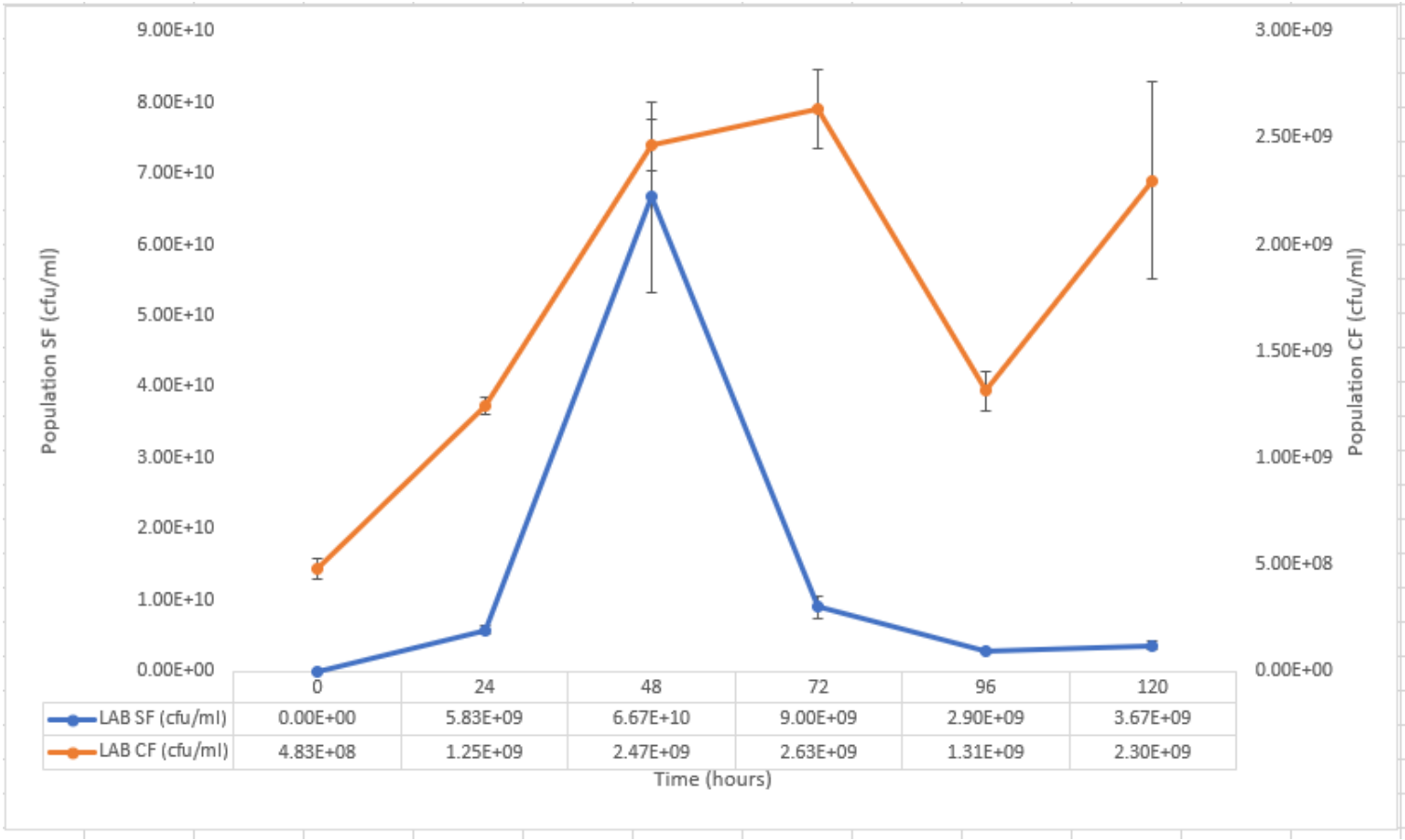
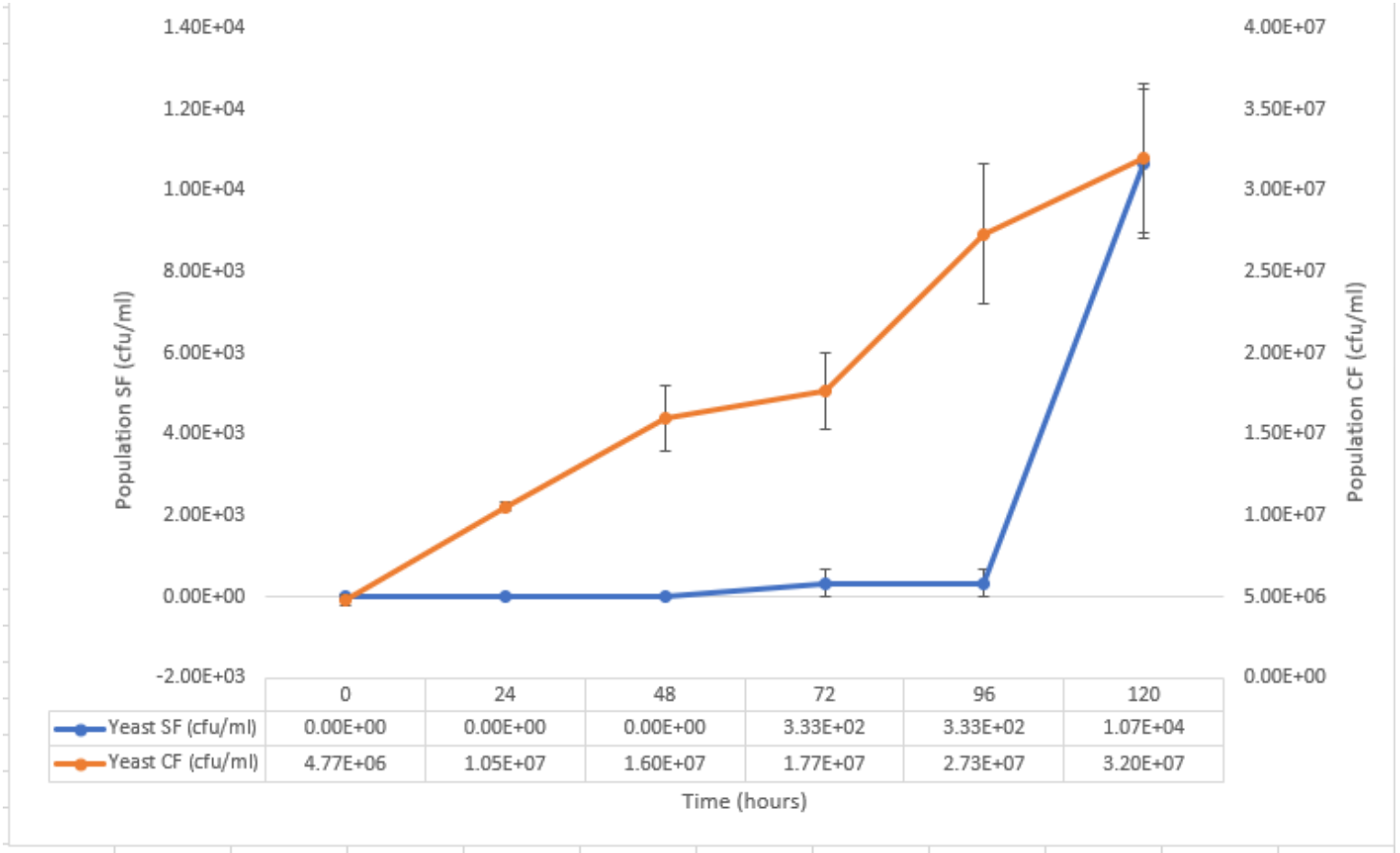


Figure 4.1a: Growth trend for LAB during Spontaneous Fermentation (SF) vs Controlled Fermentation (CF). One ml of fermented sample at intervals of 24 hours over 120 hours of fermentation was inoculated on MRS and incubated at 30 $\pm$ 2 ^{O}C for 48 hours. Data shown is the mean number of colonies $\pm$ SE of triplicate inoculations of 1 cycle of fermentation



	0	24	48	72	96	120
Yeast SF (cfu/ml)	0.00E+00	0.00E+00	0.00E+00	3.33E+02	3.33E+02	1.07E+04
Yeast CF (cfu/ml)	4.77E+06	1.05E+07	1.60E+07	1.77E+07	2.73E+07	3.20E+07

Figure 4.1b: Growth trend for Yeast during Spontaneous Fermentation (SF) vs Controlled Fermentation (CF). One ml of fermented sample at intervals of 24 hours over 120 hours of fermentation was inoculated on Rose Bengal with 10% Chloramphenicol and incubated at 30 $\pm$ 2 ^{O}C for 48 hours. Data shown is the mean number of colonies $\pm$ SE of triplicate inoculations one 1 cycle of fermentation.

Table 4.2: **The shape of LAB isolates after gram stain**

Isolate No	24 hrs (day 1)	48 hrs (Day 2)	72 hrs (Day 3)	96 hrs (Day 4)	120 hrs (Day 5)
1	No growth	rods	rods	No growth	cocci
2	rods	rods	No growth	Rods	rods
3	No growth	rods	rods	Rods	rods
4	rods	rods	rods	Cocci	rods
5	rods	rods	rods	Rods	rods
6	No growth	rods	cocci	Rods	rods
7	No growth	rods	rods	Rods	rods
8	rods	rods	rods	Rods	rods
9	rods	-	rods	Rods	rods
10	rods	-	rods	Rods	cocci
11	-	-	-	Cocci	-

No growth indicates that organism did not grow after subculture from plates with mixed culture; - indicates no isolation

4.3.2 Identification of fermentation organisms

Surprisingly, outcome of 16S rRNA sequencing of LAB isolates indicated that the 43 LAB isolates were 100 % identical to only 2 different organisms *Weissella confusa* and *Pediococcus pentosaceus* with *W. confusa* being more dominant, appearing from 24 hours of fermentation to being 8 out of the 10 isolates after 120 hours (**Table 4.3a)**. *P. pentosaceus* was detected after 72 hours till 120 hours of fermentation, though having the frequency of 1 out of 9 isolates. The 2 organisms previously identified from millet, *Lb plantarum* and *Lb brevis* (identification using BioMérieux 50 CH API®) were 100 % identical to *Lb plantarum* and *W. confusa* respectively. Though morphological and biochemical identification methods provide useful information but they are known not to be reliable and to be prone to errors, giving rise to the need for genotypic identification (Donelli, Vuotto, & Mastromarino, 2013). In this study, *W. confusa* from millet was labelled as *W. confusa* (2). 16S and 23S rRNA sequencing of the 12 yeast isolates showed 100 % similarity with *Saccharomyces cerevisiae* (**Table 4.3b)**

Table 4.3a: **16S rRNA sequencing outcome for LAB isolates**

Isolate No	24 hrs (day 1)	48 hrs (Day 2)	72 hrs (Day 3)	96 hrs (Day 4)	120 hrs (Day 5)
1		*W. confusa*	*W. confusa*		*P. pentosaceus*
2	*W. confusa*	*W. confusa*		*W. confusa*	*W. confusa*
3		*W. confusa*	*W. confusa*	*W. confusa*	*W. confusa*
4	*W. confusa*	*W. confusa*	*W. confusa*	*P. pentosaceus*	*W. confusa*
5	*W. confusa*	*W. confusa*	*W. confusa*	*W. confusa*	*W. confusa*
6		*W. confusa*	*P. pentosaceus*	*W. confusa*	*W. confusa*
7		*W. confusa*	*W. confusa*	*W. confusa*	*W. confusa*
8	*W. confusa*	*W. confusa*	*W. confusa*	*W. confusa*	*W. confusa*
9	*W. confusa*	-	*W. confusa*	*W. confusa*	*W. confusa*
10	*W. confusa*	-	*W. confusa*	*W. confusa*	Mixed culture
11	-	-	-	*P. pentosaceus*	-

- indicates no isolation

Table 4.3b: 16S and 23S rRNA sequencing outcome for yeast isolates

Isolate No	24 hrs (day 1)	48 hrs (Day 2)	72 hrs (Day 3)	96 hrs (Day 4)	120 hrs (Day 5)
1	-	-	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*
2	-	-	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*
3	-	-	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*
4	-	-	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*

- indicates no isolation

4.3.3 Microbial population during controlled fermentation

In CF, using the mixed strain starter culture containing *W. confusa, P. pentosaceus*, *Lb. plantarum, W. confusa* (2) and *S. cerevisiae*, the starting population (at 0 hours) of yeast and LAB were 4.8 x $10^6 \pm 3.3 \times 10^5$ cfu/ml and 4.8 x $10^8 \pm 4.9 \times 10^7$ cfu/ml respectively. The population of yeast continued to increase throughout the 5 days of fermentation recording a population of 3.2 x $10^7 \pm$ 4.6 x 10^6 cfu/ml at 120 hours. Unlike during SF, maximum LAB population was recorded at 72 hours of fermentation 2.6 x $10^9 \pm 1.7 \times 10^8$ cfu/ml which was lower than the maximum population of LAB during SF at 48 hours (**Figure 4.1**). By 120 hours of fermentation, population of LAB had slightly dropped to 2.3 x $10^9 \pm 4.6 \times 10^8$ cfu/ml, less than the population of culturable LAB in SF at 120 hours (3.7 x $10^9 \pm 6.7 \times 10^8$ cfu/ml. Mann Whitney U test, using SPSS version 24, shows that overall, there was a statistical difference in trend of LAB and yeast growths during SF vs CF suggesting a significantly higher ($p < 0.001$) and lower growth ($p < 0.001$) rate for LAB and yeasts respectively. However, due to low sample size (3 in each group as experiments were done in triplicates), and wide variations within groups, further Mann Whitney U tests did not show any statistical difference in the growth of LAB and yeast at all times of analyses. Differences in microbial population during SF and CF could be an indication of variabilities in the microbiota in both corns at the commencement of fermentation. Furthermore, sterilizing milled corn using the autoclave, though with no obvious change in physical conditions such as colour change, may have resulted in decreased availability of nutrients to fermentation as suggested by McAllister, Rode, Cheng, and Forsberg (1991). Friedman Test suggested that there was a significant difference in the growth of LAB and yeast cells over time. LAB during SF and CF, showed a significant change in population over time ($p = 0.02$ and 0.01 respectively). Same was recorded for yeast cell growth over time in both processes ($p = 0.03$ and 0.01 respectively). However post-hoc Wilcoxon test did not give any indication of where the significant difference lied in both organisms during spontaneous which may be due to the sample size and wide variations within groups. Nonetheless, significant differences in cell counts were observed at 0 hour vs 24 hours for LAB and yeast during controlled fermentation with $p < 0.05$ in both organisms.

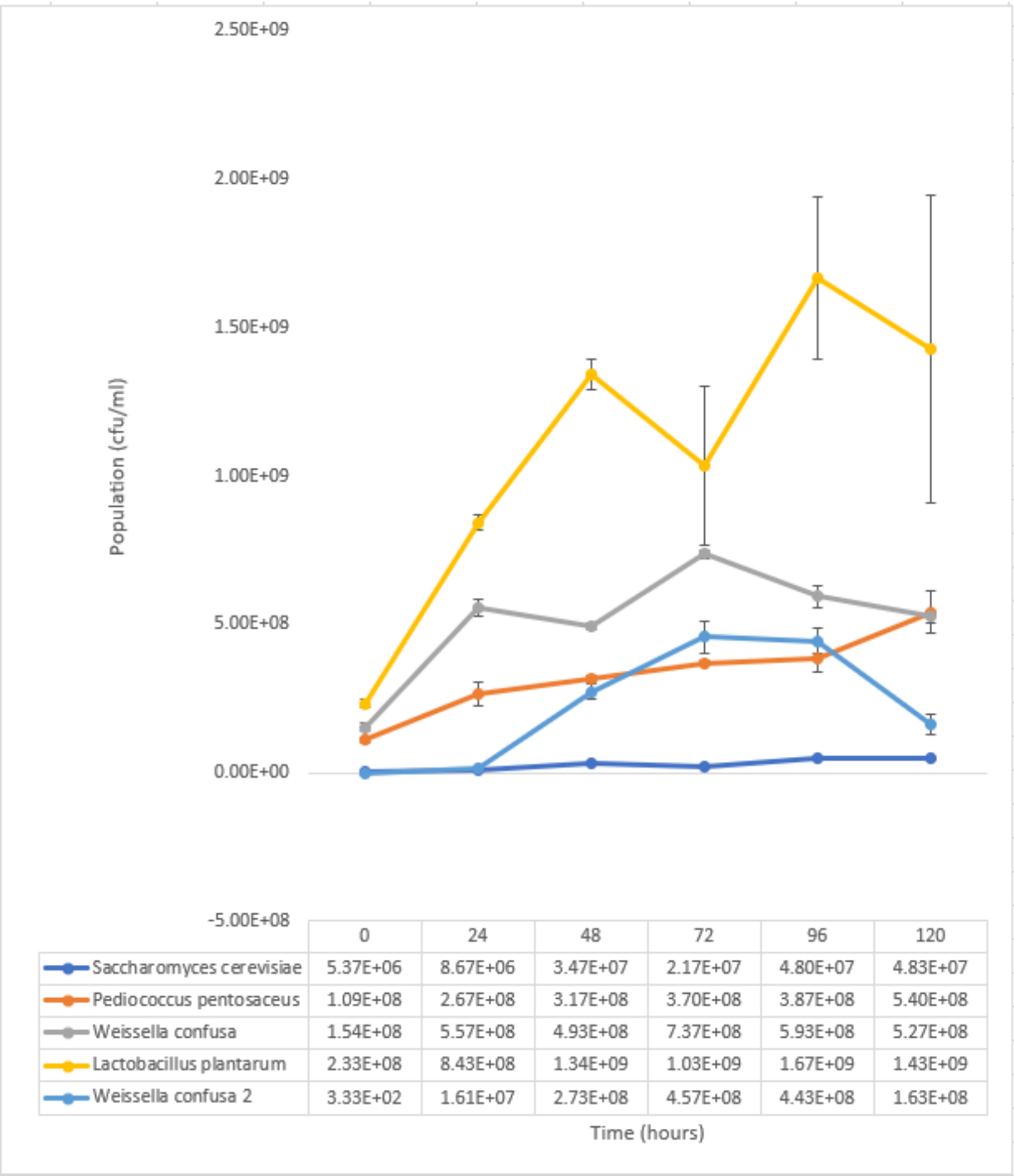
Inoculum sizes (0 hours) for *W. confusa, P. pentosaceus*, *Lb. plantarum, W. confusa* (2) and *S. cerevisiae* for single strain fermentation were 1.5 x $10^8 \pm 1.3 \times 10^7$ cfu/ml, 1.1 x $10^8 \pm 1.1 \times 10^7$, 2.3 x $10^8 \pm 1.5 \times 10^7$, 3.3 x $10^2 \pm 3.3 \times 10^2$ and 5.4 x $10^6 \pm 6.9 \times 10^5$ respectively (**Figure 4.2**). *W. confusa* and *W. confusa* (2) reached their peak growth at 72 hours of fermentation with a population of 7.4 x $10^8 \pm 1.3 \times 10^7$ and 4.6 x $10^8 \pm 5.2 \times 10^7$ respectively, after which the death phase started. At 120 hours of fermentation, viable count for both organisms were 5.3 x $10^8 \pm 2.0 \times 10^7$ and 1.6 x $10^8 \pm 3.4 \times 10^7$ respectively. On the other hand, *P. pentosaceus* continued to grow throughout the

5 days of fermentation with viable count of $5.4 \times 10^8 \pm 7.2 \times 10^7$ at 120 hours of fermentation. *Lb plantarum* exhibited the highest growth rate of all the LAB with viable counts at 72 hours, 96 hours and 120 hours being $1.0 \times 10^9 \pm 2.7 \times 10^8$, $1.7 \times 10^9 \pm 2.7 \times 10^8$ and $1.4 \times 10^9 \pm 5.2 \times 10^8$ respectively.

S. cerevisiae showed the least growth rate amongst organisms used in single strain CF. Though slowly, *S. cerevisiae* continued to grow in the first 4 days of fermentation but appeared to have reached stationary phase between 96 hour and 120 hours of fermentation with populations of $4.8 \times 10^7 \pm 1.7 \times 10^6$ and $4.8 \times 10^7 \pm 3.3 \times 10^6$ respectively. The slow growth of *S. cerevisiae* may be attributed to the carbohydrate composition of corn. *S. cerevisiae* is generally known to be non-amylolytic (van Zyl, Bloom, & Viktor, 2012), hence are unable to degrade raw starch (Yamakawa, Yamada, Tanaka, Ogino, & Kondo, 2012) which is the most abundant carbohydrate in corn. All the same, the observed level of growth of this organism may be associated with the amylolytic enzymes present in corn endosperm (Gangadharan & Sivaramakrishnan, 2009; Oyewole & Agboola, 2011).

4.3.4 pH and titratable acidity

Decrease in pH during fermentation is an indication that there is production of organic acids which do not only inhibit the growth and survival of spoilage organisms (Bouthyette, 2008) but also enhance the flavour of the fermenting food (Sekar & Mariappan, 2007). The typical pH for indigenously fermented foods is often recorded as being below 5 and with the possibility of reaching as low as 3.0 (Akabanda, Owusu-Kwarteng, Tano-Debrah, Parkouda, & Jespersen, 2014; Edema & Sanni, 2008; Hasan et al., 2014; Izah, Kigigha, & Okowa, 2016; Montet, Ray, & Zakhia-Rozis, 2014; Nwachukwu et al., 2010). pH is nonetheless dependent on the type of organisms and the type of substrate available for fermentation (Blandino et al., 2003). As expected, there was a notable drop in pH at the end of both fermentations with pH of 3.35 in SF and 4.42 in CF corn at 120 hours (**Figure 4.3**). Similarly, Nwokoro and Chukwu (2012) and Wakil and Daodu (2011) reported a final pH of 3.8 and 3.9 after 72 hours of fermentation. A lower pH in SF corn than in the CF corn may suggest that there was more production of organic acid during SF than during CF. Furthermore, titratable acidity at 120 hours of fermentation were 6.7 g/l for SF and 1.3 g/l for CF, further suggesting that there was a notable variation in the fermentation microbiota in both processes. During single strain fermentation, *Lb. plantarum* recorded the lowest pH, 4.12, at 120 hours, whilst *S. cerevisiae* recorded the highest, 5.56. *W. confusa* and *W. confusa* (2) recorded a final pH of 5.01 and 4.82 respectively which may suggest that both organisms may be different strains.



	0	24	48	72	96	120
Saccharomyces cerevisiae	5.37E+06	8.67E+06	3.47E+07	2.17E+07	4.80E+07	4.83E+07
Pediococcus pentosaceus	1.09E+08	2.67E+08	3.17E+08	3.70E+08	3.87E+08	5.40E+08
Weissella confusa	1.54E+08	5.57E+08	4.93E+08	7.37E+08	5.93E+08	5.27E+08
Lactobacillus plantarum	2.33E+08	8.43E+08	1.34E+09	1.03E+09	1.67E+09	1.43E+09
Weissella confusa 2	3.33E+02	1.61E+07	2.73E+08	4.57E+08	4.43E+08	1.63E+08

Figure 4.2: Trend of microbial population during controlled fermentation with single strain starter culture. One ml of fermented sample at intervals of 24 hours over 120 hours of fermentation was inoculated on appropriate medium and incubated at 30 $\pm$ 2 ^{O}C for 48 hours. Data shown is the mean number of colonies $\pm$ SE of triplicate inoculations of 1 cycle of fermentation.

