Division of Food Sciences

School of Biosciences

# MICROBIAL POPULATION DYNAMICS AND IMPACT ON HYDROLYSIS OF PHYTATE AND PHENOLIC COMPOUNDS DURING FERMENTATION OF *OGI* - AN INDIGENOUS FERMENTED CEREAL PRODUCT

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**ABSTRACT**

*Ogi* is a fermented food made from maize, sorghum or millet which serves as complementary food for infants and breakfast for adults in Nigeria, West Africa. This study characterized the microbial diversity of maize and sorghum grains and *ogi* produced by their natural fermentation in an attempt to understand the roles of the key microbial species and the impact of the population dynamics and selected species on changes in nutritional composition and aroma notes of *ogi* during fermentation. A combined approach of culture dependent and culture independent methods of analysis was applied to investigate the microbial community of grains and *ogi* from two different sources. Microbial diversity and viable populations varied with the source of the grain. Bacterial and fungal genera identified with the partial 16S rRNA and 26S rRNA sequence analysis respectively in maize and sorghum were *Bacillus, Enterobacter, Micrococcus, Kytococcus, Pantoea, Staphylococcus, Amycolatopsis, Methanoculleus, Aspergillus, Penicillium, Eupenicillium, Acremonium, Schizosaccharomyces, Meyerozyma, Hyphopichia,* and *Pichia* in maize grains; *Enterococcus, Enterobacter, Pantoea, Bifidobacterium, Aspergillus, Cladosporum,* and *Penicillium* in maize *ogi; Enterococcus, Enterobacter, Pantoea, Aeribacillus, Cyanobacterium, Acinetobacter, Fusarium* and *Trametes* in sorghum grains; and *Pediococcus, Lactobacillus*, *Enterococcus, Bacillus, Cladosporum* and *Penicillium* in sorghum *ogi.* Similar species were observed in both sources of maize while those of sorghum differed slightly. Predominant microbes included species of *Enterobacteriaceae* and moulds. Acetic acid bacteria were not identified as part of the diverse community. Following the predominance of moulds during the natural fermentation, preliminary screening was performed by PCR using specific biosynthetic gene primers to test whether they are the mycotoxin producing species. None of the genes tested were detected by PCR thus they may not be the toxin producing species. Starch, non-starch polysaccharide (NSP), phytate and phenolic compounds were determined in the grains and respective *ogi* to ascertain the levels of these nutritionally important components in the naturally fermented *ogi* and the impact of the varying microbial populations on the fate of these compounds during fermentation. In the

grains, the average starch and NSP contents in each case were 80.35 g/100g and 9.40g/100g in maize and 93.12 g/100g and 8.14 g/100g in sorghum. Out of the total in grain the average percentage recovery of starch and NSP respectively in the *ogi* showed 63% and 42% in maize and 58% and 27% in sorghum. Maize showed good starch and fibre (NSP) retention than sorghum after fermentation. To further understand the types and levels of polymers in NSP hydrolysis in *ogi* fermentation, HPLC analysis of the hydrolysed extract was performed. Glucose was entirely present in maize and sorghum *ogi* which represents the beta-D-glucans while arabinose and xylose (in maize only), mostly lost with the pomace, signify the arabinoxylans. Overall variations in the microbial populations of sorghum seemed causal to the difference in starch and NSP recoveries.