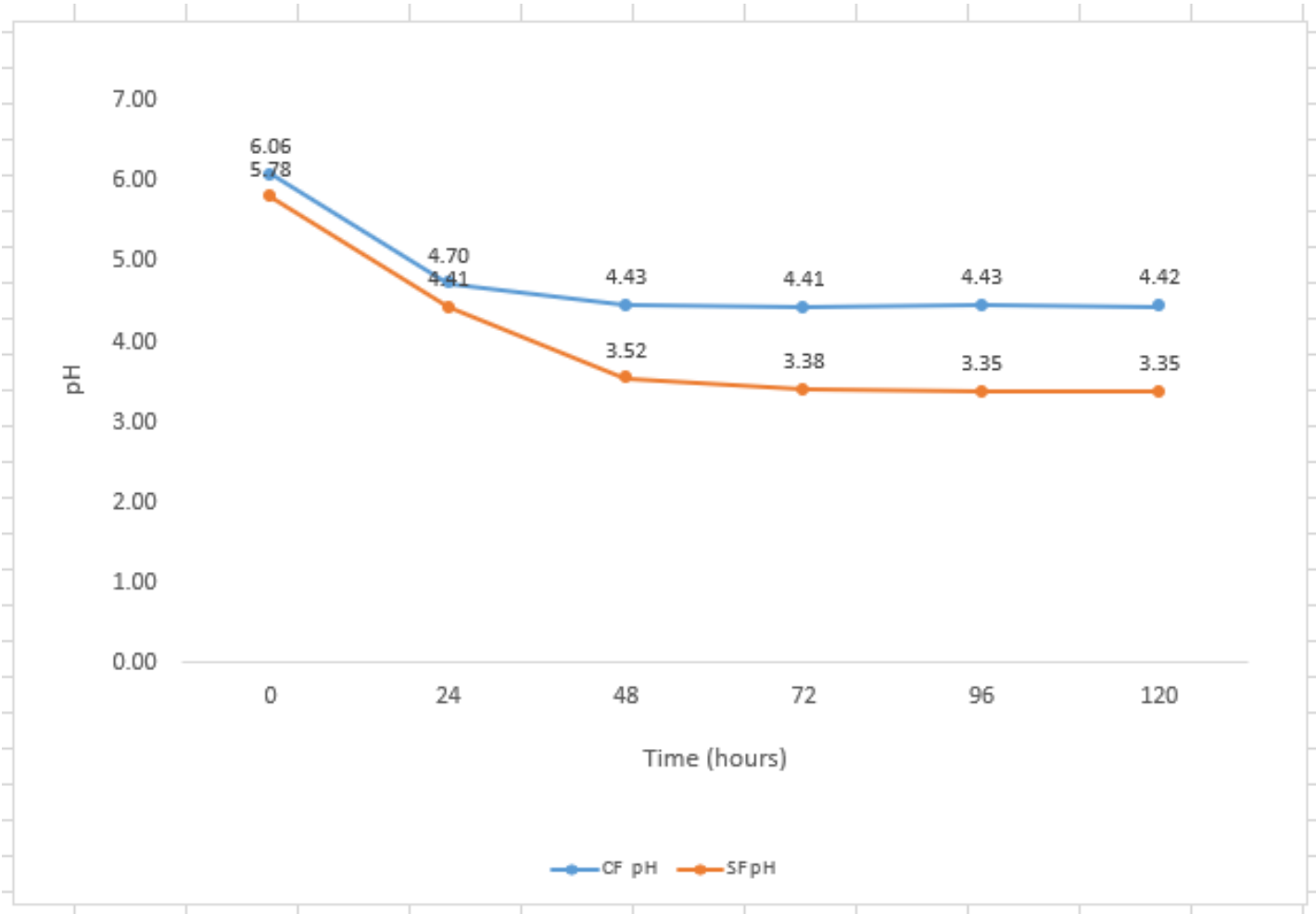


Figure 4.3a: Trend of pH change during spontaneous and controlled fermentation of corn. pH was taken during spontaneous and controlled fermentation of corn at intervals of 24 hours over 120 hours of fermentation. Data shows pH of 1 cycle of fermentation at specified time.

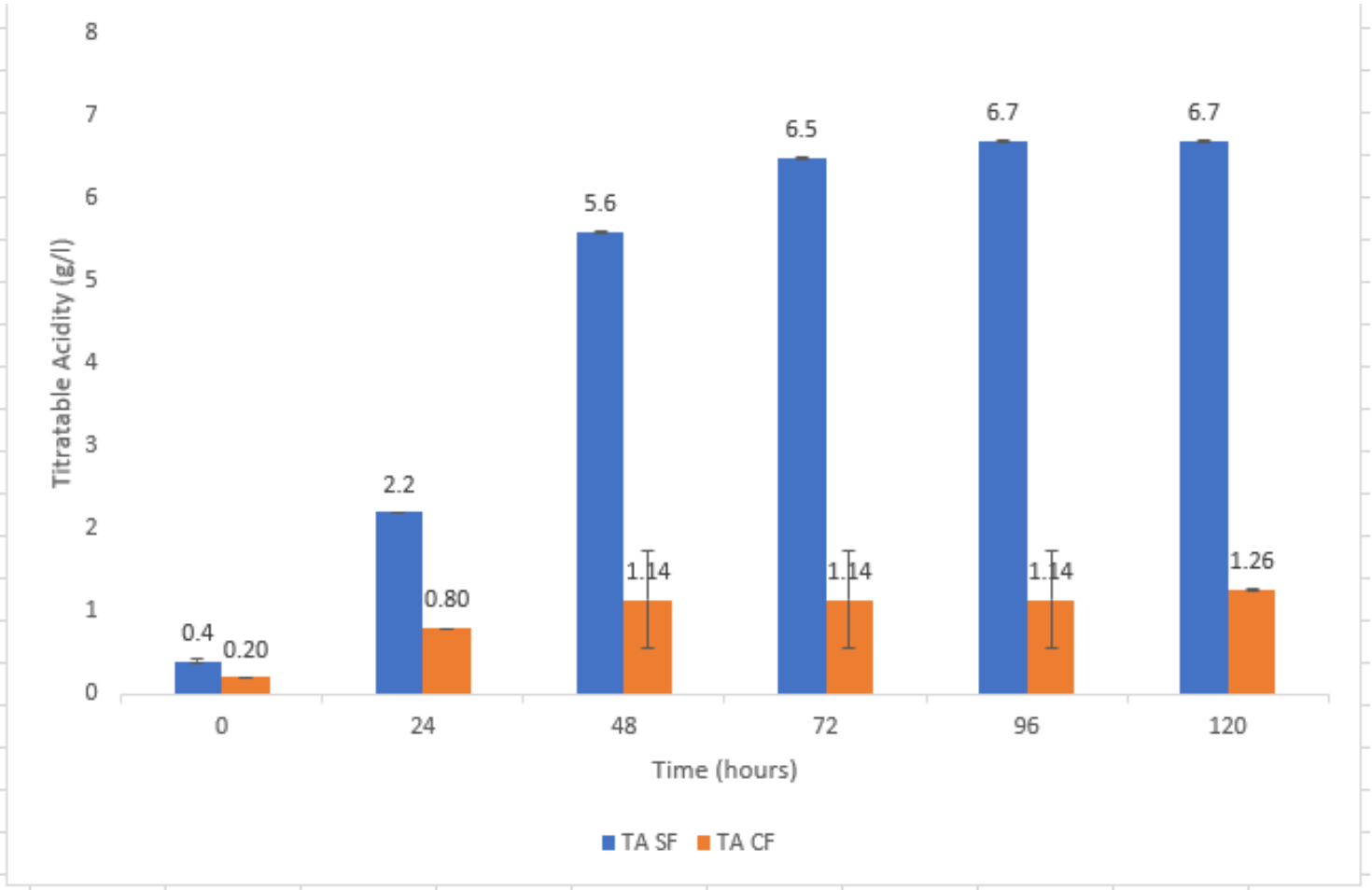


Figure 4.3b: Titratable Acidity during spontaneous and controlled fermentation of corn. Titratable acidity at intervals of 24 hours over 120 hours of spontaneous and controlled fermentation was obtained and calculated using the protocol and formula published by ISO (1998). Data shows mean titratable acidity value of triplicate analyses of 1 cycle of fermentation at stated time $\pm$ SE

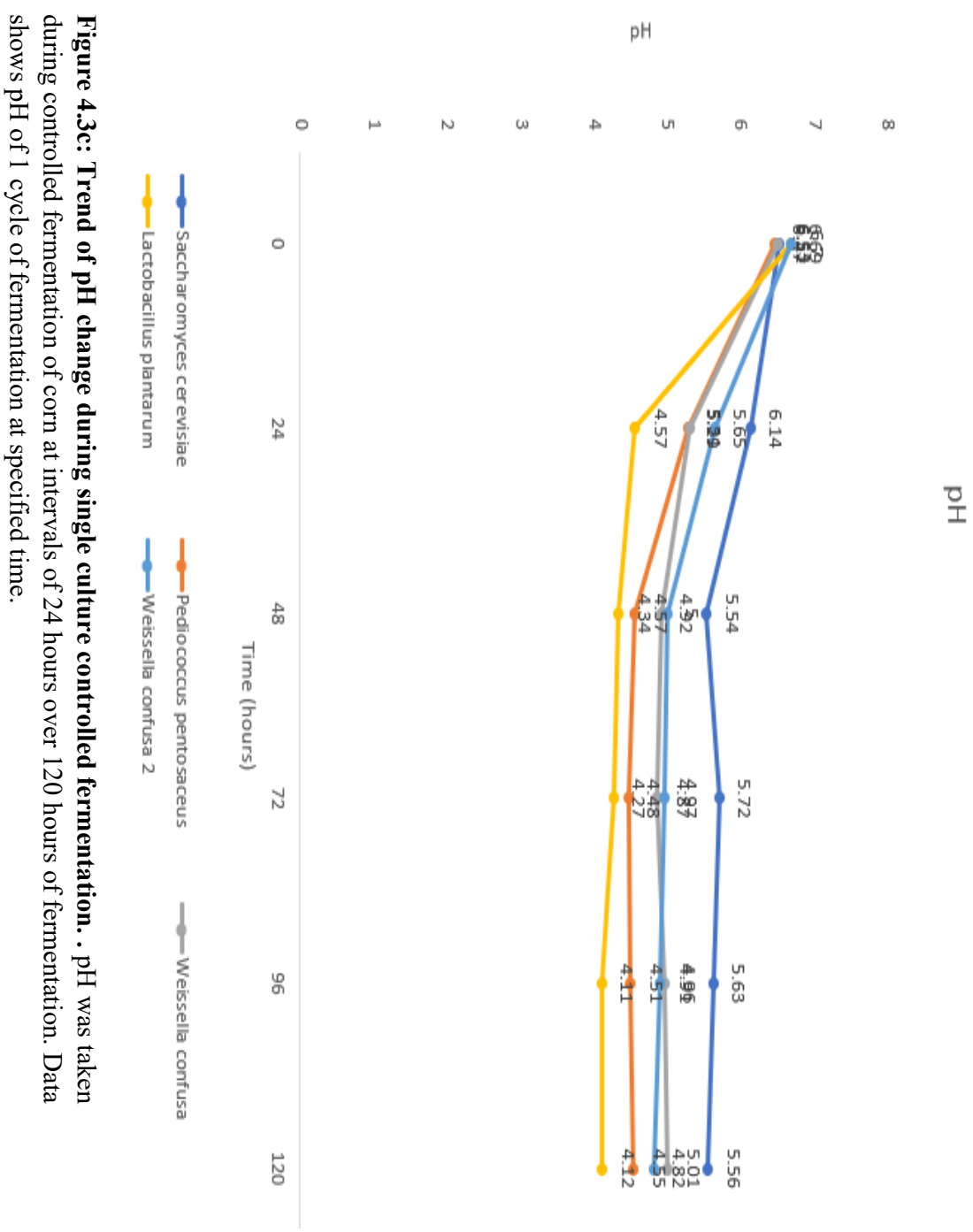


Figure 4.3c: Trend of pH change during single culture controlled fermentation. . pH was taken during controlled fermentation of corn at intervals of 24 hours over 120 hours of fermentation. Data shows pH of 1 cycle of fermentation at specified time.

4.3.5 Aromatic characteristics

SF corn is known for its aromatic smell due to the production of aromatic compounds during the process (Annan, Poll, Sefa-Dedeh, Plahar, & Jakobsen, 2003; Annan, Poll, Plahar, & Jakobsen, 2003; Jespersen, 2003). Aromatic smell produced during SF in this study was instantaneously observed from 24 hours to the end of the fermentation, however there was no observation of this aroma in any of the controlled fermentation settings. Production of aromatic compounds during

fermentation is dependent on the strains of yeast involved (Callejo, González, & Morata, 2017) and on the interaction between the organisms involved in the fermentation process (Jespersen, 2003).

4.3.6. *In-vitro* upper digestion

Survival in the harsh conditions during upper digestion is one of the many requirements for an organism to be probiotic. The pH in the human upper digestive tract is acidic in the stomach with the possibility of reaching a pH as low as 1.5 during fasting and about 3.1 after a meal (Kong & Singh, 2008) while the small intestine ranges from 6 at the duodenum to 7.4 at the terminal of the small intestine (Nugent, Kumar, Rampton, & Evans, 2001). In the current study, the pH in the stomach phase was adjusted to 3 and the intestinal phase 7, before digestion. Baseline population of all organisms was taken to be population at 120 hours. Viable count of LAB in SF after gastric and intestinal digestions were 0.07% and 0.04% of the baseline respectively (**Table 4.4**) though there was no growth of visible colonies on yeast after gastric intestinal digestion. On the hand, there was greater survival of organisms in CF. Survival of LAB and yeast after gastric digestion were 17.54% and 27.60% respectively of baseline, which was the cell count at 120 hours. After intestinal digestion, viable count of LAB and yeasts were $5.4 \times 10^8 \pm 1.2 \times 10^7$ (23.30% of baseline) and $2.7 \times 10^6 \pm 3.2 \times 10^5$ (8.54% of baseline) respectively.

Sixteen colonies from CF and 12 colonies from SF were randomly selected from MRS agar for molecular identification. All 12 organisms isolated from digested spontaneously fermented corn were 100 % identical of *P. pentosaceus* after 16S rRNA sequencing. On the other hand, 15 out of the 16 colonies from digested controlled corn fermentation showed 100 % similarity to *Lb. plantarum* and *P. pentosaceus*. Survival of *Lb. plantarum* in *in-vitro* and *in-vivo* digestion was also reported by van Bokhorst-van de Veen, van Swam, Wels, Bron, and Kleerebezem (2012) who investigated the survival of various strains of *L. plantarum* during digestion, however not all tested strain survived the harshness of the upper GIT.

Table 4.4: Viable count of organisms during *In-vitro* Static Digestion

	LAB SF	LAB CF	Yeast SF	Yeast CF
120 hours	$3.7 \times 10^9 \pm 6.6 \times 10^8$	$2.3 \times 10^9 \pm 4.6 \times 10^8$	$1.1 \times 10^4 \pm 1.9 \times 10^3$	$3.2 \times 10^7 \pm 4.6 \times 10^6$
Gastric Digestion	$2.5 \times 10^6 \pm 5.8 \times 10^4$	$4.0 \times 10^8 \pm 1.2 \times 10^7$	0.0	$8.8 \times 10^6 \pm 6.7 \times 10^5$
Intestinal Digestion	$1.6 \times 10^6 \pm 2.3 \times 10^5$	$5.4 \times 10^8 \pm 1.2 \times 10^7$	0.0	$2.7 \times 10^6 \pm 3.2 \times 10^5$

4.3.7 Antimicrobial effects against *E. coli*

S. cerevisiae and *W. confusa* (2) did not exhibit any antimicrobial characteristic against the test organisms. *Lb. plantarum*, *W. confusa* and *P. pentosaceus* showed some inhibition against both *E. coli* strains. Sterile supernatant solution from spontaneous fermentation also showed some antagonistic characteristics against the test organisms but there was no observed growth inhibition using sterile supernatant from CF (**Figure 4.4**).

The observed *E. coli* growth inhibition by the fermentation organisms and the supernatant solution from the SF corn may have been through the acidic environment produced during fermentation. The acidic environment of pH below 4 produced by LAB during fermentation, by converting carbohydrates to carboxylic acids such as lactic acids and acetic acids, is able to inhibit the growth of non-acidophilic pathogenic organisms (Blandino et al., 2003; Jalilsood et al., 2015; Salmerón, 2017). Furthermore, LAB are also able to produce antimicrobial substances such as bacteriocins, antibiotics (Epp, 2008; Prajapati & Nair, 2008; Zacharof & Lovitt, 2012) and toxins (Younis, Awad, Dawod, & Yousef, 2017), which are able to inhibit the growth pathogenic organisms, kill them or inhibit their production of protective biofilms (Bajpai et al., 2016; Jalilsood et al., 2015; Zacharof & Lovitt, 2012).

Figure 4.4: Antimicrobial effects of fermentation organisms and fermented corn against strains of *E. coli*. Holes in overlaid plates of strains of *E.coli* were filled with 100 µl of solutions of fermentation organisms and fermented products were incubate at 30 ^{o}C for 24 hours. Inhibitions zones were interpreted as antimicrobial or antagonistic activities. Data shows mean inhibition zones of triplicate experiments $\pm$ SE

4.4 Discussions

The objectives of this study were 1) to isolate and identify the microorganisms in SF milled corn and use as single and mixed culture starters in CF of milled corn; 2) to compare the growth of identified organisms in mixed culture CF vs CF single-strain vs SF; 3) To compare the antimicrobial characteristics of the fermentation organisms against strains of *E. coli*; 4) to test using

in vitro static digestion model, the survival of fermentation organisms in the upper digestive tract using a static digestion model.

4.4.1 Bacteria in fermented corn

Corn contains up to 70 % starch (Khan et al., 2014) and less than 10 % of sugars - fructose, sucrose, glucose, maltose and xylose - at varied concentrations depending on the stage of development and corn species (Russo, Collins, & Perkins-Veazie, 2004; Shaw & Dickinson, 1984; Zilic, Milasinovic, Terzic, Barac, & Ignjatovic-Micic, 2011). Three organisms, namely *W. confusa*, *P. pentosaceus*, *S. cerevisiae* were isolated during SF of corn in the current study. Over 90% of *W. confusa* are also able to ferment fructose, maltose and sucrose (Fusco et al., 2015) while *Lb plantarum* readily metabolizes sucrose, glucose and fructose into lactic and acetic acids (Filannino et al., 2014; Romano, Capece, & Jespersen, 2006). Filannino et al. (2014) identified maltose as the most preferred fermentable sugar by most strains of *P. pentosaceus*, though, Papagianni and Anastasiadou (2009) also mentioned that many strains of the organism are also able to metabolize glucose, ribose, galactose, arabinose, and fructose to lactate. The observed growth of the *W. confusa, P. pentosaceus*, *W. confusa* (2) may be due to the presence of their preferred hexose sugar in the substrate. However, the notable fast growth of *Lb plantarum* in corn may not only be associated with the presence of preferred fermentable sugar, but also its possibility of being amylolytic. *Lb plantarum* has been described as one of the few species of LAB that are known to have amylolytic activities by being able to degrade raw starch (Fossi & Tavea, 2013). Amylolytic strains of *Lb plantarum* have been isolated from cassava roots (Giraud, Brauman, Keleke, Lelong, & Raimbault, 1991; Giraud, Champailler, & Raimbault, 1994) and Nigerian fermented foods (Sanni, Morlon-Guyot, & Guyot, 2002). More recently amylolytic strains of *Lb plantarum* were reported to have been isolated from Bulgarian fermented cereal drinks (Gotcheva, Petrova, Petkova, Kuzmanova, & Angelov, 2018). Though the amylolytic activity of the strain of *Lb plantarum* used in this study was not confirmed, it may be associated with the organism's observed growth trend.

Though SF organisms and output are often unpredictable, the presence of *Lb plantarum* and *Lb fermentum* is typical in fermented corn (Decimo et al., 2017; Izah et al., 2016; Omemu, Okafor, Obadina, Bankole, & Adeyeye, 2018) and have sometimes been reported as the dominant LAB in spontaneously fermenting corn using both phenotypic (Adebayo-tayo & Onilude, 2008; Ojokoh, 2009; A. Omemu, 2011; Oyedeji et al., 2013; Rahmawati et al., 2013) and molecular (Banwo et al., 2012; Decimo et al., 2017; Oguntoyinbo & Narbad, 2012; Oguntoyinbo et al., 2011) methods of identification. The presence of *Pediococcus* spp in fermented corn is not unusual as Okeke et al. (2015) and Banwo et al. (2012) have also reported isolating *Pediococcus* spp from spontaneously

fermented white and yellow corn. In agreement with the current study, *P. pentosaceus* was identified by Banwo et al. (2012) as 1 of the 3 LAB in a fermented maize product however, the time of appearance was undetermined neither was quantity of the organism stated. Okeke et al. (2015) also identified *Pediococcus* spp (84.8% in white corn and 74.4% in yellow corn) but with *Lb paraplantarum* (15.2 and 25.6% in white and yellow maize, respectively) as the dominant LAB during SF of corn. The authors identified *P. pentosaceus* in 24 hours and 48 hours of fermentation but reported dominance of the organism in 72 hours of fermentation. By 96 hours, the organism was no longer present and had been replaced by *P. acidilactici* and *P. claussenii* as the only culturable LAB present in the corn showing changes in the microbial diversity during SF of corn.

Two studies have isolated *Weissella* spp from fermented cereals (Afolayan et al., 2017; Oguntoyinbo et al., 2011). Oguntoyinbo et al. (2011) reported isolating *W. confusa* from spontaneously fermented millet but not corn. Afolayan et al. (2017) stated that *W. paramesenteroides* as one of the 8 organisms isolated from white corn after 96 hours of fermentation though frequency of appearance, which often gives an indication of population, was not stated. *Weissella* spp have not been previously identified as a dominant organism in SF cereal. The dominant presence of the organism in the current study may be traced to the machine used for dry milling. The corn used in the current study was purchased from a local market in the south-western part of Nigeria and milled using a machine traditionally used to mill various dried food. Milling machine used in this environment is typically not sterile but it was dry cleaned before use which is the usual practice in the market before milling each dried food. *W. confusa* may therefore have been transferred from other dry foods such as millet which may have the organism as a dominant component of its microflora. Sterilizing the machine before use may have influenced the appearance or population of *W. confusa* in the study, however, it was important that traditional production chain was not interrupted for effective analyses of the traditional processing method. In addition, until 1993, *Weissella* was classified as *Leuconostoc* and *Lactobacillus* spps (Fessard & Remize, 2017). Though different in some biochemical characteristics, it is difficult to clearly differentiate between these 3 genera using phenotypical features only (Fessard & Remize, 2017) due to similar morphological characteristics particularly between *Weissella* and *Leuconostoc* (Fusco et al., 2015; Schillinger et al., 2008). It can therefore be inferred that some LAB isolated from SF corn and identified using phenotypical and biochemical test as *Leuconostoc* and *Lactobacillus* spps (Adebayo-tayo & Onilude, 2008; Omemu, 2011; Oyedeji et al., 2013) may have been *Weissella* spp.

In the study by Okeke et al. (2015), non-LAB, including *Bacillus*, *Acinetobacter*, *Enterobacter*, *Bordetella*, *Myroides*, *Alcaligenes* and *Moorella* spps, with *Bacillus* spp as the most dominant, were isolated within 48 hours of fermentation of white corn. Some of these organisms are known to be pathogenic but none of them was present after 72 hours. Similarly, *Pseudomona*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Staphylococcus* and *Bacillus* spps were isolated from fermenting corn (Bello et al., 2018), none of which was found after 72 hours. As fermentation progresses, reduction in pH could result in the inhibited growth and eventual death of non-LAB and mould, especially those that are unable to adapt to the low pH (Kigigha et al., 2016; Wolde, 2017). It is important however to note that, non-pathogenic, non-dominant, non-LAB may directly or indirectly contribute to the organoleptic characteristics such as aroma and texture of food during fermentation (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017; Esquivel-Elizondo, Ilhan, Garcia-Peña, & Krajmalnik-Brown, 2017; Murali, Srinivas, & Ahring, 2017).

4.4.2 Yeast in fermented corn

S. cerevisiae is one of the yeasts associated with fermented corn. Omemu, Oyewole, and Bankole (2007) reported isolating six types of yeasts, namely *S. cerevisiae, Candida krusei, C. tropicalis, Geotrichum fermentans, G. candidum* and *Rhodotorula graminis* during a 48 hours spontaneous fermentation of corn with *S. cerevisiae* being the dominant yeast throughout the process. *R. graminis* was isolated in the first 36 hours of fermentation. Bello et al. (2018) reported isolating *S. cerevisiae* as one of the two species of yeast isolated from SF corn though dominance was not reported while Kigigha et al. (2016) in agreement with this current study, reported it as the only yeast isolated from fermented corn and the only fungi cultured at 96 hours of fermentation. Ijabadeniyi (2007) mentioned isolating *S. cerevisiae* from 0 hours of SF of both white and yellow corns, through to the end of the process with analyses done at 24 hours interval. This outcome is comparable to the study by Omemu et al. (2007). On the other hand, the presence of *S. cerevisiae* was recorded from 24 hours of fermentation by Kigigha et al. (2016). Nevertheless, a progressive increase in population of *S. cerevisiae* all through the process was recorded by these authors. It is important to note that identification of these yeast was done using biochemical and phenotypical techniques in the mentioned studies. These methods are nonetheless known to be prone to errors, lacking reproducibility and specificity (Moraes, Perin, Silva Júnior, & Nero, 2013), hence compromising the validity identification. In the current study, microbial identification was done using molecular methods of identification minimising the risk of errors in the identification outcomes.

Variations in the time of growth of *S. cerevisiae* in the mentioned studies including the current one may be due to the variation in the strains of *S. cerevisiae* present in all the studies. Hayford and Jespersen (1999) mentioned that various strains of *S. cerevisiae* are involved in SF of corn. In their study which was aimed at undertaking fermentation and genetic profiling of 48 strains of *S. cerevisiae* isolated from various SF corn, the authors observed up to 8 different carbohydrate assimilation profiles in spite of all strains being able to ferment galactose, saccharose, raffinose and glucose. The authors further stated that all 48 strains had 4 different genotypes based on Methylation Marker Analysis (MAL) genotyping, 6 subclusters based on PCR profiling and each strain with specific chromosome profile, though some were quite related.

The plausible non-amylolytic characteristics of the yeast present in the SF corn in the current study may also explain why there was no visible growth of *S. cerevisiae* until 72 hours. The growth of *S. cerevisiae* at this point could have been stimulated by other amylolytic organisms which may have been present in the first 48 hours of fermentation and had hydrolysed starch into monosaccharides and disaccharides molecules that can then by utilised by *S. cerevisiae*. Some strains of *W. confusa*, *P. pentosaceus* isolated from fermented foods have been reported to have some level of amylolytic activities (Petrova, Petrov, & Stoyancheva, 2013; Wongratpanya & Khunajakr, 2012). In addition, non-LAB such as *Bacillus* spp and moulds such as *Aspergillus* spp, which have been associated with SF corn, have also been reported to be amylolytic (Moradi, Shariati, Tabandeh, Yakhchali, & Khaniki, 2014; Rahmawati et al., 2013; Sohail, Ahmad, Shahzad, & Khan, 2005). Therefore, the presence of plausible amylolytic *W. confusa*, *P. pentosaceus* and *Lb plantarum* in SF along with other amylolytic non-LAB in SF corn, may have made hexose sugar available to support the visible growth of *S. cerevisiae* during CF and SF in the current study. Nevertheless, sequence of microbial growth during SF in this study – the growth of LAB before yeast - was hypothesised by Hayford and Jespersen (1999).

4.4.3 SF vs CF

Overnight culture of organisms isolated from SF corn in this study, *W. confusa*, *P. pentosaceus*, *S. cerevisiae*, were inoculated, as mixed and as single starter cultures, into sterile corn for CF. Two other organisms in cryostorage, previously isolated from spontaneously millet, *Lb. plantarum* and *W. confusa* 2 were included in the mixture with the intention of replicating output of SF in a controlled setting. *W. confusa* 2 had initially been identified as *L brevis* using biochemical techniques which informed the decision to include it in the mixture starter culture. *Lb brevis* has also been isolated with SF corn by some authors, using molecular and phenotypical methods of identification (Decimo et al., 2017; Ojokoh, 2009; Rahmawati et al., 2013). Production of aromatic

compounds in the CF using mixed starter culture may however have been enhanced with the addition of *Lb brevis*, following the study by Teniola and Odunfa (2001) who compared the organoleptic properties of traditionally fermented corn with corn fermented with *S. cerevisiae* or *Lb brevis* or *S. cerevisiae* + *Lb brevis*. Using a seven-man trained panel, the authors recorded that the mean score for aroma awarded to corn fermented with *S. cerevisiae* + *Lb brevis* was statistically similar to the aroma score awarded to the SF corn. Scores awarded to other organoleptic properties were nevertheless statistically different when compared to those of SF corn. These results may yet have been different if a different strain of *S. cerevisiae* was used as implied by Callejo et al. (2017).

Characteristics of a suitable starter culture include fermentability, viability, safety, satisfactory sensory properties and genetic stability (Hati, Mandal, & Prajapati, 2013; Mohammadi et al., 2012). Among the CF using single strain starter culture, *Lb plantarum*, *P. pentosaceus* and *W. confusa* (2) had a pH of 4.12, 4.55 and 4.82 at 120 hour respectively, suggesting their fermentability characteristic. *Lb plantarum* however displayed the highest adaptability compared with other single strains by having the highest growth rate and lowest pH. This fermentability characteristic suggests that *Lb plantarum* may be ideal as a starter culture for the fermentation of corn. A similar result was reported by Ojokoh (2009) whose study suggested that CF of corn using *Lb plantarum* had lower pH and higher titratable acidity compared to the traditional SF corn. Co-culturing *Lb plantarum* with other organisms may lead to competition for fermentable sugars and a reduction in its growth rate. Also contrary to the study by Teniola and Odunfa (2001) the specie of *S. cerevisiae* in this study did not show a remarkable growth with a pH fall above 5 when used as a single strain starter culture. Yeast however showed a faster growth rate when it was co-cultured with *Lb plantarum*, *P. pentosaceus* and *W. confusa* which implies that the strain of *S. cerevisiae* used in the current study may not be ideal for single strain starter culture in the fermentation of corn, but may be ideal as a co-culture with other LAB in mixed strain starter culture.

Replicating the outcome of SF corn in a controlled setting involves mirroring the microbiota at different stages of the fermentation process in order to achieve similar chemical composition and organoleptic characteristics. The microbiota in SF changes in quantity and diversity as fermentation progresses (Bello et al., 2018; Ijabadeniyi, 2007; Okeke et al., 2015), therefore it may be challenging, or impossible, to mirror this consortium, particularly if non-dominant and non culturable organisms have significantly contributed to the composition and characteristics of the SF product. Furthermore, the scope of the current study did not include isolation and identification of non-LAB organisms mainly because of their potential of being pathogenic (Oyelana & Coker, 2012). This may have remarkably contributed to the failure to replicate the outcomes of SF in the mixed culture CF.

In a study by Annan et al. (2003) aimed at analysing aromatic compounds produced during SF of maize dough over 72 hours, the authors observed that the production of aromatic compounds commenced from 24 hours of fermentation and continued throughout the process. Compounds identified were carbonyls, alcohols, esters, acids and phenol, all of which were produced based on the interactions between LAB and yeast strains present during the fermentation process. The authors suggested that yeast strains present in the dough converted carbonyls into alcohols in the acidic environment that is created by LAB, while the increase in esters and phenol compounds produced may have been a consequence of increased population of yeast and quantity of organic acids. In the current study, even though organisms inoculated into sterile corn for CF were initially isolated from the SF corn, interaction between these organisms did not result in sensory observation of the production of aromatic compounds. Scientific aroma analysis can be done qualitatively using e-nose or quantitatively using gas chromatography-olfactometry (GC-sniffing) (Cui, Wang, Yang, Wu, & Wang, 2015). Neither of these methods was used in this study as all that was required was sensory observation of the aroma. Nevertheless, over time, odour ratings has been described as not reliable due factors such as age, gender, disease and experience that may influence olfactory performance (Kaeppler & Mueller, 2013)

4.4.4 Antimicrobial characteristics

Strains of *Lb plantarum* have been reported to produce plantaricins which have effect on a wide range of pathogenic organisms examples of which are *Carnobacteria* spp, *Clostiridia* spp, *Propionobacteria* spp, *Staphylococci* spp, *Listeria monocytogenes,* and *Aeromonas hydrophila* but also other LAB with which it co-cultures such as *Pediococcus* spp (Behera, Ray, & Zdolec, 2018; da Silva Sabo, Vitolo, González, & de Souza Oliveira, 2014; Oguntoyinbo & Narbad, 2015; Todorov, 2009). Bacteriocins produced by *P. pentosaceus* (Bajpai et al., 2016; Gutiérrez-Cortés, Suarez, Buitrago, Nero, & Todorov, 2018; Kumar, Devadas, Murugan, Krishnan, & Thayumanavan, 2018; Todorov & Dicks, 2009) and *W. confusa* (Dubey & Jeevaratnam, 2018; Goh & Philip, 2015; Kaur & Tiwari, 2017) with actions against a wide spectrum of microorganisms have also been previously reported.

Inhibition of *E. coli* using the supernatant solution SF corn was similarly reported by Abdus-Salaam, Adepoju, Olaleye, and Adeoye (2014) who further mentioned observation of the antagonistic reaction from day 2 of SF. However, it was not stated whether supernatant solutions were cell free. The variations in the inhibition outcomes observed between SF of corn and CF of corn in the current study may be due to the varied microbial population in both processes resulting

in a variation in the type or concentration of metabolite produced during fermentation. Furthermore, the plausible ability of some LAB to inhibit the growth of and the expression of bacteriocins by other LAB when they are co-cultured (Vieco-Saiz, Belguesmia, Raspoet, Auclair, Gancel, Kempf, & Drider, 2019) may also have resulted in the varied outcomes against *E. coli*.

In agreement with the current study, Davoodabadi, Dallal, Lashani, and Ebrahimi (2015) and Michail and Abernathy (2002) reported that the antimicrobial activities of *Lb plantarum* against diarrheagenic *E. coli*. Damodharan, Lee, Palaniyandi, Yang, and Suh (2015) and Bajpai et al. (2016) also reported the action of *P. pentosaceus* against *E. coli* just as Kaur and Tiwari (2017) reported *W. confusa* against *E. coli.* There was no observed antagonistic effect of *S. cerevisiae* against *E. coli* in the current study. But, 12 out of 20 strains of *S. cerevisiae* isolated from fermented dairy products in the study by Younis et al. (2017) inhibited the growth of *E. coli*. Nevertheless, *Lb plantarum*, *P. pentosaceus*, *W. confusa* and *S. cerevisiae* strains used in the mentioned studies were not of fermented corn origin and strains of *E coli* used were different from those used in the current study. Afolayan et al. (2017) reported from their study that 5 out of 6 unidentified strains of *Lb plantarum* isolated from 6 random samples of 'ogi' showed antagonistic effects against *E. coli* EC004. Due to reported large intra-species variability in microbial cells (Sela, Euler, da Rosa, & Fischetti, 2018), results from these studies may not be generalizable. Identification of organisms isolated in the current study to strain level may therefore help to make comparison with other studies more effective.

4.4.5 Survival of upper digestion

P. pentosaceus in SF and CF corn, as well as *Lb plantarum* and *S. cerevisiae* in CF corn survived the *in-vitro* upper digestion, giving the indication that these organisms may have probiotic potentials. The tolerance of strains of *Lb plantarum* and *P. pentosaceus* have previously been investigated and reported in various *in-vitro* and *in-vivo* studies (Cebeci & Gürakan, 2003; Damodharan et al., 2015; Ladha & Jeevaratnam, 2018; Ramirez-Chavarin, Wacher, Eslava-Campos, & Perez-Chabela, 2013; Valerio et al., 2006; van Bokhorst-van de Veen, Lee, et al., 2012). Also, similarly to the current study, in an in-vitro study by Pennacchia, Blaiotta, Pepe, and Villani (2008), investigating the survival of 22 strains of yeast previously isolated from fermented foods and beverages, showed that 10 yeast strains, 5 of which were *S. cerevisiae*, had up to 70% survival after 2.5 hour at 37°C exposure to gastric juice with pH of 2.5 followed by 5 h at 37°C in intestinal juice with pH of 7, containing 0.3% bile salts and 0.1% pancreatin. Though, the digestion time was longer, pH of gastric juice was lower and concentrations of bile salts and pancreatin were lower, compared to the current study. Van der Aa Kühle, Skovgaard, and Jespersen (2005) reported a

similar result, suggesting the survival of 18 strains of *S. cerevisiae* in in-vitro upper digestion. On the other hand, strains of *S. cerevisiae* used in the *in-vitro* study by Psomas, Andrighetto, Litopoulou-Tzanetaki, Lombardi, and Tzanetakis (2001) did not survive the harsh conditions of the upper digestion. It is important to note that different strains of a specie of organism may have varied responses to the same treatment (Hill et al., 2014; Folarin A Oguntoyinbo & Narbad, 2015), therefore, it is possible that not all strains of *P. pentosaceus*, *Lb plantarum* and *S. cerevisiae* may survive that conditions of the human upper GIT. Though *Lb plantarum* is commercially used as probiotic (Arasu, Al-Dhabi, Ilavenil, Choi, & Srigopalram, 2016), Cebeci and Gürakan (2003) reported that only 5 out of 13 strains of *Lb plantarum* tested for acid-bile salt tolerance showed some degree of tolerance.

4.5 Summary and Conclusion

P. pentosaceus, *W. confusa* and *S. cerevisiae* were the LAB and yeast respectively isolated from SF corn in the current study. Non-LAB were not isolated as it was not within the scope of the study. The isolated organisms as well as *Lb plantarum* which was previously isolated from SF millet in a previous study were used a mixed starter culture for CF of corn. The final pH in SF was lower (3.35) that the final pH in CF (4.42). The observed aromatic smell in SF was not present in CF. These factors may be indicative of that notable difference in the microbial communities in both types of fermentation.

Only *P. pentosaceus* survived upper digestion of the SF corn while *P. pentosaceus, Lb plantarum* and *S. cerevisiae* survived the upper digestion of the CF corn. The absence of *S. cerevisiae* after the digestion of SF corn may be attributed to the low population of the organism before fermentation. Antimicrobial activities of these organisms against 2 strains of *E. coli* were also observed. These factors may indicate the possibilities that these organisms may have probiotic potentials. Nevertheless, other tests, such as adhesion to intestinal epithelial cells, antibiotics sensitivity, bile salt hydrolase activity, viability during storage and ultimately, *in vivo* studies need to be carried out on these strains of organism for effective screening of potential probiotic properties (Joint FAO/WHO Working Group, 2007; Kechagia et al., 2013). Antagonistic effects of cell free supernatant solution from spontaneous fermentation against *E. coli* strains were also recorded. These results may suggest that the SF corn in this study may have probiotic potentials however, due to inconsistencies in end-product of SF, this result may not be generalizable. Therefore, replication of SF in a controlled environment may be necessary for a reproducible plausible probiotic effect of fermented corn

Chapter 5

Short Chain Fatty Acids in fermented corn

5.1 Introduction

Short chain fatty acids (SCFAs), also known as volatile acids, are saturated carboxylic or organic acids with 1 to 6 carbon atoms (den Besten et al., 2013; Huda-Faujan et al., 2010). In humans, aside from lactic acid, SCFAs are major end-products of the fermentation of carbohydrates that have escaped digestion, by the anaerobic caecal and colonic microbiota (Alpers, 2003; den Besten et al., 2013; Holscher, 2017; Morrison & Preston, 2016). The production of SCFAs in the human gut is a function of the gut microbiota (den Besten et al., 2013). Food intake significantly influences the composition of the gut microbiota and consequently, the concentration and type of SCFAs produced in the gut (den Besten et al., 2013). For example, an increased intake of dietary fibre is reported to be associated with an increased SCFA production in the gut (Holscher, 2017; Louis & Flint, 2017; Meier, 2009; Musso, Gambino, & Cassader, 2011). SCFA is regarded as metabolic waste product for the microbiota but serve as an important energy source for the colonocytes (den Besten et al., 2013; Meier, 2009) and providing up to 15% of the host's calorie needs (den Besten et al., 2013; Meier, 2009; Morrison & Preston, 2016; Lui et al., 2018).

About 95 % of SCFAs produced is rapidly absorbed by the colonocytes leaving just about 5% to be secreted in the faeces (den Besten et al., 2013). Precise analysis of SCFA production in human gut is almost impossible due to lack of methodology (Morrison & Preston, 2016) but, outcomes from human faecal analysis from *in-vivo* studies are used as estimates (den Besten et al., 2013; Morrison & Preston, 2016). Nonetheless, it is unclear whether outcome of faecal analysis is a replica of luminal SCFA production or concentration due to rapid absorption of the acids in the colon (den Besten et al., 2013; Morrison & Preston, 2016). Peripheral blood analysis for the estimation of colonic production of SCFA would also not be accurate as a large percentage of SCFA is used as fuel source by the colonocytes with a very small portion, largely acetate, is transported to the liver through the hepatic portal circulation with only a small amount filtering into the peripheral blood circulation (Deroover, Boets, Tie, Vandermeulen, & Verbeke, 2017). Predominantly based on faecal analyses it has been reported that the most abundant SCFAs produced in the gut are acetate, propionate and butyrate (den Besten et al., 2013; Meier, 2009; Verbeke et al., 2015) with acetate being most largely produced (up to 60%) followed by propionate (about 25%) then butyrate (about 15%) (den Besten et al., 2013; Verbeke et al., 2015; Lui et al., 2018). Acetate appears to be substrate and organism non-specific with it being an end-product for almost all of organisms in the gut some of which are lactic acid bacteria (LAB) (Louis & Flint, 2017; Morrison & Preston, 2016). On the other hand, production of propionate and more

importantly butyrate are more organism and substrate specific (Louis & Flint, 2017; Morrison & Preston, 2016), with their production being enhanced by diet rich in inulin and resistant starch (den Besten et al., 2013; Holscher, 2017; Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011). This explains why acetate appears to be the SCFA most largely produced in the human gut.

5.1.1 Exogenous sources of SCFAs and Health

SCFAs are able to provide extensive health benefits. SCFAs are substrates for gluconeogenesis, liponeogenesis, protein and cholesterol synthesis in the peripheral tissues (den Besten et al., 2013; Schwiertz et al., 2010; McNabney & Henagan (2017). Several articles have extensively reported the effects of SCFAs in the colon (Hijova & Chmelarova, 2007; Hosseini et al., 2011; Huda-Faujan et al., 2010; Morrison & Preston, 2016). They are generally known to be a fuel source to the colonocytes (den Besten et al., 2013). SCFAs in the colon stimulate sodium chloride and water absorption; mucosal cell proliferation and differentiation; mucosal blood flow; mucus production whilst also reducing the gut pH (den Besten et al., 2013; Hijova & Chmelarova, 2007; Meier, 2009; van der Beek, Dejong, Troost, Masclee, & Lenaerts, 2017). More specifically butyrate has been reported to be associated with enhance immune responses, anti-inflammatory effects, reduced lipid deposition, increased oxidation in these tissues (den Besten et al., 2013; McNabney & Henagan, 2017). Furthermore, low concentration of SCFAs in the colon has been associated to increased risk of irritable bowel syndrome, inflammatory bowel disease, cardiovascular diseases (den Besten et al., 2013; Hijova & Chmelarova, 2007; Morrison & Preston, 2016; Ohira, Tsutsui, & Fujioka, 2017), diabetes (Wen & Wong, 2017) and colon cancer (Yang et al., 2013). A study by Huda-Faujan et al. (2010) showed that there was a remarkable decrease in the faecal concentration of acetate, propionate and butyrate in people with irritable bowel syndrome compared with healthy people, nevertheless, only butyrate and propionate were of statistical significance. Out of the three mostly produced SCFAs, propionate and butyrate (especially butyrate) have been reported to most confer health benefits in the body (Louis & Flint, 2017; Morrison & Preston, 2016; van der Beek et al., 2017). Butyrate is reported to be the most preferred energy source for the colonocytes and is therefore mainly metabolized in the colon, while acetate and propionate are transported to the liver where they are metabolized into glucose, lipids, cholesterol and carbon dioxide (van der Beek et al., 2017). It has particularly been the focus of research due to its potential to enhance immune functions and influence immunological responses in the host (McNabney & Henagan,2017). Proposed mechanism for its function has been associated with the hyperacetylation of proteins that cause an alteration in gene expressions patterns and its ability to bind with fatty acid receptors which may be helpful in the regulation of inflammatory immune responses (Cleophas et al., 2019; Morrison & Preston, 2016; van der Beek et al., 2017).

Due to health benefits associated with colonic SCFA, researchers have focussed their investigations on how to enhance the concentration of SCFAs in the colon (Boets et al., 2017; Farooq et al., 2013; Holscher, 2017). And with the established link between diet, the gut microbiota and concentration of SCFAs, studies majorly concentrated on increasing the concentration of colonic SCFAs through the intake of dietary fibre (Holscher, 2017; LeBlanc et al., 2017; Meier, 2009; Morrison et al., 2006; Musso et al., 2011; Ohira et al., 2017; den Besten et al., 2013; McNabney & Henagan, 2017). Nevertheless, non-colonic beneficial effects of SCFAs have also been investigated and reported by a handful of researchers. There effects on liver, skeletal and adipose tissues when absorbed into the peripheral circulation have been suggested by den Besten et al. (2013). Two studies have also reported that SCFA acids may enhance the skin (Schwarz, Bruhs, & Schwarz, 2017) and bone health (Lucas et al., 2018) although the studies used mice subjects. Both studies did not report colonic source of the SCFAs as Schwarz et al. (2017) reported administering SCFA by subcutaneous injection while Lucas et al. (2018) administered orally in drinking water.

Dietary butyrate supplementation has also been reported to prevent and reverse obesity and insulin resistance (den Besten et al., 2013). Gao et al., (2009) reported that dietary supplementation of butyrate incorporated in high fat diet (58% of calories from fat) in mice significantly enhanced insulin sensitivity and prevented weight gain in comparison with mice that did not receive butyrate supplementation. The mechanism of these butyrate effect was related to increase in energy expenditure and induction of mitochondria function in the mice. Furthermore, a few authors have investigated and reported that dietary SCFAs may have beneficial effects in the colon (Pingitore et al, 2017; Cleophas et al., 2019; Sugiyama et al., 2010; Sabatino et al, 2005; Viernia et al., 2000). Though SCFAs are directly absorbed into the peripheral circulation from the jejunum (den Besten et al., 2013; Hussain, 2014; Iqbal & Hussain, 2009; McClements & Li, 2010), it is not impossible that some of it may escape into the colon. Chemically synthesized products such inulin-propionate ester when orally administered has been suggested to be effective for increasing concentration of colon SCFA due to its potential to deliver propionate and inulin to the colon (Pingitore et al., 2017; Polyviou et al., 2016; Zhu et al., 2018). Dietary supplementation with inulin-propionate ester, as effectively as ingestion of high fermentable fibre (inulin), was also reported to result in an increase in serum and colonic concentration of propionate, a modulation in the gut bacterial population and positive change in makers of systemic inflammation (Chambers et al., 2019). In addition, dietary butyrate supplements have been reported may be effective in decreasing the clinical symptoms, which includes diarrhoea, by increasing colonic concentration of butyrate and downregulating inflammatory biomarkers of people with Crohn's disease (Sabatino, et al., 2005). Therefore, the

plausibility that exogenous and dietary sources of SCFA, especially butyrate may significantly contribute to improved health including gut health in humans should not be ignored.

5.1.2 SCFAs in food

Similarly, during food fermentation, there is a decrease in pH which is an indication of production of organic acid during the process (Bouthyette, 2008). These organic acids make notable contributions to the organoleptic characteristics of fermented foods (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Liu, 2015; Moon, Kim, & Chang, 2018). Various articles have implied that organic acids such as include lactic, citric, caproic, valeric and SCFAs are produced during food fermentation in varied concentrations depending on the substrate and the fermentation organisms (Hayek & Ibrahim, 2013; Karovičová, 2007; Liu, 2015; Sauer, Porro, Mattanovich, & Branduardi, 2008; Zalán, Hudáček, Štětina, Chumchalová, & Halász, 2010). LAB are the most dominant organisms present during the fermentation of cereals with lactic acid being the main end product (Ali, 2010; Hayek & Ibrahim, 2013; Karovičová, 2007; Rezac, Kok, Heermann, & Hutkins, 2018; Tamang, Shin, Jung, & Chae, 2016). Nevertheless, due to the additional hydroxyl group in the structure of lactic acid, it is not classed as a SCFA (Ríos-Covián et al., 2016) (**Figure 5.1**).

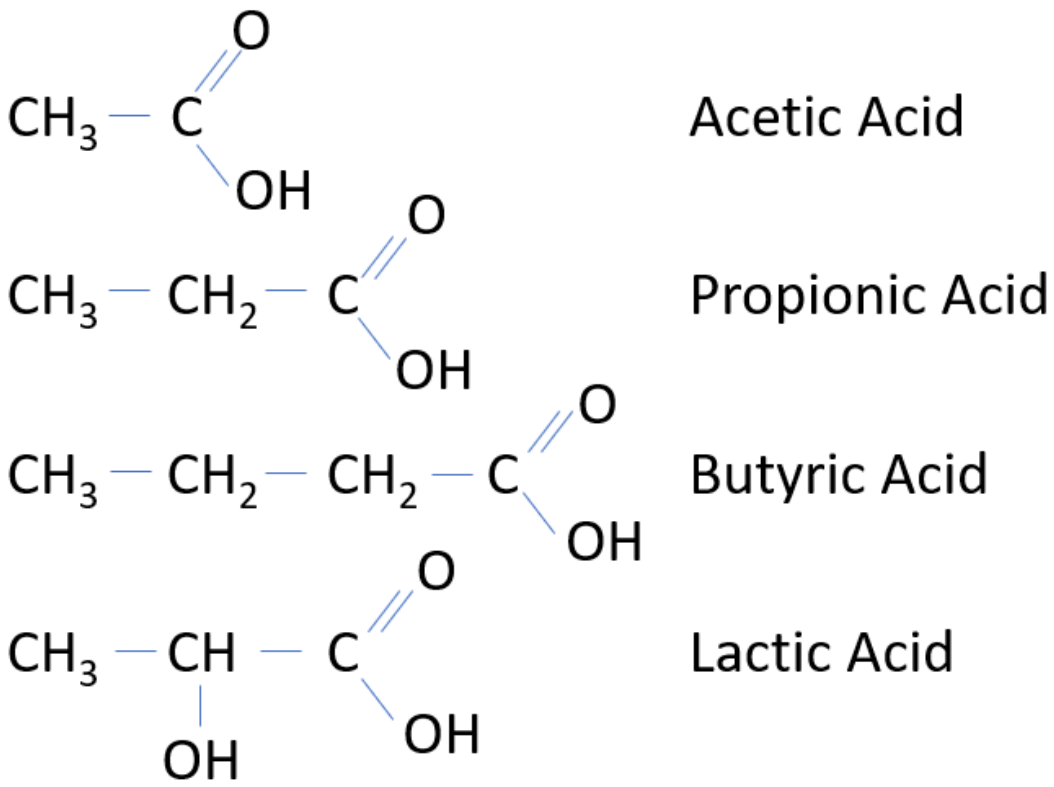


Figure 5.1: Structure of Carboxylic acids

However, some LAB are able to undergo heterofermentation and able to produce other acids such as acetic, butyric, propionic, formic, succinic acids, as end products of fermentation depending on the substrate (Farooq, Mohsin, Liu, & Zhang, 2013; Hayek & Ibrahim, 2013; Moon et al., 2018; Vaseji, Mojgani, Amirinia, & Iranmanesh, 2012; Zalán et al., 2010). Furthermore, there are other non-LAB and non-dominant bacteria such as species of *Bacillus*, *Acinetobacter*, *Enterobacter, Pseudomona*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Staphylococcus* which have been reported to be involved in the fermentation of cereal (Bello, Bello, Amoo, & Atoyebi, 2018; Karovičová, 2007; Okeke et al., 2015) may have notable roles to play in the production of organic acids in fermented foods. Quantitative and qualitative analyses of SCFAs in popular fermented foods have been previously undertaken. Vaseji et al. (2012) observed higher concentration of butyrate in probiotic yoghurt during a quantitative comparison of the concentration of the SCFA in probiotic vs non-probiotic yoghurt which had been prepared using starter cultures. Farooq et al. (2013) also stated that *Lb. acidophilus, Lb. rhamnosus, Bifidobacterium. Longum* and *B.bifidus* are able to produce acetate, propionate and butyrate during the fermentation of fibre extracted from whole millet grains. Zalán et al. (2010) also reported from their study that while lactic acid was produced by all tested LAB in 3 different media namely skimmed milk, MRS broth and Artichoke, production of SCFAs varied between organisms and was dependent on the substrate of fermentation. SCFAs have also been reported to be present in fermented cereal foods and beverages (Dertli & Çon, 2017; Erbaş, Uslu, Erbaş, & Certel, 2006; Javanainen & Linko, 1993; Kam, Aida, Sahilah, & Maskat, 2011; Karovičová, 2007; Liptáková, Matejčeková, & Valík, 2017; Muyanja, Narvhus, & Langsrud, 2012). Older studies have investigated the existence of SCFA in SF sourdough (Halm, Lillie, Sørensen, & Jakobsen, 1993; Plahar & Leung, 1982) and sourdough using starter culture (Annan, Poll, Sefa-Dedeh, Plahar, & Jakobsen, 2003). And as far as we know, only one older study has also investigated the existence of SCFAs in 'ogi' (Banigo & Muller, 1972).