Phytate was assessed based on release of total phosphorus in the samples by enzymatic and chemical methods. Recovery of phytate in the naturally fermented *ogi* ranged from 18-25% in maize and 40-48% in sorghum suggesting greater phytase activity and more nutrient bioavailability in maize *ogi* than in the sorghum. Greater activity in maize reflects the presence of phytate hydrolysing species such as *Aspergillus* in the grain. Total phenolic content (TPC) was assessed by Folin-Ciocalteu colorimetric method after direct extraction of samples by saponification. TPC in the original grains ranged from 410–437 mg GAE/100g in maize and 221–247 mg GAE/100g in sorghum. Due to the nutritional significance, the amount of phenolics that are either freely soluble or are covalently bound to the food matrix were assessed. Soluble phenolics in *ogi* ranged from 16-38% in maize and 32-49% in sorghum based on the total soluble fraction in the original grain. In all cases loss of soluble phenolics with the waste waters accounted for 12-25% and 31-39% with the pomace. Only the LAB population seemed to correlate with the release of phenolics in the natural fermentation. Given the higher value of soluble phenolics, naturally fermented sorghum *ogi* appeared to have higher antioxidant potential than the maize *ogi*.

Furthermore an attempt was made to ascertain whether the use of selected microbes would improve the antioxidant properties and aroma of *ogi* while minimizing the incidence of pathogens due to chance inoculation. Thus the impact of selected LAB

(*Pediococcus pentosaceus*) and fungi (*T. hirsuta* and *A. zeae* previously shown to have phytase activity) on changes in phytate, phenolics and aroma of *ogi* was assessed following a parallel experiment to the previous study but using autoclaved grains. Five fermentation treatments of the pure and co-cultures were investigated. Cell populations in all culture fermentations varied and reached the average maximum of log 6-9 cfu/ml. Changes in the distribution of bound and soluble phenolics were observed showing esterase activity. Leaching of phenolics was evident in all cases but was higher in the sorghum fermentations. Higher levels of soluble phenolics were recovered in pure culture fermented *ogi* using *T. hirsuta* or *P. pentosaceus* than in the natural fermentation having 76% and 45% of the original soluble fraction in maize and sorghum respectively. This suggests greater antioxidant potentials than the naturally fermented *ogi*. Pure culture fermentations using *T. hirsuta* and co-culture of *P. pentosaceus* with *A. zeae* reduced phytate by 97% and 96% in maize and sorghum *ogi* respectively showing greater phytase activity and more nutrient bioavailabilty in the *ogi* than in the natural fermentation.

The aroma profile of *ogi* was analysed by solid-phase microextraction and gas chromatography-mass spectrophotometry (SPME GC-MS). Ethyl acetate, butyl acetate and ethyl hexanoate were observed as the key active aroma components in *ogi*. The ester, methyl thiobutanoate was found to be unique to the naturally fermented *ogi* suggesting that it may have been generated by species other than the selected starter organisms. Overall in both natural and starter culture fermentations, maize *ogi* showed high relative abundance of volatile components suggesting good substrate compatibility and utilization during fermentation. Thus compounds with high threshold values may be significant in the aroma notes of maize *ogi*. *P. pentosaceus* and *T. hirsuta* in pure and in co-culture fermentations produced *ogi* with aroma notes mostly related to the naturally fermented product.

In conclusion the diversity and levels of the initial microflora and the structural composition of grain could be major factors contributing to the nutritional compositional changes in *ogi* fermentation.

## PUBLICATIONS

- Microbial diversity and hydrolysis of phytate and phenolic compounds during *ogi* fermentation. Adanma C. Innocent-Ukachi; Gregory A .Tucker; Christine E. Dodd (Paper publication: Awaiting revision).

- *Ogi* grains: Microbial diversity and lactic fermentation. Adanma C. Innocent-Ukachi* Christine E. R. Dodd and Gregory A. Tucker (Poster presentation at Sfam Summer conference Cardiff, 2013.

- Assessment of potential spoilage and pathogenic organisms in Nigerian *ogi* during natural fermentation. *A.C. Innocent-Ukachi, C.E.R. Dodd and G.A. Tucker (Poster presentation at International Union of Microbiological Societies (IUMS) conference 2014, Montreal Canada).

- Effect of bioprocessing on hydrolysis of phytate and phenolic compounds of *ogi* grains, Adanma C. Innocent-Ukachi*, Gregory A. Tucker and Christine E. R. Dodd (Oral presentation at Food Micro 2014 conference, Nantes France).