Therefore, the current study aimed to 1) investigate and compare the production of SCFAs in fermented corn during spontaneous (SF) and controlled (CF) methods of fermentation; 2) investigate SCFA production of organisms from SF during single strain CF; 3) investigate the effect of upper digestion on the SCFAs in fermented foods.

5.2 Materials and Methods

5.2.1 Sample

Aliquots of previously fermented milled corn (Chapter 4) including digested samples of fermented corn, which had been stored at -20^{O}C were thawed in the refrigerator before analyses. Samples

were thawed at $4^{O}C$ and not at room temperature in order to avoid continuous growth of organisms and to avoid loss of volatile acids.

5.2.2 Chemicals

SCFA Standard used is documented in **Table 5.1**. A total of 8 different standard solutions were prepared and used for calibration. The standard solutions contained a mixture of 6 SCFAs in the following concentrations: 0.11 x 10^{-3} M, 1.07 x 10^{-3} M, 2.67 x 10^{-3} M, 5.33 x 10^{-3} M, 7.99 x 10^{-3} M, 10.66 x 10^{-3} M, 30.0 x 10^{-3} M and 106.60 x 10^{-3} M. The standard solutions were stored at +4°C.

Table 5.1: Standard used for calibration of SCFAs

Compound	Chemical Formula	Molar Mass (g/mol)	Source product number
Sodium acetate trihydrate	$C_2H_3NaO_2.3\ H_2O$	136.08	Sigma- Aldrich CAS Number: 6131-90-4
Sodium butyrate	$CH_3CH_2CH_2COONa$	110.09	B5887 Sigma-Aldrich
Isobutyric acid	$(CH_3)_2CHCO_2H$	88.11	I1754 Sigma-Aldrich
Sodium propionate	CH_3CH_2COONa	96.06	P1880 Sigma-Aldrich
Valeric acid	$CH_3(CH_2)_3COOH$	102.13	240370 Sigma-Aldrich

5.2.3 Sample preparation

Two ml of supernatant solution of all samples were pipettes into 2.5 ml Eppendorf tubes and centrifuged for 30 minutes at 23,600 g (Ewald, 2016). Supernatant solutions were clear and free of particulates therefore no filtration was required (Fernández, Dinsdale, Guwy, & Premier, 2016). One ml of supernatant solution of samples was pipetted into the 1.5 ml auto sampler glass vials for analysis. Due to one step sample preparation and limited resources, external standards were used to obtain standard calibration in the current study (Usher, Hansen, Amoo, Bernstein, & McNally, 2015). With one step sample preparation, it was assumed that there would be minimal loss of volatile compounds (Usher et al., 2015), therefore spiking with the use of internal standards would be unnecessary. Distilled water was used as blank samples. Similarly, standards were prepared using distilled water to achieve a final volume of 20 ml of mixed standards at the concentrations mentioned.

5.2.4 Sample analysis

Zúas, Mulyana, and Budiman (2016) undertook a study aimed at validating a GC-FID method for the detection and measurement of gaseous SCFAs in a mixture of gases. The authors concluded that their GC-FID method showed good separation of target compounds, high repeatability with relative standard deviation less than 1% and good selectivity. Consequently, SCFAs in the

fermented samples in the current study was identified and quantified using a GC-FID, following a modified version of the protocol published by Skoglund (2016).

GC-FID PerkinElmer® Clarus® 480 Gas Chromatograph (GC) (Chapter 3) equipped with a split/splitless injector, built-in syringe auto sampler and a flame ionization detector (FID) was used to separate and quantify SCFAs. Two microliter sample was injected into a straight glass liner and held at 200^{O}C. Hydrogen (2 ml/min) was used as carrier and gas separation was conducted on a Perkin Elmer Elite Wax Column 30 m, 0.25 mm, 0.25 µm temp range 20-250^{O}C. Initial oven temperature of 55^{O}C was maintained for 4 min, then ramped to 130^{O}C at 50^{O}C/min and held for 5.7 minutes and finally raised to 250^{O}C at 30^{O}C/min and held for 2.5 minutes. The flow rates of air in the FID 30 ml/min. The temperature of the FID was 200^{O}C. The run time for each analysis was 20 min. Blanks were run once before each sample. Analyses of standards and samples were done in triplicate. Injection system was washed after running each sample using concentrated acetonitrile to avoid carry-over. Peak heights and areas were measured manually.

SCFA standard solutions were analysed to obtain retention times (RT) for each SCFA. This information was then used for SCFA identification in samples. Chromatograms were received from the FID on the computer screen. Area under peaks was taken as response of the SCFA in the column. Response from the standard solutions of known concentration was used for calibration. Instrument calibration is necessary to establish a relationship between the response obtained from the instrument and the known concentration of the test compound (Prichard & Barwick, 2003). Concentrations of SCFAs in the samples were then estimated using the calibration chart (Prichard & Barwick, 2003).

5.2.5 Statistical analysis

Concentration of SCFA in samples was not normally distributed, and was therefore subjected to non-parametric tests. Mann Whitney U test was carried out to compare analyte production during SF vs CF

5.3 Results

5.3.1 Retention Times (RT)

Mixtures of eight standards were prepared in previously mentioned concentrations of all 6 SCFAs. Acetate was the first SCFA to be detected, with earliest RT of 10.008 min, while valeric acid was not detected until after 14 mins. The mean RT ($\pm$ SD) for acetate, butyrate, propionate, isobutyrate and valeric acid at for all tested concentrations were 10.087 $\pm$ 0.041, 12.212 $\pm$ 0.037, 10.914 $\pm$

0.047, 11.209 ± 0.048 and 14.328 ± 0.012 (**Figure 5.2**) respectively. RT for all standards were normally distributed. As a result, on this occasion, the SD as a measure of variability was used as it best expresses the differences of the RTs from the mean RT of each standard (Lee, et. al., 2015). A shift in RT of up to 1.7 % was observed between the lowest concentration and the highest of all standards with peaks appearing early as concentration increased. RT in chromatography has been described as irreproducible (Wilson, Barnes, & Boswell, 2014) as it is affected by controllable variables such as gas flow rate, temperature in the oven and the column and uncontrollable experimental (Dolan, 2014; Wilson et al., 2014). In this current study, controllable factors and conditions for analyses were digitally set using the control panel before initiation of analyses and frequently checked to ensure that there were no variations or changes during analyses. However, uncontrollable factors such as inaccurate dimensions of the column, interaction of analytes with the column, complexity of the sample and column degradation may also cause variations in RT during analyses (Dolan, 2014; Wilson et al., 2014). Furthermore, as it appears with the standards in this current study, increased concentration of test compound may also result in a shift of RT (Dolan, 2014; Rood, 1997). Increased concentration of analyte may contribute to the further degradation of the column which may compromise the accuracy of the RT as the analyses progresses (Rood, 1997). Dilution of the analyte may rectify this however, samples were not diluted in the current study as researcher was uncertain of the concentration of SCFAs that may be contained in the samples. There was therefore, the need for a wide range concentrations of standards for effective calibration.

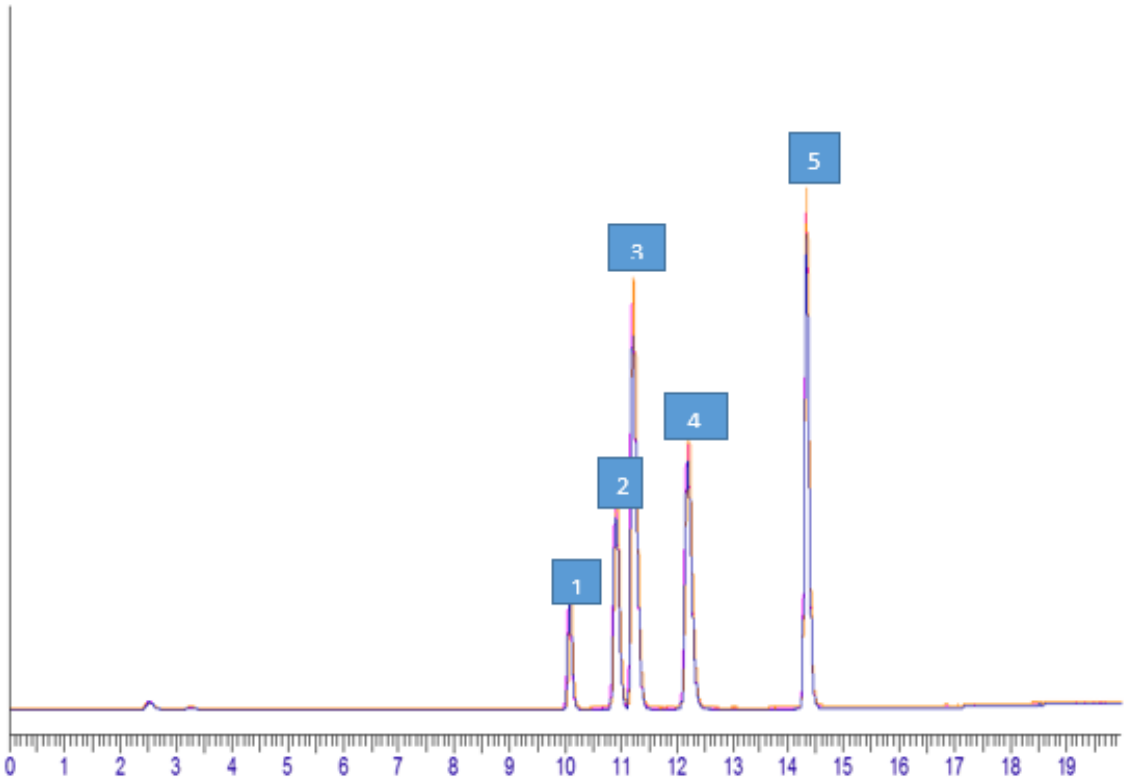


Figure 5.2: Chromatograms for external standards with retention times (RT). Figure shows chromatograms of the time taken for each external standard to be detected by the Flame ionization detector (FID) attached to the gas chromatograph (GC) and the detector signal (chromatograph peaks). Data shows the overlay of 3 chromatograms and each peak represents the following external standard: 1) Acetate; 2) Propionate; 3) Isobutyric acid; 4) Butyrate; 5) Valeric acid.

5.3.2 Linearity of Calibration

The degree or linearity of the relationship between the response of the instrument and the concentration of the test compound is measured using the linear regression equations (Prichard & Barwick, 2003). In the current study, there was a wide calibration range with concentrations ranging from 0.11 x 10^{-3} M to 106 x 10^{-3} M. In order to avoid a high degree of leverage and to obtain higher degree of accuracy (Prichard & Barwick, 2003), equation for linear regression was determined using 2 levels of concentrations: 0.11 x 10^{-3} M – 7.99 x 10^{-3} M and 5.33 x 10^{-3} M – 106.00 x 10^{-1} M, each based on 5 points, following a method described by Banel, Jakimska, Wasielewska, Wolska, and Zygmunt (2012). Coefficient of regression (R^2) for all calibrations ranged from 0.990 to 0.999 which was indicative of the strong association between the response and the concentration of the analyte and that the response detected from the GC-FID was dependent on the concentration of the SCFAs in the standard solutions (**Figures 5.3**). Concentration of SCFA in sample was calculated using the formula below.

$$\textbf{Concentration of SCFA} = \frac{\textbf{Response – intercept}}{\textbf{Slope}}$$

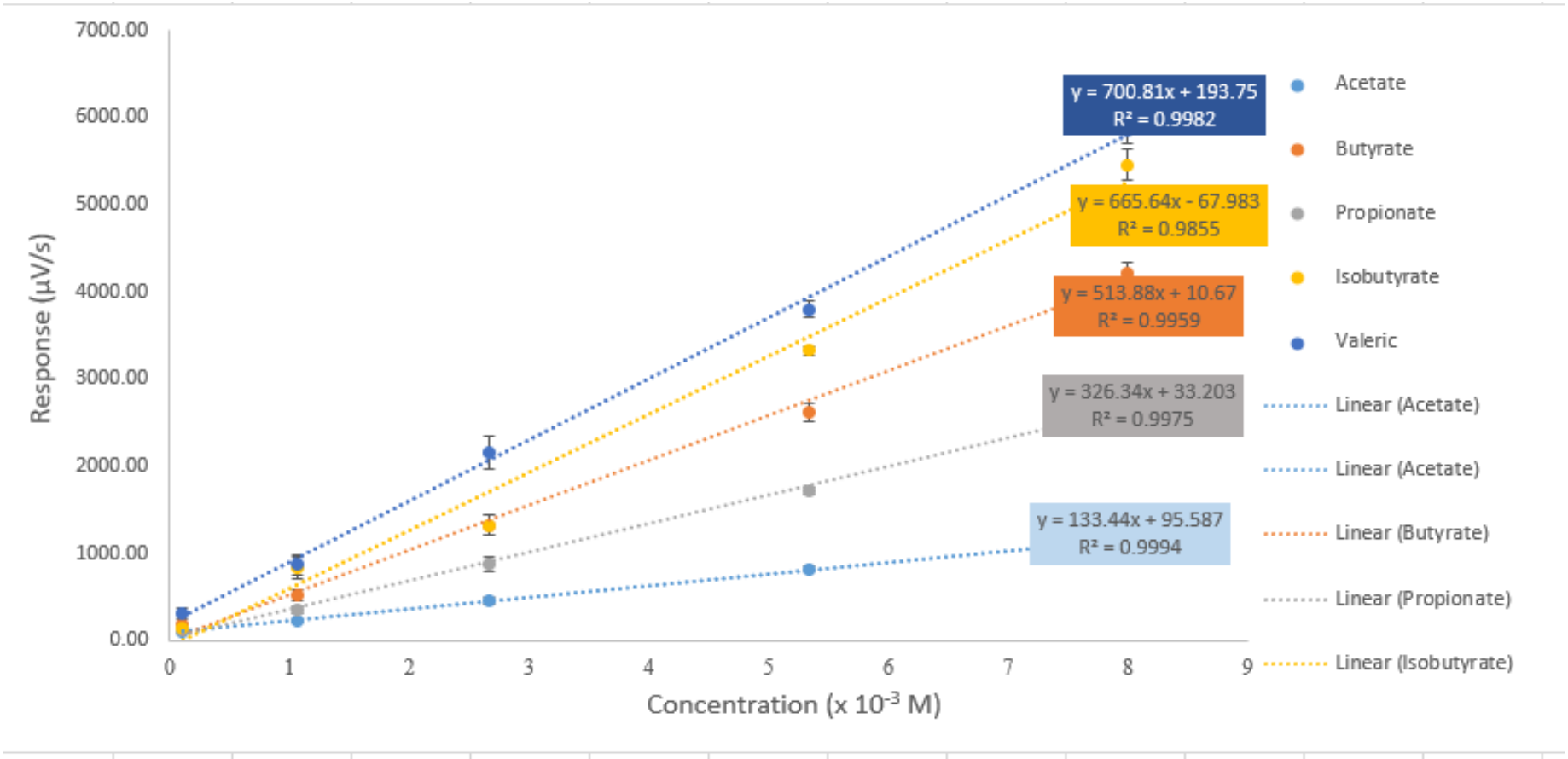


Figure 5.3a: Low Concentration Calibration Chart. Figure shows linearity between the response of the GC-FID and lower 5 levels of concentrations (0.11 x 10^{-3} M, 1.07 x 10^{-3} M, 2.67 x 10^{-3} M, 5.33 x 10^{-3} M, 7.99 x 10^{-3} M,) of each external standard. Data shown is the mean response of triplicate analyses $\pm$ SE

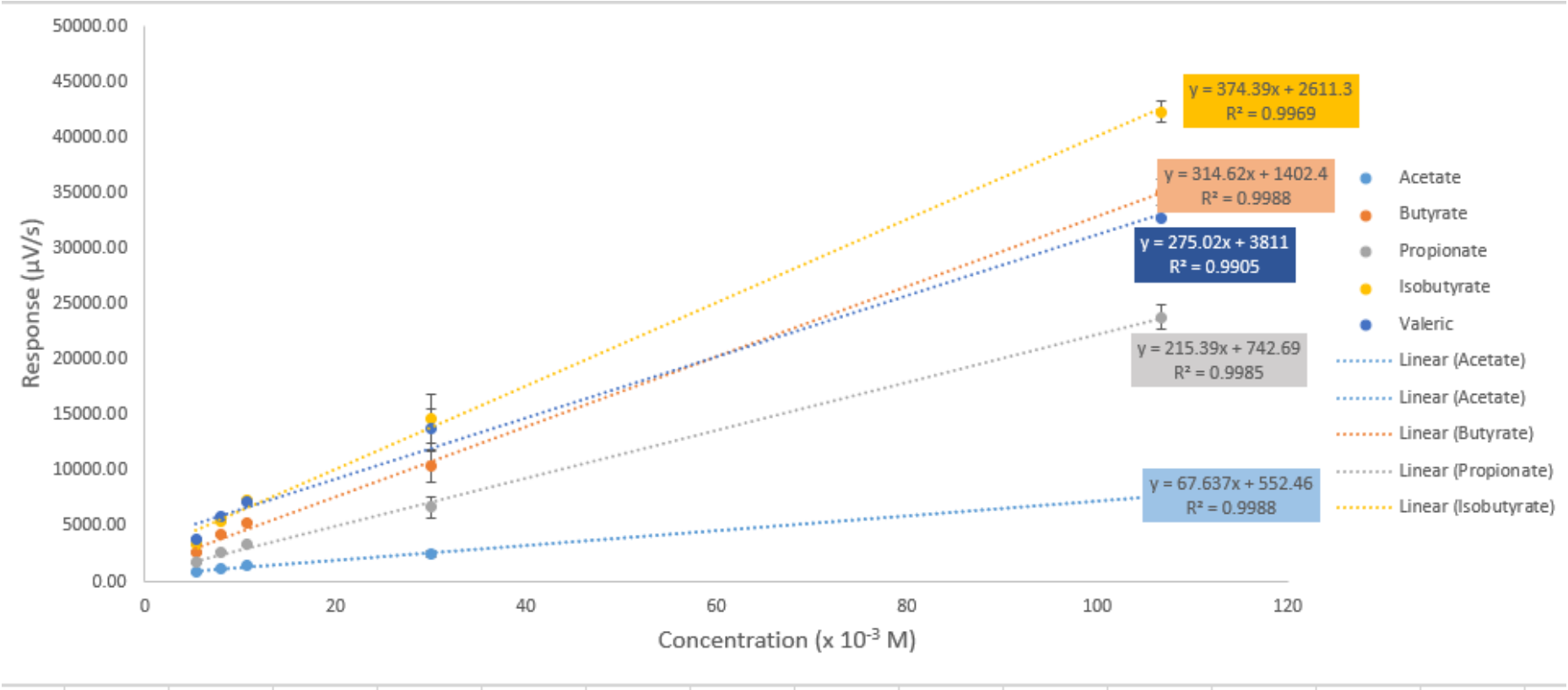


Figure 5.3b: High Concentration Calibration Chart. Figure shows linearity between the response of the GC-FID and higher 5 levels of concentrations (5.33 x 10^{-3} M, 7.99 x 10^{-3} M, 10.66 x 10^{-3} M, 30.0 x 10^{-3} M and 106.60 x 10^{-3} M) of each external standard. Data shown is the mean response of triplicate analyses $\pm$ SE

5.3.3 Percentage Recovery (%R)

The relationship between the expected concentration and actual concentration of an analyte gives information on the accuracy of the analytical method employed (Kalra, 2011). Calculating the recovery percentage of the analyte is a method employed to express this accuracy (Betz, Brown, & Roman, 2011; Horwitz, 2002; Kalra, 2011; Kayesh et al., 2013). Although percentage recovery is dependent on the concentration of the analyte (Betz et al., 2011; Horwitz, 2002; Kalra, 2011; Kayesh et al., 2013), a general percentage recovery range of between 80% and 120% may be considered appropriate in analytical chemistry (Erwin & Freek, 2001; Yin, 2015). Percentage recovery is calculated using the following formula:

$$\%R = \frac{\text{Recovered Concentration} \times 100}{\text{Injected Concentration}}$$

In this study, the accuracy of this method was evaluated at all the concentrations. Percentage recovery was evaluated based on mean response of triplicate analyses at each concentration (**Table 5.2**). Percentage recovery for the lowest concentration of all the SCFAs was either close to the lower acceptable limit (acetate and isobutyrate) or outside the acceptable percentage recovery range (butyrate, valeric acid and propionate). Furthermore, %R for valeric acid and isobutyrate at 30 M and 10.66 M respectively were on boarder line of the upper limit of the acceptable range. These may be indicative of the probable need for method adjustment (Horwitz, 2002) which may range from adjustment of gas flow rate to change in column size, for analyses of these SCFAs at these levels of concentration. Nevertheless, %R for at least 7 out of the eight concentrations of SCFAs using this protocol was within the acceptable range.

Table 5.2a: **Percentage recovery for Acetate**

Standard Concentration (x 10^{-3} M)	**Response (Area µV/s)**	**Recovered Concentration (x 10^{-3} M)**	**% Recovery**
0.11	107.75	0.09	81.82
1.07	228.84	1.00	93.46
2.67	461.80	2.74	102.62
5.33	819.17	5.42	101.69
7.99	1151.15	7.91	99.00
10.66	1399.30	12.52	117.45
30.00	2472.57	28.39	94.63
106.66	7785.06	106.93	100.25

Response is mean value of triplicate analyses

Table 5.2b: Percentage recovery for Butyrate

Standard Concentration (x 10^{-3} M)	Response (Area µV/s)	Recovered Concentration (x 10^{-3} M)	% Recovery
0.11	179.67	0.33	300.00
1.07	525.53	1.00	93.46
2.67	1324.94	2.56	95.88
5.33	2623.03	5.08	95.31
7.99	4223.45	8.20	102.63
10.66	5311.29	12.42	116.51
30.00	10353.58	28.45	94.83
106.66	35041.23	106.92	100.24

Response is mean value of triplicate analyses

Table 5.2c: Percentage recovery for Valeric Acid.

Standard Concentration (x 10^{-3} M)	Response (Area µV/s)	Recovered Concentration (x 10^{-3} M)	% Recovery
0.11	315.09	0.17	154.54
1.07	869.38	0.96	89.72
2.67	2159.49	2.81	105.24
5.33	3801.95	5.15	96.62
7.99	5855.74	8.08	101.13
10.66	7121.22	12.04	112.95
30.00	13725.61	36.06	120.02
106.66	32722.68	105.13	98.57

Response is mean value of triplicate analyses

Table 5.2d: Percentage recovery for Propionate.

Standard Concentration (x 10^{-3} M)	Response (Area µV/s)	Recovered Concentration (x 10^{-3} M)	% Recovery
0.11	130.52	0.30	272.72
1.07	353.63	0.98	91.59
2.67	879.59	2.59	97.00
5.33	1715.39	5.16	96.81
7.99	2690.21	8.14	101.88
10.66	3409.76	12.38	116.14
30.00	6679.91	27.57	91.90
106600	23818.38	107.14	100.45

Response is mean value of triplicate analyses

Table 5.2e: Percentage recovery for Isobutyrate

Standard Concentration (x 10^{-3} M)	Response (Area µV/s)	Recovered Concentration (x 10^{-3} M)	% Recovery
0.11	151.15	0.09	81.82
1.07	833.66	1.13	105.61
2.67	1930.08	2.81	104.97
5.33	3323.51	4.94	92.68
7.99	5455.81	8.20	102.63
10.66	7408.90	12.81	120.17
30.00	14718.39	32.34	107.80
106.66	42292.14	105.99	99.37

Response is mean value of triplicate analyses

5.3.4 Limits of variation (Normalisation of data)

RTs from samples were compared with RT for standards for plausible identification of SCFAs present in fermented samples. Following the shifts in RT during analyses of the standards, limits of variation was set based on lowest and highest retention times and the standard deviation (SD) for all SCFAs (**Table 5.3**). Hence, limits were set as follows

Lower limit = lowest RT – SD

Higher limit = highest RT + SD.

Table 5.3: Limits of variation for identification of SCFAs in samples

Standard	Lowest RT (min)	Highest RT (min)	SD	Lower limit (min)	Higher limit (min)
Acetate	10.008	10.173	0.041	9.967	10.214
Butyrate	12.149	12.307	0.037	12.112	12.344
Propionate	10.838	11.020	0.047	10.791	11.067
Isobutyrate	11.129	11.313	0.048	11.081	11.361
Valeric acid	14.315	14.351	0.012	14.303	14.363

5.3.5 SCFAs in samples

There were 20 samples in total each of which was analysed in triplicate. Based on RT of standards (**Table 5.4**), only acetate was detected in samples of CF corn, while acetate and butyrate were found in SF corn samples. Detection of butyrate in SF and not CF corn may be due to the variation of the microbial community in both types of samples (Li et al., 2017).

Table 5.4a: **SCFA in CF corn using single strain starter culture and undigested corn samples.**

Sample (After 120 hours of fermentation)	RT (min)	SCFA
CF using *Weissella confusa*	10.216 + 0.005	Acetate
CF using *Pedicoccus pentosaceus*	10.123 + 0.064	Acetate
CF using *Saccharomyces cerevisiae*	10.130 + 0.052	Acetate
CF using *Weissella confusa* 2	10.176 + 0.016	Acetate
CF using *Lactobacillus plantarum*	10.135 + 0.072	Acetate
Undigested unfermented	10.189 + 0.036	Acetate
Digested unfermented	10.133 + 0.066	Acetate

RT is the mean of triplicate analyses of each sample $\pm$ SD

Table 5.4b: **SCFA in CF using mixed strain starter culture**

Time of Controlled Fermentation	**RT (min)**	**SCFA**
0 hours	10.167 + 0.009	Acetate
24 hours	10.156 + 0.024	Acetate
48 hours	10.127 + 0.036	Acetate
72 hours	10.127 + 0.079	Acetate
96 hours	10.098 + 0.043	Acetate
120 hours	10.139 + 0.074	Acetate
Digested	10.125 + 0.055	Acetate

Starter culture consisted of *W. confusa, P. pentosaceus, S. cerevisiae, W. confusa* 2, *L. plantarum* (Chapter 4). RT is the mean of triplicate analyses of each sample ± SD

Table 5.4c: **SCFAs in SF corn.**

Time of Spontaneous Fermentation (hour)	**RT (min)**	**SCFA**
24 hours	10.057 + 0.049	Acetate
	12.163 + 0.073	Butyrate
48 hours	10.050 + 0.033	Acetate
	12.140 + 0.091	Butyrate
72 hours	10.030 + 0.065	Acetate
	12.165 + 0.045	Butyrate
96 hours	9.944 + 0.055	Acetate (RT outside set limits)
	12.121 + 0.033	Butyrate
120 hours	9.868 + 0.011	Acetate (RT outside set limits)
	12.112 + 0.004	Butyrate
Digested	10.019 + 0.133	Acetate
	12.293 + 0.004	Butyrate

RT is the mean of triplicate analyses of each sample ± SD

The intercept and slope values from the calibration curves were used to estimate the concentration of detected analyte in the quantity injected into the GC-FID. The calibration curve used for the analyses was dependent on the level of response area of the analyte observed. For acetate, low concentration calibration was applied to response areas < 1151.15 μV/sec, and high concentration calibration was applied when response areas were > 1151.15 μV/sec. Similarly, for butyrate, low concentration calibration was applied to response areas < 4223.45 μV/sec, and high concentration calibration was applied when response areas were > 4223.45 μV/sec.

5.3.5.1 Acetate in samples

Acetate was detected in unfermented corn and at 0 hours of controlled fermentation. The presence of acetate in these sample is not uncommon as acetate is known to be ubiquitous in natural environment (Bernal, Castaño-Cerezo, & Cánovas, 2016; Deroover et al., 2017) and this ubiquitous characteristic makes it easily transferrable (Deroover et al., 2017). Acetate may be contained in soil or fertilizers that may have been used during corn cultivation (Njukeng, Elambo, Ejolle, & Schnug, 2013) and which could have easily been transferred to corn grains. Also, though dry cleaned before use, it is not impossible to have acetate contamination from the milling machine used for the corn in this experiment. As earlier mentioned, the machine was generally used for dried foods, including dried fermented cassava. Furthermore, acetate is also known to be an exogenous carbon source to various bacteria and fungi (Bernal et al., 2016; Sun et al., 2018). This gives the likely explanation to why concentration of acetate was highest in unfermented corn in comparison to all single strained fermented corn, after 120 hours of the process (**Figure 5.4**), suggesting that the organisms utilized acetate as a readily available energy source or for the production of other fatty acids (Esquivel-Elizondo, Ilhan, Garcia-Peña, & Krajmalnik-Brown, 2017). This plausible explanation was further exhibited during the first 48 hours of CF with mixed strain starter culture made up of *W. confusa, P. pentosaceus, S. cerevisiae, W. confusa* (2), *Lb. plantarum*. However, acetate concentration started to increase at 72 hours becoming higher by over 20% at 120 hours compared with its concentration at 0 hours. This may have resulted from a change in the growth dynamics of the fermentation organisms, with other sources of energy made available to the organisms after 48 hours.

During SF on the other hand, there was a consistent decrease in the concentration of acetate throughout the fermentation process (**Figure 5.4**) also suggesting acetate as the preferred carbon source or the interconversion reaction of acetate into other carboxylic compounds (Esquivel-Elizondo et al., 2017). Notably, there was higher concentration of acetate throughout the process compared with samples of the other type of fermentation and the sterile corn. Mann Whitney U test

showed that the concentration of acetate during SF was significantly larger than during mixed culture CF ($p < 0.0001$). The concentration of acetate at 0 hour of SF was not obtained, however its concentration at 24 hours of SF when compared with 24 hours of CF and the concentration of the sterile unfermented corn, suggests a noteworthy loss during the process of heat sterilization. Corn was sterilized at $121^{O}C$ and with acetic acid being volatile and having a boiling point of $118^{O}C$ (Young, 2008), therefore the loss of acetate due to evaporation during sterilization would be inevitable. Furthermore, there was a shift in the RT of acetate outside the limit of variation which may be an indication of some degree of inaccuracy in the response obtained from the detector.

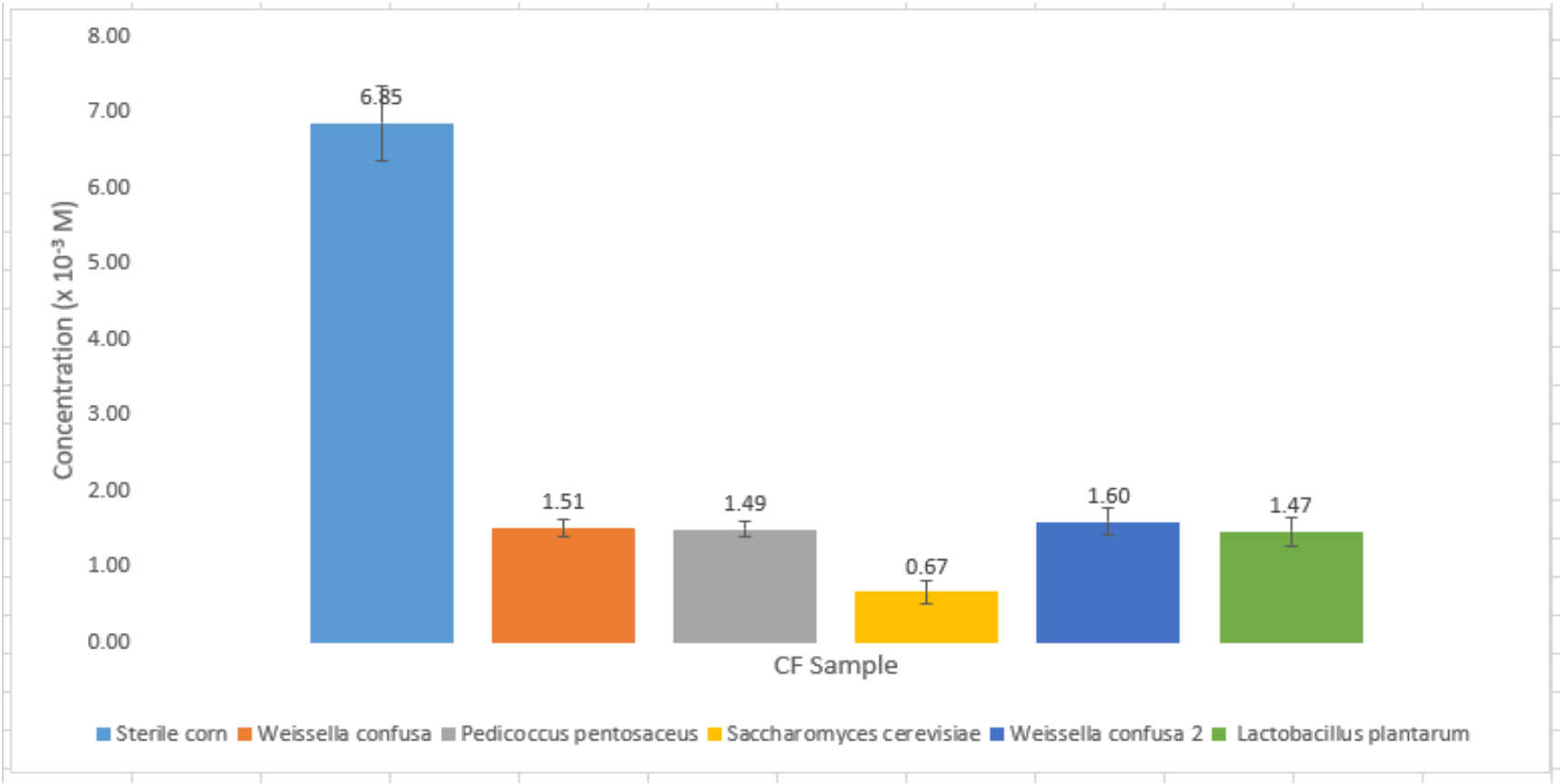


Figure 5.4a: Concentration of Acetate after 120 hours of single organism fermentation. Concentration in samples was calculated based on the response detected from the GC-FID, slope and intercept of the appropriate calibration curve (as previously explained), formula in **section 5.3.2**. Data on the graph is the representation of the mean concentration of triplicate analyses of each sample $\pm$ SE

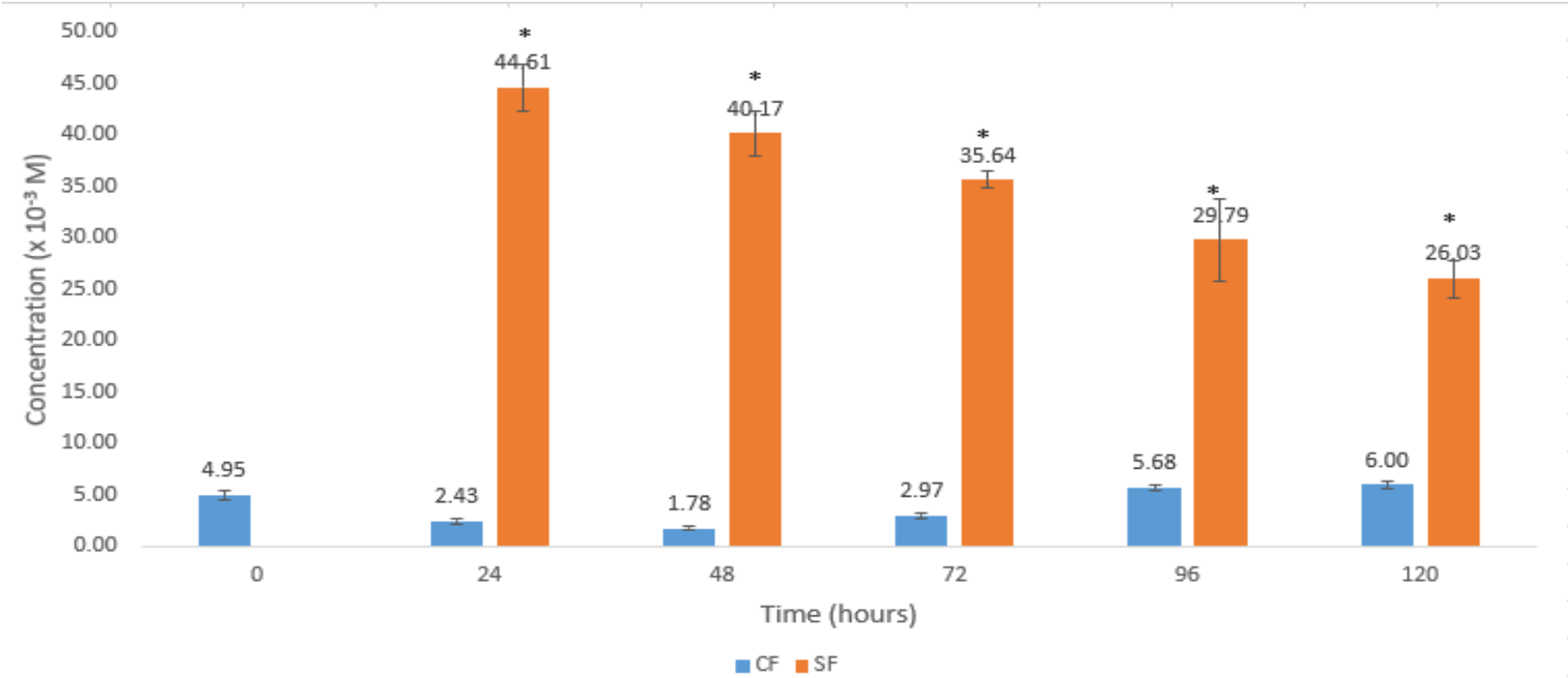


Figure 5.4b: Concentration of Acetate during SF vs CF fermentation. Samples of supernatant solutions were collected at 24-hour intervals during 120 hours of fermentation. Concentration in samples was calculated based on the response detected from the GC-FID, slope and intercept of the appropriate calibration curve (as previously explained), formula in **section 5.3.2**. Data on the graph is the representation of the mean concentration of triplicate analyses $\pm$ SE. *$p < 0.0001$

5.3.5.2 Butyrate in samples

Butyrate was not detected in sterile corn hence its presence in SF could be related to the outcome of metabolic activities of fermentation organisms which convert carbohydrates into butyrate or acid-utilizing organisms which convert lactate and acetate into butyrate (Esquivel-Elizondo et al., 2017; Morrison et al., 2006; Morrison & Preston, 2016). At 24 hours of fermentation, concentration of butyrate was 13.68 M and had more than doubled (29.25 M) at 48 hours of fermentation with no remarkable change in concentration at 120 hours (**Figure 5.5**). Production of butyric acid may have contributed to the observed aromatic smell at 24 hours (as reported in the Chapter 4) as butyric acids and its esters are known to considerably contribute to the flavour and aroma characteristics of fermented foods (Blandino et al., 2003; Kam et al., 2011; Longo & Sanromán, 2006; McFeeters, 2004; Routray & Mishra, 2011).

5.3.6 Effect of simulated digestion on SCFA

There was 17%, 112% and 442% increase in acetate after the upper digestion of SF, CF and sterile corns respectively (**Figure 5.6**). Though in limited quantities, cereals, including corn, may contain up to 7% lipids, which is predominantly found in the germ (Liptáková et al., 2017). Digestion of these lipids result in the release of free fatty acids (Hussain, 2014; Iqbal & Hussain, 2009). There was a larger increase in acetate in sterile corn after upper digestion (30.27M) than in the fermented samples (4.52M for SF and 6.7M for CF) following the same treatment. This may suggest fat metabolism by the organisms (Li et al., 2017) or oxidation of fat by cereal enzymes such as lipoxygenase during fermentation to generate volatile compounds (Liptáková et al., 2017; Marko, Rakická, Mikušová, Valík, & Šturdík, 2014), thereby reducing the amount of fat available in the fermented samples for digestion. On the other hand, there was over 85% loss of butyrate after upper digestion of SF corn. At 30^{O}C, vapour pressure for acetic and butyric acids are 1.5 kPa (11 mmHg) and 0.057 kPa (0.43 mmHg) respectively (ILO-ICSC, 2019) while boiling point for acetate (118^{O}C) is also lower than that of butyric acid (163.8^{O}C) suggesting that acetate may be more volatile than butyrate. Nevertheless, the reason for the loss of butyrate after upper digestion is unknown.

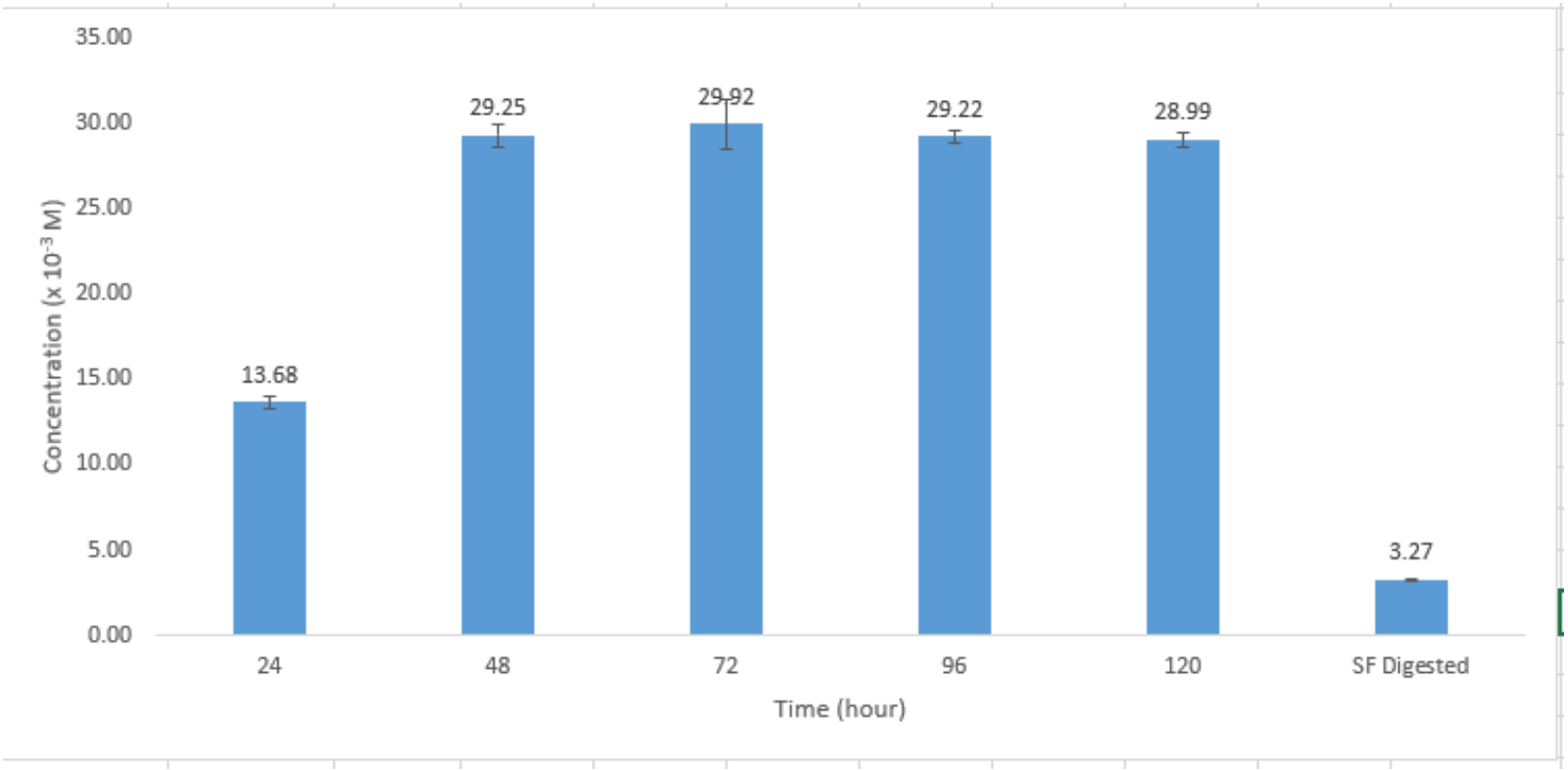


Figure 5.5: Concentration of butyrate during fermentation and after digestion. Samples of supernatant solution were collected at 24-hour intervals during 120 hours or spontaneous fermentation and after simulated digestion. Concentration of butyrate in samples was calculated based on the response detected from the GC-FID, slope and intercept of appropriate calibration curve (as previously explained), formula in section 5.3.2. Data on the graph is the representation of the mean concentration of triplicate analyses one cycle of fermentation at each time $\pm$ SE

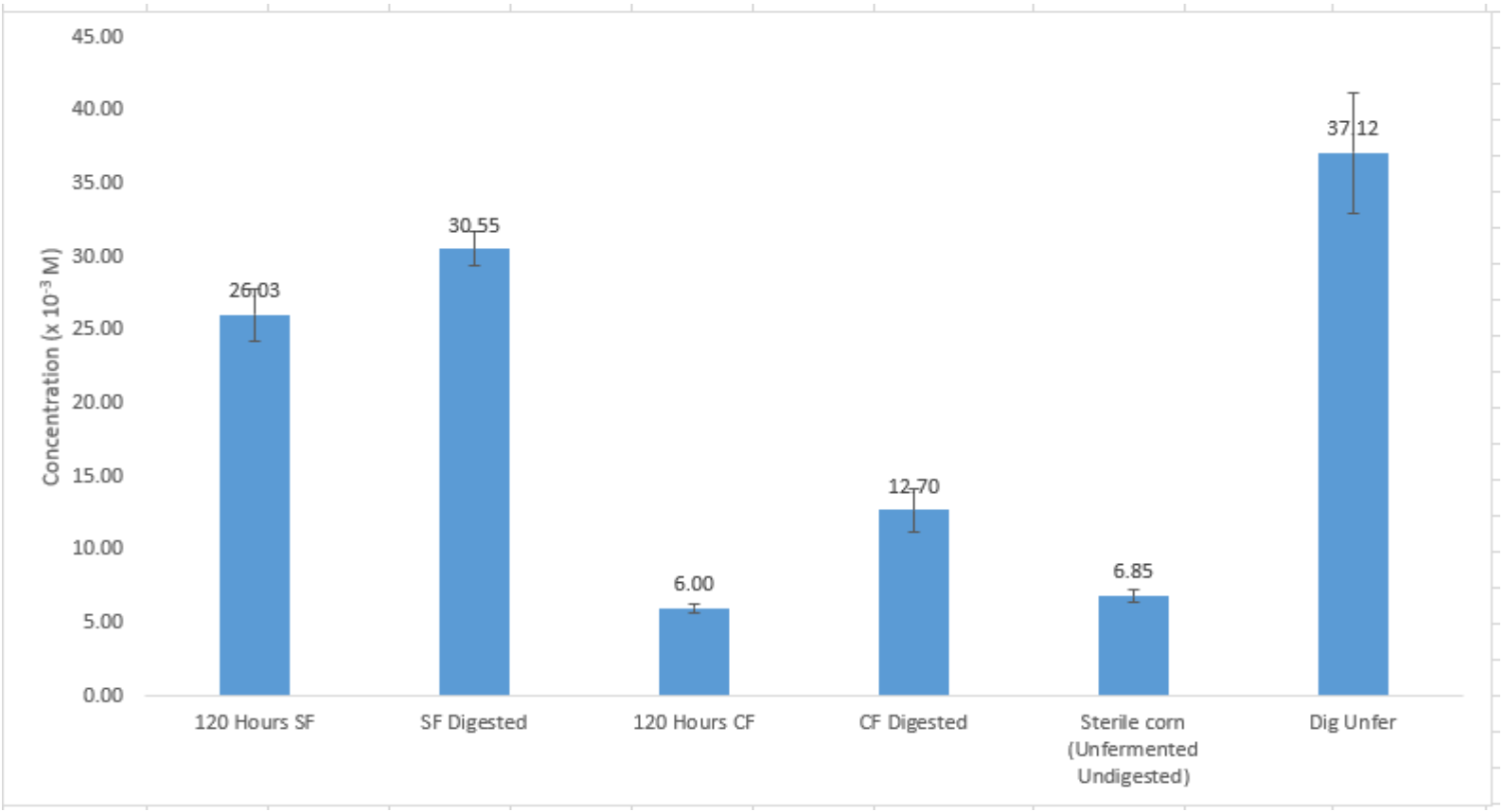


Figure 5.6: Comparison of concentrations of Acetate Concentration in sample was calculated based on the response detected from the GC-FID, slope and intercept of the appropriate calibration curve (as previously explained), formula in **section 5.3.2**. Data on the graph is the representation of the mean concentration of triplicate analyses of each sample $\pm$ SE

5.4 Discussion

The aims of this study were to 1) investigate and compare the production of SCFAs in fermented corn during SF vs CF; 2) investigate SCFA production of organisms from SF during single strain CF; 3) investigate the effect of upper digestion on the SCFAs in fermented foods.

Though lactic acid is the major output of cereal fermentation (Morrison & Preston, 2016), its presence in the fermented samples in the current study was not investigated as it is not classed as a SCFA (Ríos-Covián et al., 2016).