- Impact of selected microorganisms on phenolic compounds during fermentation of *ogi.* Adanma C. Innocent-Ukachi*, Gregory A. Tucker and Christine E. R. Dodd (Poster presentation at Sfam Summer conference Dublin, 2015).

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## LIST OF ABBREVIATONS

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BHI | Brain heart infusion |
| CFU | Colony forming unit |
| HPLC | High performance liquid chromatography |
| LAB | Lactic acid bacteria |
| *Lb* | *Lactobacillus* |
| MRS | deMann Rogosa and Sharpe |
| NAD | Nicotinamide adenine dinucleotide |
| NCTC | National collection of type cultures |
| NSP | Non-starch polysaccharide |
| OD | Optical density |
| OAA | Oxaloacetic acid |
| PEP | Phosphoenolpyruvate |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PHF | Phytate hydrolysing fungi |
| PSM | Phytate specific medium |
| PSML | Phytate specific medium (liquid) |
| PSMS | Phytate specific medium (solid) |
| UNFMCC | University of Nottingham Food Microbiology Laboratory Culture Collection |

Symbol

| | |
|---|---|
| § | Section |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.0 Background

Food fermentation has existed for many years and serves as a processing and preservation technique. Although still practiced at the household level in most developing countries, fermentation technology in recent years has become more useful in achieving safe and stable products with high sensory qualities (Plessas *et al*., 2011). In Africa, fermented foods are widely consumed and they form a major dietary staple, mostly in the rural and semi urban communities. Foods that are most commonly fermented include tubers (e.g cassava), cereal grains (maize, sorghum, millet, rice), legumes (locust bean) and milk (Oyewole, 1997). Cereals are widely utilized as food and account for as much as 77% of total caloric consumption in some African countries (Odunfa, 1993; Galati *et al*., 2014). A large proportion of the cereal grains produced are processed by fermentation before consumption and they serve as weaning foods for infants and dietary staples for adults.

*Ogi,* or *akamu* as generically known respectively in Yoruba and Igbo ethnic groups of Nigeria in West Africa, is a fermented starchy food made from maize (*Zea mays*), Sorghum (*Sorghum vulgare*) or millets especially pearl millet (*Pennisetum glaucum*) and finger millet (*Eleusine coracana*). The naming of *ogi* and its colour depends on the type of cereal such as *ogi-baba* (sorghum *ogi,* brown), *ogi-gero* (millet *ogi,* grey) and *ogi* (maize, white or yellow). Traditionally *ogi* is processed by natural fermentation of the grain by steeping in water for 72 h at 28 ± 2$^{o}$C. Fermented grains are milled in a hammer mill and sieved through a muslin cloth with water to obtain the slurry which is allowed to settle at ambient temperature for 24-48 h (souring stage) before decanting the water. However, fermentation organisms which survive till the end of the process proliferate further during the souring stage (Omemu, 2011). Spoilage of *ogi* due to

chance inoculation by spoilage organisms and the tropical humid conditions has also been a source of concern (Teniola and Odunfa, 2002). Although prolonged souring presents the risk of growth of spoilage organisms, this process gives *ogi* its unique aroma characteristics being dominated by yeasts and some lactic acid bacteria (LAB) species (Odunfa and Adeyele, 1985). Prior to consumption, *ogi* is prepared by reconstituting in cold water (8-10% w/v) and gelatinizing with boiling water (100$^{o}$C) to form a gruel or alternatively cooked to form a stiff gel known as *agidi,* both of which serve as food for infants and adults. Traditionally *ogi* is stored by soaking in water which is changed daily to minimize spoilage. Under this condition the product is able to be kept for 5-7 days.

Food fermentation is usually classified based on the major products of the fermentation process such as lactic acid, acetic acid, alkaline and alcohol fermentations (Steinkraus, 1997, Blandino *et al*., 2003) which in turn depend on the diversity, population changes and metabolic activities of the microorganisms responsible. The major population found during natural fermentation of cereal grains include the following genera of bacteria, *Bacillus, Lactobacillus, Streptococcus,* and *Leuconostoc*; moulds, *Aspergillus* and *Penicillum*; and yeasts, *Debaryomyces* and *Saccharomyces* (Anthony and Chandra, 1997). Natural fermentation is usually initiated by a mixed flora resulting by chance and results in a variety of metabolic processes and hence variations in product taste, texture and aroma characteristics. The natural grain microbial flora accounts for the group which initiates the fermentation. However, this tends to change from a mixed population of LAB, yeasts, moulds and other bacterial species to a mostly acid tolerant group such as LAB which produce acids resulting in a drop from neutral pH 7.0-7.5 to acidic pH 3.0–5.0. Apart from the intrinsic grain flora a major part of the microbial community emerge at the onset of steeping while some of the intrinsic group (mostly moulds) disappear at the later stage of fermentation due to changes in process conditions such as population density and metabolic activities (Odunfa, 1985).

Culture-dependent and culture independent identifications of bacterial isolates revealed LAB as one of the major groups of organisms associated with fermentation of *ogi* (Blandino *et al*., 2003; Oguntoyinbo *et al*., 2011). Predominant LAB species identified with *ogi baba* (sorghum *ogi*) include *Lactobacillus plantarum* and *Lactococcus lactis*. (Odunfa and Adeyele, 1985). Identification by molecular techniques detected *Lactobacillus fermentum*, *Lb. amylolyticus*, *Lb. helveticus, Lb. delbrueckii* and *Weissella confusa* in maize and sorghum *ogi* (Oguntoyinbo *et al*., 2011). LAB provide antimicrobial properties by producing substances such as organic acids (Schnürer and Magnusson, 2005), diacetyl, hydrogen peroxide, reutericyclin (Leroy and De Vuyst, 2004) and bacteriocins (Oyewole, 1997). However, the efficiency of the antimicrobial properties of LAB has been reported to depend on factors such as the initial microflora of the grains and level of contamination during fermentation (Iwuoha and Eke, 1996).

Yeast and mould populations are important during traditional fermentation of cereal grains (Jespersen *et al*., 1994). The mycotoxigenic potentials of moulds have been a source of concern in traditionally fermented foods (Kpodo *et al*, 1996). Although, moulds tend to disappear or reduce by up to 60% within 24 h of natural fermentation (Odunfa and Adeyele, 1985, Halm *et al*., 1993; Jespersen *et al*., 1994), mycotoxins can be retained in the final product. The presence of aflatoxin and fumonisins upto 1.83-3.65 ng/g and 1.74-2.28 µg/g respectively have been reported in maize products after 72h of fermentation (Fandohana *et al*., 2005). Fungal species were mostly identified during souring of *ogi* and some of these have been associated with spoilage (Odunfa and Adeyele, 1985; Dike and Sanni, 2010). Although not previously detected as part of the grain microbiota, the yeast *Debaryomyces hansenii* was found to emerge after 48 h (Odunfa and Adeyele, 1985) following the decrease in pH during fermentation of sorghum *ogi. Saccharomyces cerevisiae, Candida krusei, C. tropicalis* and *C. mycoderma* form the dominant species during souring and contribute to flavour acceptability of *ogi* (Akinrele, 1970; Jespersen, 2003).

The symbiotic existence of yeast and LAB in traditionally fermented cereal-based foods enhance their metabolic activities and favours the development of aroma compounds as these organisms have been reported to synthesize groups of flavour compounds such as organic acids, terpenes, lactones, aldehydes and esters (Janssens *et al*., 1992). The presence of spoilage yeasts which utilize organic acids produced by LAB, results in an increase in pH of the fermentation medium favouring the growth of undesirable bacterial species which breakdown lactic acid to acetic, butyric or propionic acid (Franco and Peréz-Díaz, 2012). Utilization of lactic acid by *Lactobacillus* species in response to stress during fermentation and subsequent conversion to acetic acid and alcohol has also been reported (Oude-Elferink *et al*., 2001).