5.4.1 SCFA in CF vs SF

Acetate and butyrate were detected in SF corn in the current study. Similarly, apart from lactic acid, Banigo and Muller (1972), identified acetic and butyric acids as the carboxylic acids with the largest concentration detected in SF corn 'ogi' out of a total of 11 acids identified. Conversely, propionate was detected along with acetate and butyrate as the major carboxylic acids in traditional maize sourdough (solid-state fermentation) by Plahar and Leung (1982) and Halm et al. (1993). Annan et al. (2003) reported not detecting butyrate in SF maize sourdough, but acetate was the major carboxylic acid detected. Output of spontaneously or traditionally fermented foods is unpredictable and dependent on environmental factors such as geographical location (Katongole, 2008; Li et al., 2017). Maize dough is a traditional meal in Ghana with a slight difference in production method compared to ogi (submerged fermentation) which may explain the variations in the production of carboxylic acid in both fermented maize products. Furthermore, the microbial community in the substrate is also an important determinant in production of carboxylic acid and other aromatic compounds during fermentation (Li et al., 2017) and variations in these microbial consortium may result in variations in fermentation output. Plahar and Leung (1982) also reported that moisture content in the maize dough was associated with the type and concentration of carboxylic acid produced during SF. This provides another plausible explanation for the variations in the outcomes of the mentioned studied, including the current study.

In the study by Özcelik, Kuley, and Özogul (2016), which was aimed at investigating the production of organic acids in fish infused broth and in MRS broth by LAB after 4 days of fermentation, the authors showed that production of organic acids including SCFAs was greatly influenced by the type of substrate. The authors reported that *Lb plantarum* (FI8595) produced 753.11 ± 57.52 mg/ml of butyric acid with no acetic acid detected in MRS broth; produced 558.72 ± 28.33 mg/ml and 56.46 ± 4.89 mg/ml of butyric and acetic acids respectively in anchovy infused broth; produced 27.52 ± 1.66 mg/ml and 261.65 ± 11.81 mg/ml of butyric and acetic acids

respectively in sea bass infused broth. Production of SCFA may be also be dependent on the strain of organisms involved the fermentation process. Kahouli et al. (2015) investigated the bio-production of SCFAs 6 different strains of *Lb reuteri* reporting that, only 3 out of the 6 test strains produced acetic, propionic and butyric acids in the tested bio-sample (simulated intestinal fluid) at varied concentrations.

Though production of SCFA is influenced by the type of substrate (Zalán et al., 2010; Özcelik et al., 2016), other factors such as the type of strains used during single strain and the interaction within the microbial consortium of mixed culture CF (Zalán et al., 2010) may have resulted in the absence of butyrate in the CF. While not all LAB may be directly butyrogenic, they may be able to produce other compounds which become precursor or substrate for other organisms to convert to butyrate as authors have suggested this indirect roles of LAB in the production of butyrate in the human colon (George et al., 2018). Various studies have suggested that lactic acid, acetic acids or ethanol produced by LAB and yeast can be converted to butyrate by major butyrate-producing bacterial species in the human colon such as *Anaerostipes caccae*, *Eubacterium hallii* and *Anaerostipes coli* which belong to the phylum *Firmicutes* (Esquivel-Elizondo et al., 2017; Moens, Verce, & De Vuyst, 2017; Muñoz-Tamayo et al., 2011). Organisms used as starter culture in CF were isolated from the SF corn however there was production of butyrate in SF which was not detected in all CF samples. This therefore indicates that there were other organisms during SF, which though may not have been dominant, may have significantly contributed to the production of butyrate. Contamination of the corn with butyrate producing organisms from soil, fertilizer or the milling machine is also not impossible. Clostridia for example, are often described as "butyric acid spores" and have some pathogenic species namely *Clostridium tetani*, *Clostridium botulinum* and *Clostridium perfringens* (Drouin & Lafrenière, 2012). Though they are found to be contaminants in dairy products, they are ubiquitous by nature and can be isolated from a wide range of sources (Drouin & Lafrenière, 2012; Ljungdahl, Hugenholtz, & Wiegel, 1989). They can be found in the soil and in the fertilizer, hence can easily be transferrable to plants and farm produce including corn kernels (Drouin & Lafrenière, 2012). Clostridia are able to produce butyrate from carbohydrate but they are also able to convert acetate and lactate to butyrate (Drouin & Lafrenière, 2012; Heyndrickx, 2011; Ljungdahl et al., 1989).

5.4.2 Effect of digestion SCFA in fermented corn

In the current study, upper digested resulted in an increase in the concentration of acetate after digestion but a notable decrease in the concentration of butyrate. Digestion was done using a standardised static *in-vitro* model at 37^{O}C, but the reason for the loss of butyrate is unknown.

However, the use of a dynamic *in vitro* digestion model may be more reliable for a correct estimation of the bioavailability of SCFAs from a digested food substance (Alegría, Garcia-Llatas, & Cilla, 2015). During digestion, free fatty acids, including SCFAs from dietary sources are absorbed from the small intestine through simple diffusion directly into the portal vein and delivered to the liver after which it is transported to the peripheral circulation (Hussain, 2014; Iqbal & Hussain, 2009; McClements & Li, 2010). From the colon, undissociated SCFAs are absorbed into the peripheral circulation via passive diffusion while dissociated SCFA anions are absorbed through active transport (den Besten et al., 2013).

5.5 Summary and Conclusion

Strains of *W. confusa, P. pentosaceus, S. cerevisiae, W. confusa* (2), *Lb. plantarum* used in the current study showed the potential to produce acetate during the CF of 'ogi' as single strain starter culture and as a mixed starter culture. Acetate was also detected in SF corn in the current along with butyrate. This shows the potential for fermented corn to be a exogenous source of SCFA depending on the microbial community involved in the fermentation process. The current study also showed that digestion of fermented corn resulted in an increase in the concentration of acetate but a decrease in the concentration of butyrate. Nevertheless, the use of dynamic *in vitro* digestion model may be a more reliable method of investigating bioavailability of SCFAs after digestion (Alegría et al., 2015).

Some articles have also investigated and reviewed the concentration of carboxylic compounds, including SCFAs produced fermented foods however the scope has been with respect to their effects on the organoleptic characteristics of the fermented food, though the possible health benefits of these carboxylic compounds have been acknowledged (Dertli & Çon, 2017; Halm et al., 1993; Hayek & Ibrahim, 2013; Javanainen & Linko, 1993; Kam et al., 2011; Lee et al., 2010; Leite et al., 2013; Longo & Sanromán, 2006; Marko et al., 2014; Muyanja et al., 2012).

As far currently known, studies that have investigated the effect of these SCFAs present in fermented foods on the systemic or peripheral concentration of the carboxylic acids in the consumers are not available. The effects of the intake fermented foods containing SCFAs on colonic concentration of these compounds and possible derivable immune and metabolic health benefits as previously discussed have not been investigated *in vivo*. The current study suggests the fermented corn may be a source of exogenous and dietary SCFAs. Nevertheless, more studies investigating the effect of this exogenous source on systemic concentration and gut health may be beneficial.

Chapter 6

Nitric oxide from ingestion of fermented corn

6.1 Introduction

Nitric oxide (NO) is a reactive signalling molecule involved in several biological, physiological and pathological processes in the human body (Luiking, Engelen, & Deutz, 2010). Though short-lived, the molecule is able to dissolve in lipids which enhances its ability to diffuse quickly through cells, initiating intercellular and intracellular signals (Müller & Schier, 2011; Shah, Lyford, Gores, & Farrugia, 2004; Thomas, 2015). NO is quickly oxidized into nitrite (NO_2^-) and nitrate (NO_3^-) in the presence of oxygen and in aqueous state (Hetrick & Schoenfisch, 2009; Tiso & Schechter, 2015; Tripathi, Tripathi, Kashyap, & Singh, 2007; Weitzberg, Hezel, & Lundberg, 2010) making its quantitative and qualitative analyses difficult (Csonka et al., 2015; He & Frost, 2016; Hetrick & Schoenfisch, 2009). Therefore quantification of NO is estimated from the analyses of its metabolites such as nitrite and nitrate (Hetrick & Schoenfisch, 2009; Luiking et al., 2010).

NO may either be endogenously synthesized in the body, or exogenously supplied (Levine, Punihaole, & Levine, 2012; Luiking et al., 2010). Endogenous NO may be from enzymatic and from non-enzymatic sources. Enzymatic sources are catalysed by different isoforms of the Nitric Oxide Synthase (NOS) (Thomas, 2015; Tripathi et al., 2007). There are 3 main isoforms of NOS including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS); each of which has its own functions (Chen, Pittman, & Popel, 2008; Förstermann & Sessa, 2011; Luiking et al., 2010; Shah et al., 2004). Neuronal NOS (nNOS) is primarily produced in central and peripheral neurons as well as in the testes, skeletal muscles, in epithelial cells of various organs, macula densa cells in kidneys and in pancreatic islet cells (Förstermann & Sessa, 2011). The functions of NO derived from nNOS include blood pressure regulation, modulation of penile erection, muscle relaxation, modulation of the growth and development of the nerve tissue, whilst also influencing memory and learning (Förstermann & Sessa, 2011; Tripathi et al., 2007). NO from iNOS is mainly expressed in response to any form of attack to the body such as infection, inflammation and tumour (Förstermann & Sessa, 2011; Hosking, 2009; Tripathi et al., 2007). Though predominantly produced by the immune cells (Hosking, 2009; Tripathi et al., 2007), non-immune cells are also able to express iNOS for the production of NO in response to cytokine production, an example of which can be seen in cytokine-activated endothelial cells in response to tumor cells (Förstermann & Sessa, 2011). eNOS is expressed majorly by the endothelial cells but they have also been reported to be produced by other cells such as cardiac myocytes, platelets, immune cells and the epithelial cells of the kidney (Förstermann & Sessa, 2011; Hosking, 2009).

Homeostatic regulation of cardiovascular functions, protection against onset and progression of atherogenesis, are examples of functions of NO derived from eNOS (Förstermann & Sessa, 2011).

Non-enzymatic generation of NO in the human cells involves the reduction of nitrite and nitrate into NO (Kim & Hur, 2017; Tiso & Schechter, 2015; Zweier, Samouilov, & Kuppusamy, 1999). The process is initiated when there is a notable reduction in the concentration of NO in the cells often resulting from an impairment in the enzymatic production process using the NOS (Luiking et al., 2010; Zweier et al., 1999). NO is sourced using NOS-independent pathways during diseases such as ischaemia (Luiking et al., 2010; Zweier et al., 1999) or in acidic conditions, mostly in stomach, with saliva being the source of nitrite in the stomach (Bender & Schwarz, 2018; Zweier et al., 1999).

The mentioned pathways of NO signalling can be seen in almost all biological processes in the human body (Chen et al., 2008; Förstermann & Sessa, 2011). Hence, various studies and articles have investigated and reviewed the function, association or involvement of NO in various physiological and pathological processes in the human body (Chen et al., 2008; Hosking, 2009; Kochar, Chandewal, Bakal, & Kochar, 2011; Luiking et al., 2010; Thomas, 2015). Apart from being involved in the immune function by controlling the body's response to infections, inflammation and cancer (Förstermann & Sessa, 2011; Tripathi et al., 2007; Shah et al., 2004), NO has been recorded to control reproduction in males and females by influencing spermatogenesis and ovulation respectively (Dixit & Parvizi, 2001; McCann & Rettori, 1996; Rosselli, Keller, & Dubey, 1998); participate in the cardiovascular functions in the body by controlling the mechanism and the rate at which the heart pumps, howbeit also controlling how the heart recovers from diseases such as infarction and ischemia (Levine et al., 2012; Rastaldo et al., 2007; Strijdom, Chamane, & Lochner, 2009); and regulate motility and vascular processes in the gastrointestinal tract (GIT).

Decreased and eventual inadequate production of NO in the body, termed endothelial dysfunction, is known to be an early signal for the onset and progression of chronic disease (Bryan, 2015). Decreased bioavailability of NO, which may be linked with smoking and age, may also be associated with illnesses such as cardiovascular diseases (Steffen et al., 2012). Hence, exogenous sources of NO are used as alternative sources of NO for the treatment and management of such diseases (Balotf et al., 2018; Bondonno, Croft, & Hodgson, 2016; Bryan, 2015; Qu et al., 2016). Dietary sources of nitrate and nitrite including drinking water and vegetables (Bondonno et al., 2016), have been identified as useful exogenous sources of NO (Bondonno et al., 2016; Nathan S. Bryan, 2015). Vegetables including celery, cress, spinach and lettuce contain over 250 mg of nitrate

per 100 mg while mushrooms, potatoes, eggplants and tomatoes contain less than 20 mg of nitrate per 100 mg (Bondonno et al., 2016). Dietary intake of nitrate/nitrite has been suggested to help improve athletic performance, due to improved respiratory, cardiac and muscular functions in athletes (Kerley, 2017; Stanaway, Rutherfurd-Markwick, Page, & Ali, 2017).

A lower prevalence of cardiovascular diseases has been reported in populations that follow the Mediterranean Diet compared with populations following western diet (Bloomfield et al., 2015; D'Alessandro & De Pergola, 2015; Widmer, Flammer, Lerman, & Lerman, 2015). Though the Mediterranean Diet contains less amount of saturated fat, larger amount of various types of phytochemicals and fibre compared with the western diet (Pernice, Vitaglione, Sacchi, & Fogliano, 2007) all of which have been reported to reduce the risk of cardiovascular diseases (Pagliaro, Santolamazza, Simonelli, & Rubattu, 2015), the Mediterranean Diet also contains over 20 times of nitrate compared with the western diet (Bondonno et al., 2016). Therefore, the theory of a protective association between high nitrate intakes from the Mediterranean Diet and the risk of cardiovascular diseases may be correct. Nevertheless, there are no epidemiological studies that have investigated this plausible protective association possibly due to the difficulty in weighing dietary contributions to plasma or urinary concentrations of nitrate/nitrite (Bondonno et al., 2016; Hord, Tang, & Bryan, 2009).

Even though NO is known to have regulatory and protective functions in the body it may also play cytotoxic roles such as pro-inflammatory roles, inducing toxic reactions against the cells producing it, when production is in high concentrations (Hosking, 2009; Levine et al., 2012; Rosselli et al., 1998; Shah et al., 2004; Tripathi et al., 2007). Regulatory and protective effects often require pico- to nanomolar levels of NO concentrations whereas higher concentration may be cytotoxic (Levine et al., 2012). As a result, the Acceptable Daily Intake (ADI) of nitrate and nitrite ion according to Joint Food and Agricultural Organization (FAO)/World Health Organization (WHO) are 3.7 mg and 0.06 mg respectively per kilogram body weight (European Food Safety Authority, 2008; Hord et al., 2009). But the WHO recommends a minimum daily fruit and vegetable intake of 400g (Agudo, 2005), suggesting a daily nitrate intake of up to 1000 mg, which is same for those following the Dietary Approaches to Stop Hypertension (DASH) diet (Bondonno et al., 2016). This not only calls to question the recommended limits set by FAO and WHO (Hord et al., 2009) but also suggests that dietary nitrate/nitrite intake of up to 1000 mg may not result in the cytotoxic effects that may result from endogenous supplies of NO in the body. A few studies have suggested the possible toxic effect of high intake of dietary nitrate/nitrite intake, however, results have been inconsistent (Hord et al., 2009). Nevertheless, dietary nitrate/nitrite intake is not recommended by

America Academy of Paediatrics to infants below 3 months due to its associated risk to methemoglobinemia (Hord et al., 2009)

Aside from, water and vegetables, fruits and processed meat have also been mentioned as the dietary sources of nitrate/nitrite (Bondonno et al., 2016; Hord et al., 2009). Abdulrazak, Otie, and Oniwapele (2014) also reported that cereal grains may be potential sources of dietary nitrate and nitrite. Fermented food products are frequently consumed in various countries in the world (Chilton, Burton, & Reid, 2015). Fermented fruits, vegetables are very popular and important cuisine in Asia (Swain, Anandharaj, Ray, & Parveen Rani, 2014) and fermented cereal foods and beverages are frequently consumed in Africa (Achi & Asamudo, 2018). Yet, as far as we know, there is dearth of information about fermented products being possible dietary sources of nitrite and nitrate with only a handful of studies investigating NO concentrations in fermented vegetables, sausage and corn (Ezeagu, 1996; Kim & Hur, 2018; X. Wang, Ren, Liu, Zhu, & Wang, 2013; Yan, Xue, Tan, Zhang, & Chang, 2008; Yang, Liu, Xi, & Tang, 2004).

Therefore, this current study aimed to 1) quantify the concentrations of nitrate and nitrite in spontaneous (SF) and controlled fermented (CF) corn 2) To investigate the effect of fermented corn, spontaneous and controlled, on the NO concentration in plasma.

6.2 Materials and Methods

6.2.1 Sample preparation

Aliquots of previously fermented milled corn using spontaneous and controlled methods at 120 hours of fermentation including digested samples of fermented corn, which had been stored at -20^{O}C (Chapter 4) were thawed before analyses. Unfermented undigested corn was sterilized as previously described (in chapter 4) and kept on working bench at room temperature until further analysis. All frozen samples were thawed at room temperature as indicated in instructions of the assay kit used. Blood sample from a healthy volunteer was supplied by Cambridge Biosciences. Blood sample was stored at 5^{O}C upon delivery until day of analysis when it was kept on the working bench and brought to room temperature. Invitrogen^{TM} Nitric oxide assay kit, purchased from ThermoFisher Scientific UK, was used for NO analysis of samples. The kit estimates NO but determining the concentrations of nitrite and nitrate in the sample using colorimetric technique (Griess reaction). Corn was mixed with blood at ratio 1:1 to investigate the effect of corn on NO concentration in the plasma. All samples were mixed with the 1 X Reagent Diluent at ration 1:2 as indicated on the kit's information sheet before analysis. All samples containing blood were filtered

through 10,000 MWCO filter at 4000 rpm for 45 mins as indicated on the kit's information sheet before analysis.

6.2.2 Standards

Standards were prepared as described on the kit's information sheet. Standard solutions were made at 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 3.125 µM nitrite concentrations and 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 3.125 µM nitrate concentrations.

6.2.3 Sample analysis

Solutions of samples and standards, including blanks were pipetted into labelled 96 wells flat bottom microplate as instructed in the kit's information sheet. Microplates were analysed using the Synergy™ HTX Multi-Mode Microplate Reader. The reader was set to shake 3 times within 5 seconds and optical density was measured at wavelength 570 nm, the closest to the wavelength of 540 $\pm$ 20 nm which was recommendation on the kit's information sheet. KC4 Data Analysis Software was used for data collection, analysis and exporting from the plate reader. Optical density from the nitrite and nitrate standard solutions of known concentration were used for calibration following steps stated on the kit's information sheet. This calibration shows the relationship between the optical density and the known concentrations of the standards. The closer to 1 the coefficient of regression (R^2), the stronger the relationship between both variables (Prichard & Barwick, 2003). Nitrite and nitrate concentrations in the samples were then estimated using the calibration chart (Prichard & Barwick, 2003). Each sample and all standards were analysed in triplicate.

6.2.4 Statistical analysis

Due to the sample size (Faber & Fonseca, 2014; Sullivan & Feinn, 2012), descriptive statistics were completed to compare the concentrations of nitrate and nitrite in SF vs CF corn and to estimate the effect of corn on the nitrate and nitrite concentrations in blood plasma.

6.3 Results and Discussion

6.3.1 Linearity of Calibration

The coefficient of regression (R^2) for nitrate and nitrite were 0.9936 and 0.9996 respectively (**Fig 6.1**) showing a strong association between the optical density and the concentrations of the test compounds. Concentration of nitrite and nitrate in the samples were calculated following the steps described in the kit's information sheet.

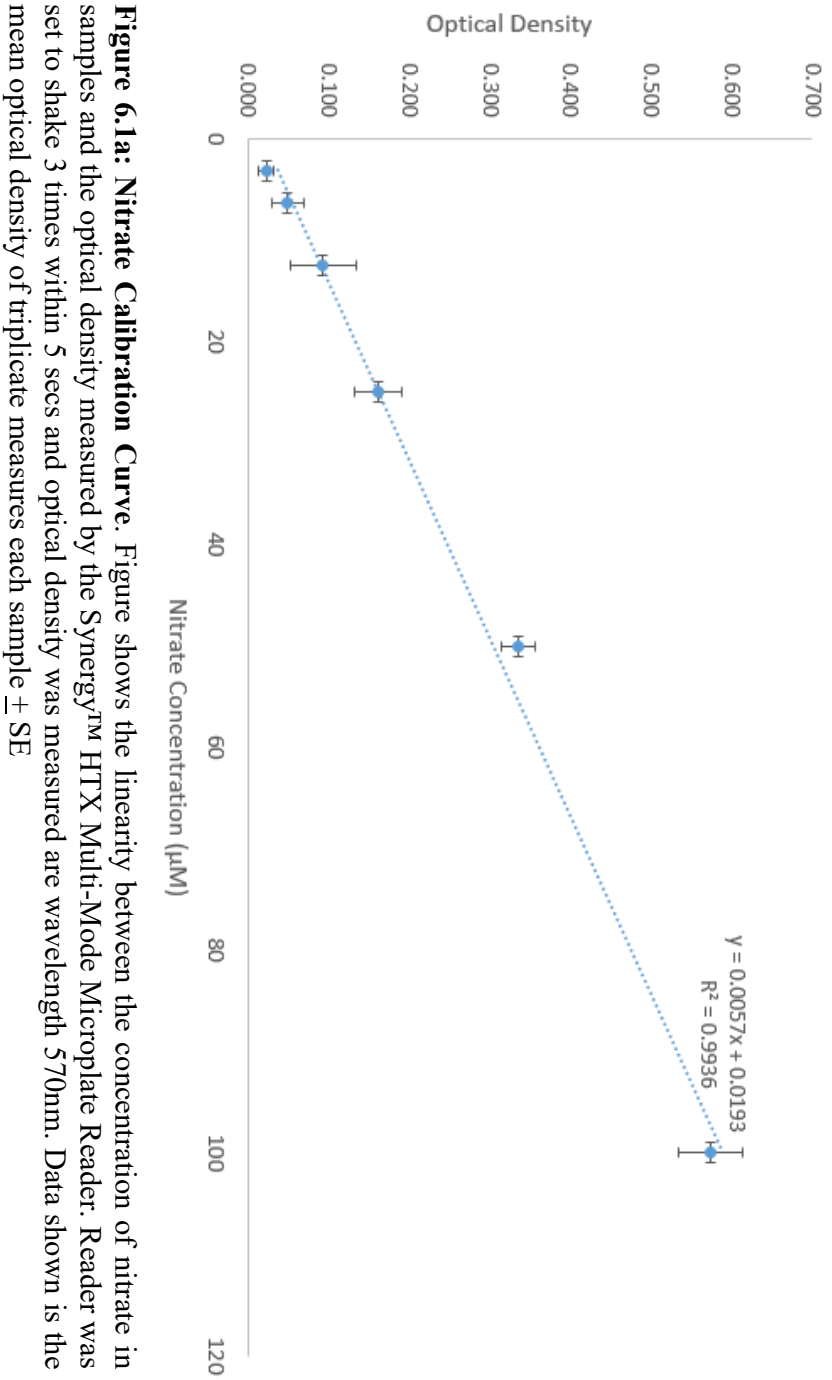


Figure 6.1a: Nitrate Calibration Curve. Figure shows the linearity between the concentration of nitrate in samples and the optical density measured by the Synergy™ HTX Multi-Mode Microplate Reader. Reader was set to shake 3 times within 5 secs and optical density was measured are wavelength 570nm. Data shown is the mean optical density of triplicate measures each sample $\pm$ SE

Figure 6.1b: Nitrite Calibration Curve. Figure shows the linearity between the concentration of nitrate in samples and the optical density measured by the Synergy™ HTX Multi-Mode Microplate Reader. Reader was set to shake 3 times within 5 secs and optical density was measured are wavelength 570nm. Data shown is the mean optical density of triplicate measures each sample ± SE

6.3.2 Percentage Recovery (%R)

The accuracy of the assay kit was evaluated by calculating the percentage recovery. As expected, percentage recovery varied with change in concentration (Betz, Brown, & Roman, 2011; Horwitz, 2002; Kalra, 2011). Nevertheless, percentage recovery for both compounds across all

concentrations were within the acceptable range of 80% and 120% (Rosenberg & Ariese, 2001; Yin, 2015) except for the minimum nitrate concentration (3.125 µM) (**Table 6.1**).

Table 6.1a: **Percentage recovery for Nitrite.**

Nitrite Standard Concentration (µM)	Optical Density	Recovered Concentration (µM)	% Recovery
3.125	0.008	2.52	80.77
6.25	0.018	7.29	116.58
12.50	0.029	12.52	100.19
25	0.060	27.52	109.14
50	0.103	47.76	95.52
100	0.217	102.05	102.05
200	0.429	203.00	101.50

Optical density is mean value of triplicate analyses

Table 6.1b: Percentage recovery for Nitrate.

Nitrate Standard Concentration (µM)	Optical Density	Recovered Concentration (µM)	% Recovery
3.125	0.023	0.65	20.77
6.25	0.050	5.39	86.18
12.50	0.094	13.11	104.84
25	0.162	25.04	100.14
50	0.336	55.56	111.12
100	0.575	97.49	97.49

Optical density is mean value of triplicate analyses

6.3.3 NO in fermented samples

There were 10 samples and triplicate analyses were undertaken for each sample. The concentration of nitrite and nitrate found in unfermented undigested milled corn were 31.89 μM and 50.77 μM respectively (**Figure 6.2**). Detection of nitrite and nitrate in corn has been previously reported by (Abdulrazak et al., 2014; Erkekoğlu & Giray, 2009). Using spectrometry, Abdulrazak et al. (2014) reported detecting about 72.6 μM and 0.76 μM of nitrate and nitrite in grounded white corn.