Apart from the impact on organoleptic properties of food, fermentation offers other important benefits such as improving the bioavailability of micronutrients and removal of toxic substances from foods (Sanni, 1993; Iwuoha and Eke, 1996). Phytochemicals found naturally in plants, such as the antioxidants (including phenolic compounds), flavour compounds and anti-nutrients such as phytate, are of great importance to the processing and nutritional value of foods of plant origin.

Phenolic compounds are secondary plant metabolites and include several aromatic organic compounds which contribute to flavour and colour of cereal foods (Wall and Blessin, 1970; Asami *et al*., 2003; Mattila *et al*., 2005). Precursors of aroma compounds such as ortho-quinones are enzymatically released from phenolic compounds and further converted by non-enzymatic reactions (Almonds, 2009). Amongst the phenolic compounds found in cereal grains, such as maize and sorghum mostly used in fermentation of *ogi*, phenolic acids predominate. The major phenolic acids found in maize and sorghum include ferulic and gallic acids (Hahn *et al*., 1983; Mattila *et al*., 2005). Phenolic acids, some of which have been reported to have potential health benefits, can pose a challenge for bioavailability from the diet since in many cases they may be insoluble in nature due to their esterification and linkage to cell wall polymers (Okarter, 2011). However, phenolic acids can be degraded by heat

or microbial enzymes (esterases) (Crepin *et al*., 2004; Almonds, 2009). Esterases are phenolic hydrolases and are found to be a component of the hemicellulolytic system of several microorganisms (Topakas *et al*., 2007). For instance LAB and some mould species have been reported to produce esterases which can hydrolyse phenolic compounds releasing soluble phenolics (Matthews *et al*., 2007). Some common microbial esterases include: feruloyl and p-coumaroyl esterases which hydrolyse ferulic and p-coumaric acids (Donaghy and McKay, 1997; Topakas *et al*., 2007) and cinnamoyl esterase mostly found in rumen or intestinal microorganisms (Guglielmetti *et al*., 2008). Ferulic acid esterases are found to be produced mostly by fungal species such as *Aspergillus* in submerged fermentation using lignocellulose from maize bran as the carbon source (Topakas *et al*., 2007).

Cereal grain is a good source of phosphorus needed in human nutrition. Phosphate and inositol in plant seeds and grains formed during maturation are stored in the form of phytate or phytic acid (myo-inositol hexakisphosphate - $IP_6$) (Oomah *et al*., 2008). Phytate, being a highly negatively charged ion, has great affinity for food components with opposite charges such as minerals, trace elements and proteins, making them unavailable (Maenz, 2001; Steiner *et al*., 2007). Phytate is hydrolysed by phytase (myo-inositol hexakisphosphate phosphohydrolase) to release phosphorous thus improving its bio-availability in plant foods. Sources of phytase include plants and microorganisms. Several bacterial and fungal strains are known to produce phytase however mould species (mostly *Aspergillus*) are used in the commercial production of phytase (Pandey *et al*., 2001).

Cereal grains have also been noted to offer other nutritional challenges, such as having low lysine and tryptophan and their relatively low mineral levels (Nout, 2009), and this contributes to the low nutritional quality of *ogi*. The nutrient content of *ogi* depends on the variety of cereal grain used and on the processing techniques. During processing of *ogi*, proteins, water soluble vitamins and minerals are lost to the steeping and wash water which can adversely affect the nutritional quality (Banigo and Muller, 1972). Research efforts have been made to identify suitable processing

techniques in order to minimize nutrient losses in *ogi*. Banigo and Muller (1972) reported that a reduction in protein loss could be achieved by using an improved wet milling technique. Bioprocessing such as fermentation has been reported to improve nutrient bioavailability (Agte *et al*., 1997). Fortification of *ogi* using other protein-rich plant food sources such as soybean or okra (Aminigo and Akingbala, 2004) and the use of fermentation (Chavan and Kadam, 1989; Kazanas and Fields, 1981; Wakil *et al*., 2008) to improve protein quality have been studied. Recently there has been a growing interest in the use of starter cultures in *ogi* fermentation to enhance the activities of desirable microorganisms in order to increase product quality (Odunfa *et al*., 2001; Teniola and Odunfa, 2001). In addition to nutrient and sensory qualities, ways of improving the functionality of beneficial non-nutrient compounds during *ogi* fermentation needs to be investigated.

Knowledge of the microbial diversity in *ogi* is important in order to understand those species which may be important in fermentation, may minimize the effect of spoilage organisms and improve the nutritional quality and consistency of *ogi* production. Classical identification of microbial species, based on traditional culturing techniques, and changes during natural fermentation of *ogi* have been reported (Akinrele, 1970; Odunfa and Adeyele, 1985; Iwuoha and Onyekwere, 1996; Blandino *et al*., 2003). There is minimal information on microbial characterization of *ogi* using molecular techniques (Omar and Ampe, 2000, Oguntoyinbo *et al*., 2011; Oguntoyinbo and Narbad, 2012). Although cultural characterization is time consuming, a combination with molecular approach could be more efficient to have a wide overview of the organisms present in *ogi*.

The significance of the major LAB and yeast species isolated from *ogi* fermentation on protein quality and aroma characteristics has been identified (Odunfa *et al*., 2001; Teniola and Odunfa, 2001; Omemu *et al*., 2007), but the role of these groups for esterase and phytase activities and their influence on levels of phenolics and phytate during *ogi* fermentation is yet to be established. This study aimed to characterize the microbial diversity of maize and sorghum grains and identify species which may be

important in *ogi* fermentation. Key organisms involved in *ogi* fermentation and their roles during the hydrolysis of phytate and phenolic compounds, release of aroma compounds and potentials as starters would also be identified.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Cereal grains

Cereal grains are fruits of grasses belonging to the family of *Gramineae*. Cereals are produced and consumed in all parts of the world and form important sources of energy. Cereals are used for both human and animal nutrition. Maize, rice, wheat and barley are mostly produced worldwide (Table 2.1). Total estimates of global production of cereal grains ranged from 1.9–2.7 billion tons in 1993–2013. The production of sorghum is only about 1/15$^{th}$ of the total amount of maize (FAOSTAT, 2015).

**Table 2.1**: World †production of some cereal grains

| Cereal | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|---|---|---|---|---|---|
| Maize | 728 | 713 | 706 | 790 | 830 | 820 | 851 | 887 | 877 | 1018 |
| Sorghum | 58 | 59 | 57 | 62 | 66 | 56 | 60 | 57 | 56 | 62 |
| Paddy rice | 607 | 635 | 640 | 656 | 688 | 686 | 701 | 722 | 734 | 740 |
| Wheat | 632 | 626 | 602 | 612 | 683 | 686 | 649 | 699 | 671 | 615 |
| Barley | 153 | 138 | 139 | 134 | 154 | 151 | 123 | 132 | 133 | 143 |
| Millet | 29 | 30 | 31 | 33 | 34 | 26 | 32 | 27 | 26 | 29 |
| Rye | 17 | 15 | 12 | 15 | 18 | 18 | 11 | 13 | 14 | 16 |
| Oat | 26 | 23 | 25 | 25 | 23 | 19 | 22 | 21 | 23 | 23 |

†Million tons; values retrieved from FAOSTAT (2015)

Cereal grains are rich in carbohydrate, protein, vitamins (E and B- complex), iron, trace minerals and dietary fibre (Haard, 1999). About 50% of daily dietary protein and energy requirement is provided through consumption of cereals (Haard, 1999). The major carbohydrate components in cereal grains include starch, water soluble or insoluble dietary fibre and free sugars (Charalampopoulos *et al*., 2002). They contain

both structural (cellulose, hemicellulose and pectin) and non-structural carbohydrates (starch, sugars and fructosan).