Nitrate and nitrite were also detected in SF and CF corns after 120 hours however, though at lower concentrations in comparison to unfermented corn (**Figure 6.2**). There was 68% and 45% decrease in nitrite and 54% and 40% decrease in nitrate after 120 hours of SF and CF respectively.

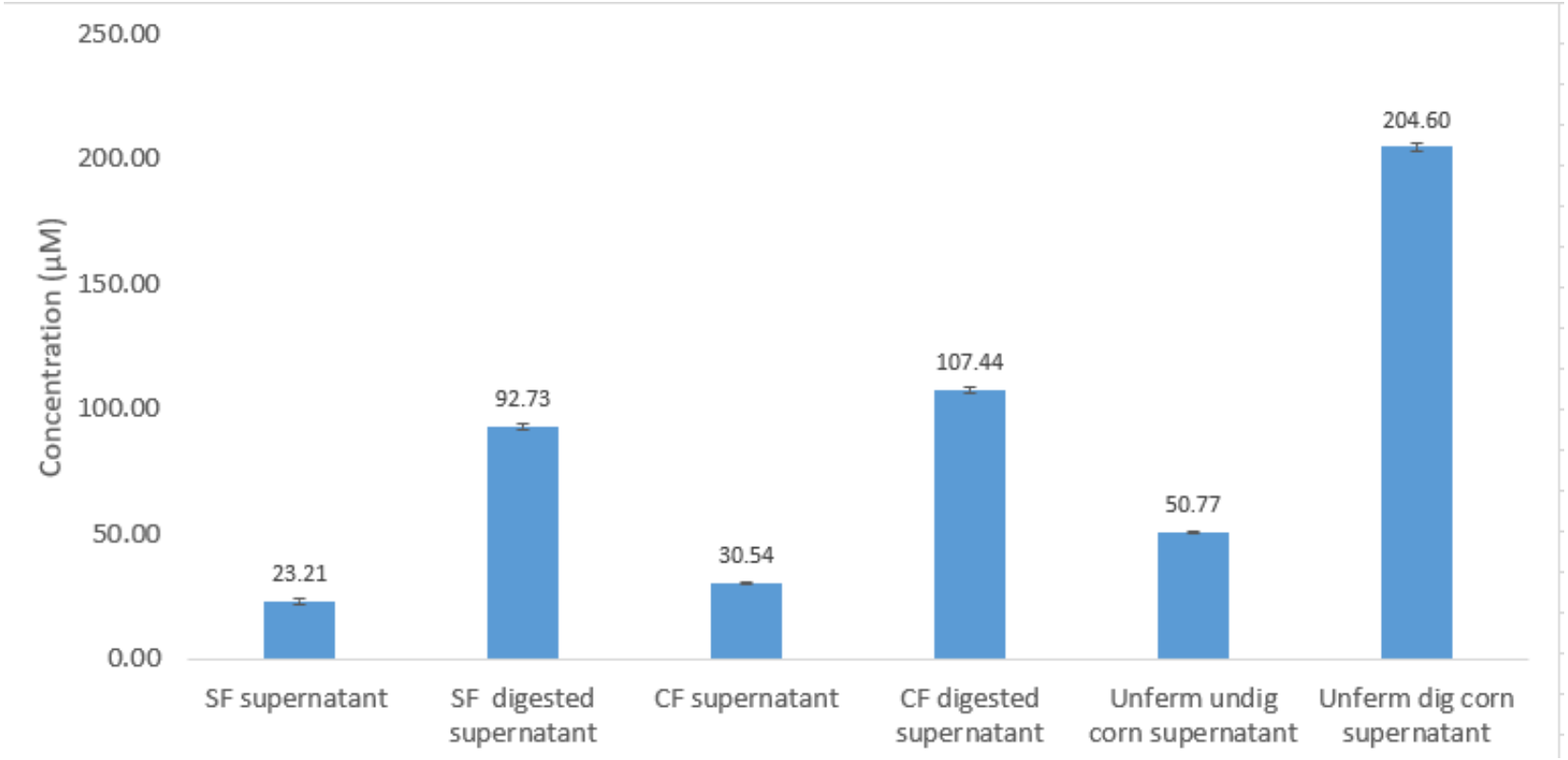


Figure 6.2a: Nitrate Concentration of Corn Sample Concentration of nitrate in sample was calculated based on the optical density, slope and intercept of the calibration curve. Data on the graph is the representation of the mean concentration of triplicate analyses of each sample ± SE

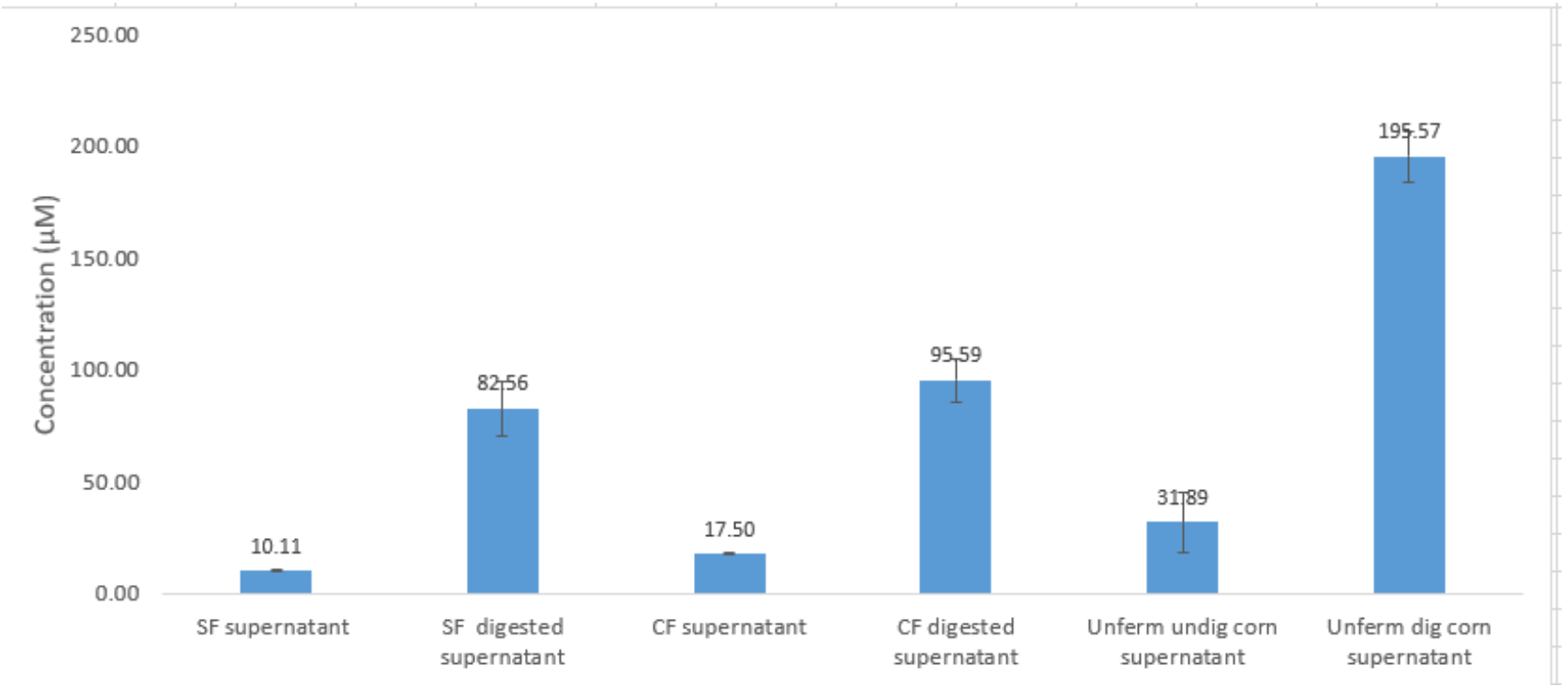


Figure 6.2b: Nitrite Concentration of Corn Sample Concentration of nitrate in sample was calculated based on the optical density, slope and intercept of the calibration curve. Data on the graph is the representation of the mean concentration of triplicate analyses of each sample ± SE

6.3.4 NO after digestion

Increased concentration of nitrite and nitrate after upper digestion was observed for all corn samples in the current study. The concentration of nitrite increased from 10.11 µM to 82.56 µM in SF corn, 17.50 µM to 95.59 µM in CF corn and 31.89 µM to 195.57 µM in unfermented corn. Also, concentration of nitrate increased from 23.21 µM to 92.73 µM in SF corn, 30.54 µM to 107.44 µM in CF corn and 50.77 µM to 204.60 µM in unfermented corn.

Results also show that there was higher concentration of nitrate and nitrite in CF vs SF corns. Concentration of nitrate and nitrite in CF vs SF were 30.50 µM vs 23.21 µM and 17.50 µM vs 10.11 µM respectively. The concentration of nitrate and nitrite in plasma before addition of fermented corn samples were 20.94 µM and 6.23 µM respectively (**Figure 6.3**). According to Hord et al. (2009) fasting plasma concentrations for nitrate and nitrite are 0.02-50 µM and 0.1-0.5 µM. It may therefore be inferred that fasting plasma was not used in the current study. There was an increase in the plasma concentration of nitrate and nitrite with digested CF corn effecting greater increase in both compounds compared with SF corn.

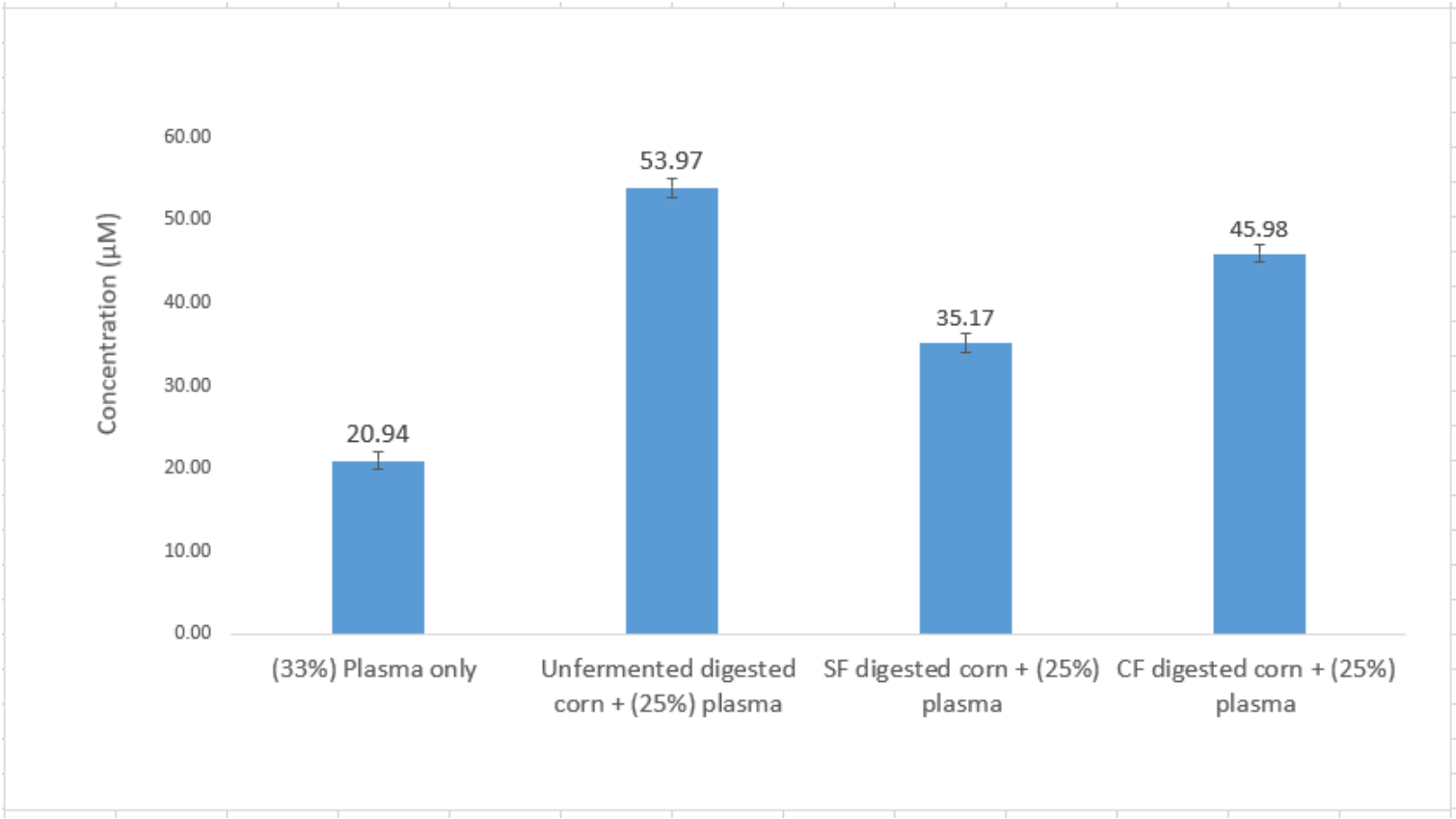


Figure 6.3a: Nitrate Concentration of Plasma Concentration of nitrate in sample was calculated based on the optical density, slope and intercept of the calibration curve. Data on the graph is the representation of the mean concentration of triplicate analyses of each sample ± SE

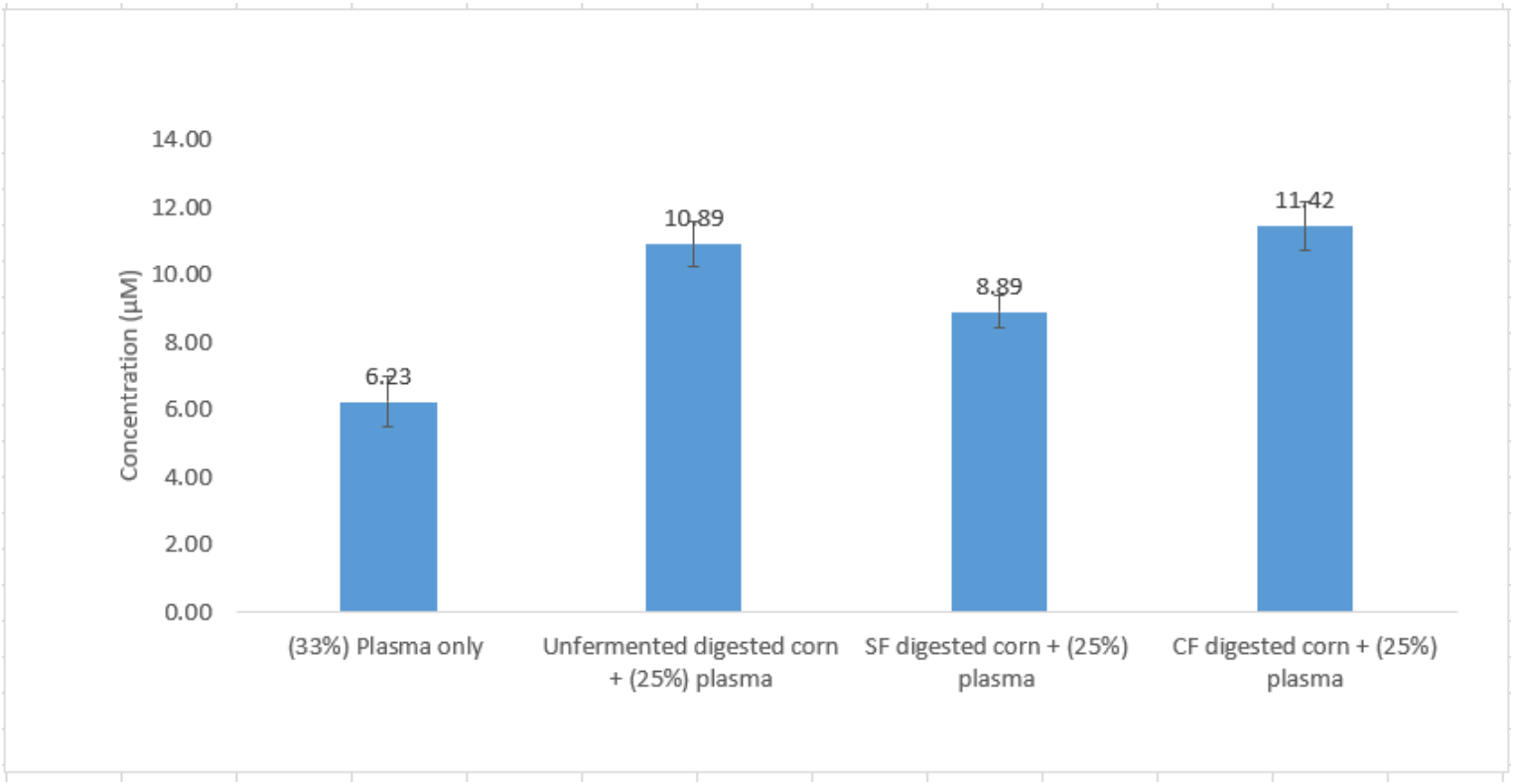


Figure 6.3b: Nitrite Concentration of Plasma Concentration of nitrate in sample was calculated based on the optical density, slope and intercept of the calibration curve. Data on the graph is the representation of the mean concentration of triplicate analyses of each sample $\pm$ SE

6.4 Discussion

The objectives of this study were to 1) quantify the concentrations of nitrate and nitrite in spontaneous (SF) and controlled fermented (CF) corn 2) To investigate the effect of fermented corn, spontaneous and controlled, on the NO concentration in plasma

Factors such as geographical location, type of soil used for cultivation, type and frequency of fertilizer and manure used during cultivation and source of water used during irrigation would influence the concentration of nitrate and nitrite in a plant produce (Abdulrazak et al., 2014; Uwah, Abah, Ndahi, & Ogugbuaja, 2009). As a result, there may be some variations in the concentrations of nitrate and nitrite between studies. Erkekoğlu and Giray (2009) showed large variations in the concentration of nitrite in the 51 samples of corn obtained in 4 regions of Turkey with minimum and maximum detected nitrite concentrations being 83.6 µM and 2,606 µM. The corn used for this current study was purchased from a local market in Ogun State, Nigeria, however the specific location of its cultivation is unknown.

At the end of 120 hours of fermentation, the concentration of nitrate and nitrite in SF corn were 23.21 µM and 10.11 µM respectively. Also, the concentration of nitrate and nitrite in CF corn, were 30.54 µM and 17.50 µM. Nitrate and nitrite depletion during fermentation has been reported Yang et al. (2004), Yan et al. (2008), Wang et al. (2013), Kim and Hur (2018) although none of them used corn as the substrate of fermentation. Ezeagu (1996) reported detecting, from 50 samples of fermented corn obtained from various locations in Ibadan, Nigeria, nitrate ranging from 67.7 µM to 1612 µM and nitrite ranging from 0.5 µM to 1.3µM. However, the authors noted that the concentration of nitrate consistently decreased while the nitrite concentration consistently increased over time. Nitrate was said to have decreased by 80% and nitrite increased by 200% after 8 days of storage. Depletion of nitrite and nitrate during fermentation has been attributed to the activities of LAB though the exact mechanism of depletion is unknown (Kim & Hur, 2018; Wang et al., 2013; Yan et al., 2008) A possible mechanism may be the activities of nitrite and nitrate reductase which some authors have reported to be produced organisms that can undergo denitrification, examples of which include *Lb casei*, *Lb plantarum* and *E.coli* (Bello, Bello, Amoo, & Atoyebi, 2018; Liu et al., 2014; Tiso & Schechter, 2015; Wang, Wu, Yang, Li, & Qi, 2013). Though *E.coli* is not a LAB, it may be involved in spontaneous fermentation of corn as reported by Bello, Bello et al. (2018). Nitrite is reduced by the nitrite reductase to NO which is difficult to quantify but may be estimated if amount of nitrite loss is known (Tripathi et al., 2007). Furthermore, the variation in the degree of depletion in both compounds in both fermentations may be associated with the variations is the microbial communities. In the study by Oh, Oh, and Kim (2004), five

LAB isolated from kimchi, a fermented vegetable product from Korea, showed varied degree depletion of nitrite during their growth in MRS broth. The authors however reported an increased depletion of nitrite with an increase in temperature, with all organisms showing up to 90% of nitrite depletion in 48 hours of growth at 30^{O}C and 36^{O}C. In the current study, fermentation was done at 30^{O}C which may have contributed to the degree of observed depletion.

Results of the effect of digestion reported in this current study are contrary to those reported in a study by Kim and Hur (2018) which was aimed at investigating the concentration of nitrite after *in-vitro* digestion of fermented sausage. The authors reported a statistically significant decrease in the concentration of nitrite in 6 different types of fermented sausages after upper digestion in comparison with the concentration before digestion. According to the authors, the most significant reduction occurred in the stomach phase. During human physiological process, dietary nitrate is reduced to nitrite by the oral microbiome which made up of over 300 species of organisms including species of genera *Streptococcus, Neisseria, Rothia, Prevotella, Actinomyces, Granulicatella, Porphyromonas* and *Haemophilus* (Hezel & Weitzberg, 2015; Yamashita & Takeshita, 2017). Saliva which contains nitrate and has also been enriched with nitrite in the mouth is then swallowed resulting in the delivery of nitrite in the stomach, where nitrite is disproportionated by stomach acid into NO (Bryan & Petrosino, 2017; Hezel & Weitzberg, 2015; Qu et al., 2016; Tiso & Schechter, 2015). A large portion of the nitrite is however delivered into the small intestine where is it absorbed into the systemic circulation (Bryan & Petrosino, 2017; Hezel & Weitzberg, 2015; Qu et al., 2016; Tiso & Schechter, 2015). In the current study, concentration of nitrate and nitrite at the oral and stomach phases were not determined, therefore the phase of digestion resulting in a change in concentration of these compounds in unknown. Kim and Hur (2018) theorised in their study that changes in the pH in the simulated stomach and small intestine, 1.5 and 8.0 respectively, may have resulted in the deprotonation of nitrite hence the decrease in nitrite concentration during in-vitro digestion. In the current study, pH stomach and small intestinal phases were 3.0 and 7.0 respectively (chapter 4). However, the different substrate used in both studies (sausage vs corn) may have contributed to the contrary outcomes. Another plausible explanation for the inconsistent result may be in the variations in the composition of the digestive fluids used in both studies. Unlike the study by Kim and Hur (2018), ammonium carbonate ($(NH_4)_2CO_3$) was a component of the simulated salivary and gastric fluids used for *in-vitro* digestion in the current study. Ammonium carbonate is easily decomposed to ammonia and carbon dioxide in water with temperature from 36^{O}C (O'Neil, 2013). Digestion in the current study was done at 37^{O}C, which may have stimulated the decomposition of ammonium carbonate.

Ammonia may then have been oxidized into nitrate and nitrite causing a notable rise in their concentrations in all the digested samples in comparison with the undigested samples.

Fermented corn is traditionally consumed in the western and southern parts of Africa (Achi & Asamudo, 2018; Ezekiel et al., 2018; Kohajdová, 2017). It is usually cooked before consumption and often traditionally used as weaning food (Abu-Ghannam & Rajauria, 2015; Achi & Asamudo, 2018; El Sheikha & Montet, 2014; Ezekiel et al., 2018) but in the South-West of Nigeria, uncooked fermented corn is traditionally administered to children and consumed adults for the treatment of acute gastrointestinal diseases such as diarrhoea (Afolayan, Ayeni, & Ruppitsch, 2017; Shittu, Ajayi, Bankole, & Popoola, 2016). Therefore, the knowledge of what happens to the concentration of nitrate and nitrite available in cooked and uncooked fermented corn as well as after upper digestion is essential to determining its efficacy of increasing blood plasma concentration of nitrite and nitrate following its ingestion. In addition, studies investigating the contribution of nitrate and nitrite to the blood plasma from the ingestion cooked and/or uncooked fermented corn may provide more insight on the risk of development methemoglobinemia (Hord et al., 2009), especially in children. In the current study, the use of simulated digestion fluids that contained ammonium may affect the validity of the concentration of nitrite and nitrate in blood plasma upon addition of fermented corn. The ammonium present in the digestive fluids may have contributed to the notable increase in the plasma concentrations of nitrate and nitrite following *in-vitro* upper digestion. *In-vivo* methods of investigation may be more effective to investigate the effect of fermented corn, cooked and uncooked on blood plasma. Ezeagu (1996, 2006) proposed that daily intake of 100 g of fermented corn for a child may be regarded as safe as the concentrations of nitrate and nitrite in 100 g of fermented corn may be within the recommendations of 3.7 mg and 0.06 mg of nitrate and nitrite respectively per kilogram body weight by FAO/WHO. Nevertheless, this proposal may not be generalizable due to possible variations in the concentration of nitrate and nitrite in corn from various locations of cultivation as reported by Erkekoğlu and Giray (2009).

6.5 NO in the gut

NO is supplied into the gut through the activities of the iNOS (Salzman, 1995). Non-enzymatic endogenous supply of NO has also been reported in the large intestine by the activities of the gut microbiome (Vermeiren et al., 2009; Tiso & Schechter, 2015). Vermeiren et al., (2009) and Tiso & Schechter, (2015) explain that production of SCFAs in the colon, creating an acidic environment with a pH over 4.2, causes the synthesis of ammonia and NO from dietary nitrate and nitrite by gut microorganisms such as LAB, Bifidobacteria, *Bacillus* spp and *E.coli*. Apart from regulation of the motility in the gut, other essential roles of the NO in the human gut include the maintenance of the

integrity of the gastric epithelium and influencing the production of the mucus barrier (Lanas, 2008). It is said to be a crucial signally molecule for immune responses in the human gut (Vermeiren, Van de Wiele, Verstraete, Boeckx, & Boon 2009). Mourad, Turvill and Farthing (1999) have also suggested that NO may play an active and important role in maintaining fluid/electrolyte balance in the large intestine. As previously explained, compromised secretory mechanism, altered motility of the GIT, decreased mucus production – compromising the integrity of the mucosal wall in the large intestine are underlaying causes of diarrhoea (Sweetser, 2012). It can therefore be inferred that adequate supply of NO may be instrumental in the management or prevention of these causes.

6.6 Summary and Conclusion

The immune functions of NO in the human gut including antimicrobial, anti-inflammatory, antiviral, modulation of production of cytokines, cytotoxic effects, stabilising the intestinal mucosal epithelial cells and fluid regulation (Hosking, 2009; Kochar et al., 2011; Kojouri, Hassanpour, Taghavi, & Taghadosi, 2012; Tripathi et al., 2007) give it potential for the treatment and prevention of diarrhoea from microbial infections or antibiotics induced. Excessive induction of iNOS and over endogenous production of NO has been reported to promote inflammatory responses and tissue dysfunction in the colon (Salzman, 1995; Lanas, 2008; Roediger, 2008). Kochar et al. (2011) in their study suggested that increased production of NO may be associated with bile-salt induced diarrhoea in the colon. Also, Kukuruzovic, Brewster, Gray, and Ansteyt (2003) also concluded in their study that increased endogenous production of NO in the colon may contribute to impaired mucosal barrier, hypokalaemia and malnutrition as a cost for its gut-protective and antimicrobial effects preventing severity, children with diarrhoea. Nevertheless, so far, no study has suggested that dietary sources of nitrate and nitrite produce similar effects. On the contrary, though with reference to *in vivo* animals and *in vitro* studies, Lanas, (2008), Vermieren et al., (2009) and Tiso & Schechter (2015) suggest that dietary nitrate and nitrite is able to increase the mucus production and enhance immune responses such as anti-inflammatory responses in the gut, resulting in an enhanced gut health. From the current study, fermented corn may be able enhance the mucosal integrity and immunological responses by being an exogenous source of NO. It is however unknown if such dietary sources of NO may have such lethal effects or what dosage would be termed as safe intake in disease conditions such as diarrhoea, making obvious the need for research on the effect of dietary sources of NO in human immune-gastro processes.

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Chapter 7

Summary and Conclusion

7.1 Objectives of study

The aim of this study was to investigate the potentials for 'ogi' to be a functional food in the management of gastrointestinal diseases by investigating the mechanisms through which it may be able to confer the health benefits when consumed. In order to achieve this, the following were investigated

1. Probiotic potential of 'ogi' using the spontaneous and controlled methods of fermentation
2. Production of SCFAs by fermentation organisms during spontaneous and controlled fermentation
3. Potential of 'ogi' to modulate or influence the immune response

7.1.1 Production of 'ogi'

Processes involved in the traditional production of 'ogi' include cleaning, soaking, wet milling and soaring. Fermentation commencing during soaking, with the pH dropping as low as 3.8 (Oyedeji, Ogunbanwo, & Onilude, 2013) (**Figure 7.1**), and continues during soaring with the possibility of pH dropping even lower as seen in the current study. This traditional method was modified during the current study, by using whole dry milled corn grains (**Figure 7.2**) as previously described by Akanbi, Ade-Omowaye, Ojo, and Adeyemi (2003) and Akingbala, Onochie, Adeyemi, and Oguntimein (1987).

Wet milling and sieving as seen in the traditional method, results in the significant reduction of the fibre (Ijabadeniyi, 2007). The use of whole corn and the elimination of the sieving step in the current study not only increases the fibre content of ogi but also results in up to 32% increase in protein content in comparison with traditionally produced 'ogi'(Antai & Nzeribe, 1992; Farinde, 2015; Ijabadeniyi, 2007). Though the use of whole grain may provide nutritional gain, the viscosity and rheological properties may be compromised (Akanbi et al., 2003; Akingbala et al., 1987) which may cause it to be less preferable than the traditionally prepared 'ogi' to the consumer (Farinde, 2015).

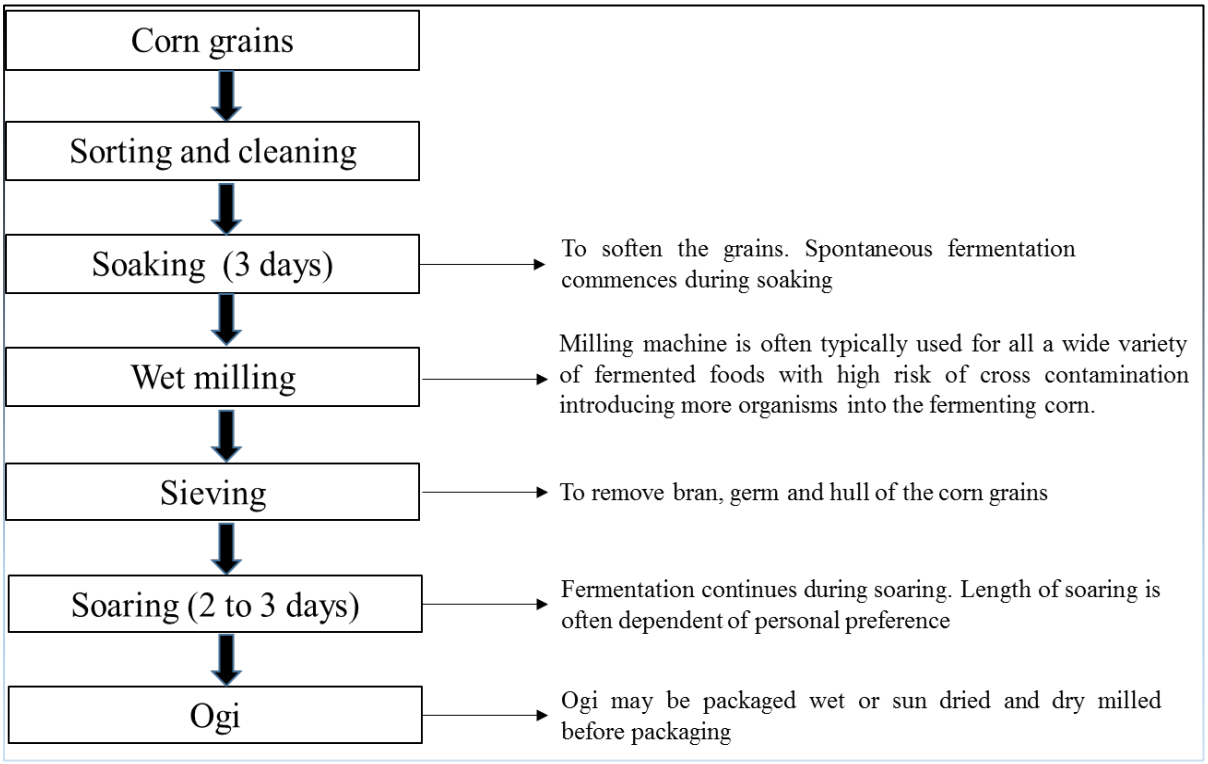

Figure 7.1a: Traditional method of 'ogi' production.

Adapted from Omemu, A., & Bankole, M. (2015). Consumer's knowledge, attitude, usage and storage pattern of ogi-a fermented cereal gruel in south west, Nigeria. Food and Public Health, 5(3), 77-83.

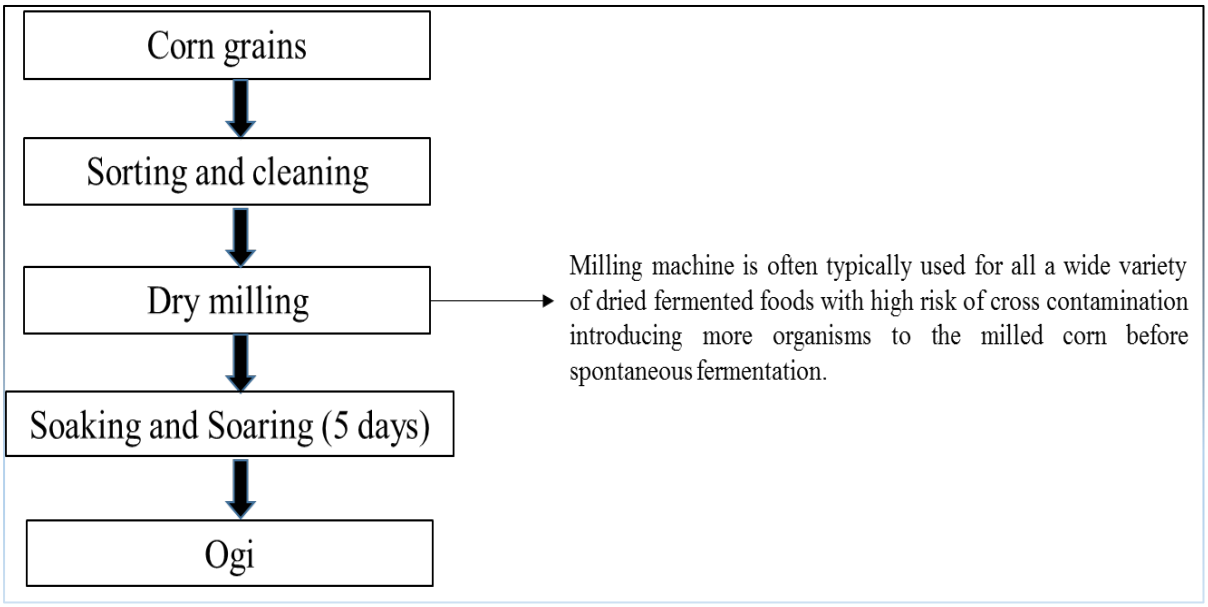

Figure 7.1b: Modified method of 'ogi' production (produced by the author).

7.1.2 SF vs CF

In the current study, the 57 LAB and yeast isolates from spontaneously fermented corn were identified as three namely *W. confusa*, *P. pentosaceus* and *S. cerevisiae*. Sequence-based genotypic techniques were used for the identification of isolated organisms in the current study which enhances the accuracy of their identities. *Lb plantarum* and *Lb fermentum* are typically isolated from spontaneously fermented corn (Decimo et al., 2017; Izah, Kigigha, & Okowa, 2016; Omemu, Okafor, Obadina, Bankole, & Adeyeye, 2018) but were not identified in the current study. Corn was purchased and milled in South-West Nigeria but was transported to the UK for fermentation and analyses. According to Dong, Jiang, Yu, Liu, and Zhang (2010) microbial ecosystem responds to changes in environmental conditions and human activities. The authors in their study reported that the wet and warm climate correlated with a high abundance and diversity of microorganisms while cold and dry climate correlated with a notable decrease in microbial load and diversity on the Tibetan Plateau. Variations in environmental factors such as temperature and atmospheric pressure, water may have induced abiotic and biotic stresses (Bartlett, 2002; Khalid, 2011; Papadimitriou et al., 2016) on the milled corn's indigenous microflora, resulting in limited microbial diversity and population.

LAB and yeast mainly are the main organisms associated with spontaneous fermentation of cereal including corn (Tamang, Watanabe, & Holzapfel, 2016). Also, with SF being unpredictable and uncontrollable, the potential risk of growing pathogenic organisms cannot be ignored. The laboratories at the University of Chester are licenced to accommodate only Group 1 biological agents (unlikely to cause human disease) based on the Health and Safety Executive (2013) classification. Though non-LAB, non-yeast are often lost as fermentation progresses (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Jalilsood et al., 2015; Salmerón, 2017), possible adaptability of unwanted organisms to the sub-lethal inimical stresses created by acids, metabolites, antibiotics or limited nutrients during fermentation (Giraffa, 2004) is should not be overlooked. With these considerations in mind, the scope of the current study was limited to isolating LAB and yeast in the SF corn sample. There was therefore a notable difference in the microbial diversity and population and in the biochemical and sensory characteristics of CF and SF corns in this study.

7.1.3 Probiotic potential of 'ogi'

P. pentosaceus was recovered in both SF and CF corn after *in vitro* upper digestion which suggests that this strain of LAB may be probiotic. The strain of *P. pentosaceus* isolated from this study is however unknown. *P. pentosaceus* KID 7, *P. pentosaceus* OF31 are examples of strains isolated from fermented cereal foods (Adesulu-Dahunsi, Jeyaram, & Sanni, 2018; Damodharan, Lee,

Palaniyandi, Yang, & Suh, 2015). The probiotic potential of various strains of *P. pentosaceus,* some of which were isolated from a wide range of fermented foods, have also been reported in other *in vitro* and *in vivo* studies (Chelliah et al., 2018; Damodharan et al., 2015; Ladha & Jeevaratnam, 2018; Shukla & Goyal, 2014; Torres-Aguilar, Rodriguez-Fragoso, Garcia-Vazquez, & Reyes-Esparza, 2017; Zommiti et al., 2018).

S. cerevisiae was recovered in CF corn but not in SF corn after *in vitro* upper digestion. Since the same strain of *S. cerevisiae* was present in both types of fermented corn, and based on baseline percentage (8.54%) of *S. cerevisiae* that was detected in SF corn, it may be inferred that the absence of *S. cerevisiae* in digested SF may be due to low population of the organism in SF corn pre-digestion. *Lb plantarum* present in CF corn but not detected in SF corn also survived *in vitro* upper digestion. Probiotic potentials of strains of these organisms which originated from fermented cereal foods have also been reported in other *in vitro* and *in vivo* studies (de Lima et al., 2017; Giri, Sen, Saha, Sukumaran, & Park, 2018; Ogunremi, Sanni, & Agrawal, 2015; Oguntoyinbo & Narbad, 2015; Rao, Kumar, & MY, 2017). Species of *Weissella* have received some significant attention in the research environment with the theory that they may have probiotic potentials (A. Adesulu-Dahunsi, A. Sanni, et al., 2018). In the current study, *W. confusa* did not also survive the upper digestion. Though other strains of *W. confusa* of fermented food origin may have probiotic potentials (A. Adesulu-Dahunsi, A. Sanni, et al., 2018; Sharma, Kandasamy, Kavitake, & Shetty, 2018), the strains used in the current study did not appear to have the potential.

Furthermore, *Lb plantarum* and *P. pentosaceus* exhibited some inhibition against the strains of *E. coli* used in the current study while the strains of *S. cerevisiae* and *W. confusa* isolated from SF corn did not show any inhibition. Cell free supernatant solution of SF corn but not CF also showed some inhibitions against *E. coli* strains. Lack of inhibition of *E. coli* by *S. cerevisiae* does not negate it's probiotic potential as the strain may be able to confer other forms of health such as anti-inflammatory effects (Foligné, Dewulf, Vandekerckove, Pignède, & Pot, 2010), adhesion to the intestinal epithelium (Tiago et al., 2012).

Results from this study suggests that 'ogi', depending on the organisms involved in the spontaneous fermentation, may have probiotic potential and may also be influenced by environmental factors.

7.1.4 SCFAs in 'ogi'

SCFAs were present in SF and CF corns. Analyses of single strain CF and SF showed that there was a consistent decrease in the acetate that was present in unfermented corn during fermentation. It may be indicative that isolated organisms - and those which were not isolated but were present

in SF - may have utilised the acetate as the ready energy source. Nevertheless, there was an increased concentration of acetate after 120 hours fermentation in mixed strain CF corn. There was production of butyrate from 24 hours of fermentation. After *in vitro* upper digestion, acetate was still detected in both SF and CF corn, and butyrate in SF corn. Free fatty acids available in the small intestine are absorbed directly into the portal veins and delivered into the liver (Hussain, 2014; Iqbal & Hussain, 2009; McClements & Li, 2010), it is therefore unclear whether dietary SCFA escape into the large intestine, with the possibility to significantly increase their concentrations in the colon.

SCFAs are produced in the colon by gut microbiota (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016) and health benefits derived from SCFA produced in the colon have been widely reported (Hijova & Chmelarova, 2007; Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011; Huda-Faujan et al., 2010; Morrison & Preston, 2016). Nevertheless, SCFA may also be supplied to the body via other means. In vivo studies by Schwarz, Bruhs, and Schwarz (2017) and Lucas et al. (2018) have shown the possibilities that SCFA administered subcutaneously and orally may enhance skin and bone health respectively. Therefore, there is need for *in vivo* studies to investigate the effect of dietary SCFAs on health more particularly, gastrointestinal health.

Increased intake of dietary fibre has been associated with increased production of SCFA in the colon resulting in enhanced health (Holscher, 2017; Louis & Flint, 2017; Meier, 2009; Musso, Gambino, & Cassader, 2011). Though the fibre content of 'ogi' was not investigated in the current study, depending on the type of milling machine used and hole size of the sifting material, 'ogi' may have a notable fibre content, with the unsifted 'ogi' containing having even higher fibre content in comparison with the sifted type (Akingbala et al., 1987; Antai & Nzeribe, 1992; Farinde, 2015). The fibre upon delivery into the colon may consequently result in an increased concentration of SCFAs with.

7.1.5 Prebiotic potential of 'ogi'

The prebiotic functions of exopolysaccharide (EPS) produced during food fermentation has been discussed by various authors such as Ripari (2019) and Zhou, Cui, and Qu (2018). Though not investigated in the current study, some organisms associated with corn fermentation have been reported to produce EPS. Various strains of all the species of LAB isolated is the current study have been reported to produce ESP in various other studies. Production of ESP by strains of *P. pentosaceus* have been reported by Smitinont et al. (1999) and Yasutake et al. (2016) though reported strains did not originate from fermented corn. ESP production of strains of *W. confusa*

was also reported by Adesulu-Dahunsi et al. (2018), Benhouna et al. (2019) and Malang, Maina, Schwab, Tenkanen, and Lacroix (2015). Though the strains tested in the mentioned studies were isolated from fermented foods, none of them was isolated from fermented corn. Malang et al. (2015) reported that all 110 strains of *W. confusa* tested produced dextran and 18 strains produced fructan. Nevertheless, these strains were isolated from spontaneously fermented cassava and milk. Temperature, carbon source, length of fermentation and pH are important factors that influence EPS production during food fermentation (Ripari, 2019). Though the strains of *W. confusa* in the current did not survive the upper digestion, it is not impossible that they produced some ESP during the corn fermentations. Notable growth was observed when both strains were used as single strains starter culture, suggesting that the temperature and fermentation conditions were ideal for EPS production during fermentation.

Similarly, *Lb plantarum* has also been reported to be another LAB which produces ESP with an ideal substrate (Ripari, 2019; Zhou et al., 2018). More specifically, strains of *Lb plantarum* isolated from spontaneously corn have been reported to produce ESP during fermentation of corn (Adebayo-tayo & Onilude, 2008; Adesulu-Dahunsi, Jeyaram, Sanni, & Banwo, 2018; Ishola & Adebayo-Tayo, 2012). Also, ESP produced by strains of *Lb plantarum* may have other functional properties such as antimicrobial and antidiabetic characteristics (Adesulu-Dahunsi et al., 2018; Sasikumar, Vaikkath, Devendra, & Nampoothiri, 2017).

Based on the species of LAB isolated from spontaneously fermented corn in the current study, suggesting that 'ogi' may have prebiotic potentials may be pertinent.

7.1.6 Potential to enhance immune response - Nitric Oxide (NO) in 'ogi'

NO was present in the corn used in the current study which was in agreement with other studies that have detected NO in raw corn though in varied concentrations (Abdulrazak, Otie, & Oniwapele, 2014; Erkekoğlu & Giray, 2009). Nevertheless, there was a notable decrease in the concentration of NO after fermentation probably due to activities of nitrite and nitrate reductase which may have been secreted by the fermentation organisms (Bello, Bello, Amoo, & Atoyebi, 2018; Liu et al., 2014; Tiso & Schechter, 2015; Wang, Wu, Yang, Li, & Qi, 2013). The concentration later increased after digestion in the current study but this may be due to the ammonium that was contained in the digestion fluids used in the *in vitro* digestion. There was an observed increase in the concentration of NO in the blood plasma and this may also be associated with the composition of the digestion fluids used in the study. Nevertheless, the prospect of fermented corn being a dietary source of NO is not refuted. The benefits of dietary NO have been

theorized to enhance cardiac, muscular and respiratory functions (Kerley, 2017; Stanaway, Rutherfurd-Markwick, Page, & Ali, 2017). However there are no studies addressing the effect of dietary NO on immune functions. Endogenously synthesized NO is known to enhance immune function in the body so it is not impossible that dietary NO may have same effect.

7.2 Limitations of study

Though the processing method of 'ogi' was modified in this study such that it may contain a higher amount of fibre compared with the traditional method of process, due to limited resources, fibre identification and quantification was not undertaken in the current study. Such analysis would have given more insights on 'ogi's' prebiotic potential and its possibility to increase the quantity of SCFA in the colon. Though some strains of isolated organisms have been reported to be able to product EPS (Adebayo-tayo & Onilude, 2008; Adesulu-Dahunsi, Jeyaram, Sanni, & Banwo, 2018; Ishola & Adebayo-Tayo, 2012), due to limited resources, EPS production of the strains of organisms used in the current study during the fermentation of corn was not investigated. With microbial production of ESP being characteristically multifactorial (Ripari, 2019), testing for ESP production by the strains of organisms used in this study in the production of 'ogi' would have been useful in the investigation of prebiotic potential of the fermented product.

The possibility to reproduce the end product of spontaneously fermented corn in a controlled environment is essential to be able to effectively investigate the components of the fermented food product that may enhance the gut health. However, undertaking this study emphasized how multiple factors, including geographical location, source of water and weather, could significantly influence the characteristics of the end product of spontaneous fermentation. This was evident in the differences in characteristics of the SF and CF corn in this current study. Due to limited resources, triplicate analyses of SF and CF were undertaken using 1 cycle of fermentation each. This practice was observed in studies by Van der Meulen et al. (2007), Nwachukwu, Achi, and Ijeoma (2010), Banwo, Sanni, Tan, and Tian (2012) and Oguntoyinbo and Dodd (2010). With the unpredictability of spontaneously fermented products, the current study may have benefitted from more cycles of spontaneous fermentation. It is probable that independent spontaneous fermentations and isolation of non-LAB, may have yielded wider microbial diversity which may consequently have yielded a closer replica of spontaneous fermentation of corn in a controlled setting, an important objective of this study. Nonetheless, the inclusion of a strain *Lb plantarum*, a species of LAB previously isolated from fermented millet and typical associated with SF corn in CF corn was aimed at having an end product that would be similar to that of SF corn.

7.3 Future research

The challenge of not being able to reproduce the spontaneous fermentation in a controlled setting does not negate the potential benefits that may be derived in SF corn. Neither does it imply that practices and beliefs around fermented corn that have been passed across generations in Nigeria cannot be founded on research. It may however be an indication that the approach to research may need to change from trying to identify and characterise the effects or potential benefits derived from the specific component of the fermented product to having a broader and more holistic approach to investigating the efficacy of the product in promoting gut health. This may involve investigating the effects of the complex matrix of the food product on various aspects of the gut health including the management of diarrhoea which is the scope of the current study. It may involve shifting focus from specific end products of fermentation including fermentation organisms, to comparing effects that may be derived from varied species of corn and varied methods of fermentation, to allow for the plausible observation of collaborative and connected mechanisms that may yield notable and consistent health benefit to the consumer.

7.4 Conclusion

The potential for fermented corn to be a functional food was investigated in this thesis. *W. confusa, P. pentosaceus* and *S. cerevisiae* were LAB and yeast cells isolated from spontaneously fermented corn. *P. pentosaceus* and *S. cerevisiae* showed some probiotic potentials by surviving the upper digestion. *W. confusa,* on the other hand did not survive the upper digestion. *Lb. plantarum*, though not isolated from 'ogi' in the current study , is often associated with spontaneously fermented corn and therefore used as a starter culture along with *W. confusa, P. pentosaceus* and *S. cerevisiae,* in controlled fermentation. *Lb. plantarum* also showed some probiotic potential by surviving the upper digestion and also showing some antimicrobial effects against *E. coli* along with *P. pentosaceus.* Furthermore, though not investigated in the current study, strains of all isolated organisms have been suggested to produce ESP which may have prebiotic effects on the consumer (Ripari, 2019; Zhou et al., 2018; Malang et al., 2015; Yasutake et al. 2016). These information therefore suggests that 'ogi' may have the functional potential of being probiotic and prebiotic.

Conversely, SCFAs and NO were detected in fermented corn and in digested fermented corn in the current study. This makes 'ogi' a possible dietary source of these substances with the potential to deliver these substances in the colon to enhance gut health and immune function in the body of the consumer however more studies are required.

The lack of predictability of the outcome of fermentation makes the outcome of the current study unlikely to be generalisable. Albeit, some common characteristics such as the presence of *Lb plantarum* (a known probiotic LAB) may be present in spontaneously fermented corn in areas where 'ogi' is traditionally produced and consumed. Further studies identifying other common features in a large variety of spontaneously fermented corn may be useful in determination of the functionality of 'ogi' in enhancing the health of the consumer. Also 'ogi' may have repeatable and expectable health benefit if a holistic approach is employed by investigating the effectiveness of the complex matrix of the fermented product

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