Non absorbable starch and products of starch degradation are classified as resistant starches (Englyst and Cummings, 1990). Resistant starches may form part of the dietary fibre (Englyst *et al*., 1982). The non-starch polysaccharides (NSP) -are usually less than 10% of original grain weight (Kumar *et al*., 2012) and these constitute the dietary fibre (Englyst and Cummings, 1990). It is the number and types of monomeric units that differentiates starch from non-starch polysaccharides (Kumar *et al*., 2012). Components of hemicellulose may include sugar acids such as glucuronic and galacturonic acids, hexoses such as glucose and galactose and pentoses like xylose and arabinose (Wall and Blessin, 1970). Hydrolytic enzymes which act on hemicellulose include xylan esterase, α–l-arabinofuranosidase, α-4-O-methyl glucuronosidases and phenolic acid (ferulic and p-coumaric) esterases (Kirk and Cullen, 1998). Hydrolysis of hemicellulose mainly yields four sugars namely: xylose, arabinose, glucose, and galactose (Wall and Blessin, 1970). Xylan and other hemicelluloses are hydrolysable by acid (Pérez *et al*., 2002).

The amount of NSPs in cereal foods depends on the original grain content and method of processing. Viscosities of NSPs depend on the structure (linked compounds such as phenolics), concentration and solubility and are important in determining their digestion. Water soluble NSPs are highly viscous, have a low degree of branching and a high content of ferulic acid (Kumar *et al*., 2012). High viscosity reduces the rate of substrate utilization and efficiency of digestive enzymes resulting in low nutrient absorption. Processing methods such as cooking, bran dehulling and use of microbial enzymes have been employed to improve the nutritional quality of foods rich in NSPs (Hoebler *et al*., 1998).

Maize, sorghum and millet grains lack gluten-like proteins. These proteins consist mainly of hydrophobic amino acids and others such as glutamine (Charalampopoulos *et al*., 2002), which may be important in flavour development during fermentation. Nevertheless these grains contain other substrates which are convertible into flavour

compounds by microorganisms or precursors of flavour through proteolytic activities in combinations with other enzyme (malt) activities (Kohajdová and Karovičová, 2007).

Fermentation reduces carbohydrate, non-digestible polysaccharides and oligosaccharides and increases essential amino acids, lysine, methionine and tryptophan (Adams, 1990; Kohajdová and Karovičová, 2007, Odunfa *et al*., 2001).

### 2.1.1 *Ogi* grains

#### 2.1.1.1 Maize (*Zea mays*)

Maize constitutes one of the largest cereal grain productions in the world and is a major food crop in Africa. The total maize production in Africa accounted for 6.97% of the world's total production in 2013 and Nigeria produced 8,694,900 tons (12.2%) of Africa's total production (FAOSTAT, 2015). Maize grains vary widely and are classified based on the characteristics of the endosperm as flint, waxy, dent, or flour maize. Flint maize is characterized by a hard, glassy endosperm with a smooth hard pericarp while the flour maize has soft, starchy endosperm with a thin pericarp. Dent maize is physically identified by the collapsed ends of the grains and also possesses a soft and starchy endosperm (Žilić *et al*., 2011). Waxy type is a hybrid derived from the normal maize and contains 100% amylopectin compared to 75% in the latter (Ptaszek *et al*., 2009). White, yellow, red or multi-coloured maize types are also cultivated. However, the white and yellow maize are mostly cultivated in Nigeria. Phenolic compounds (mostly phenolic acids and flavonoids) and carotenoids are present in coloured maize kernel. Carotenes give a yellow coloured maize (Cortés *et al*., 2006; Žilić *et al*., 2012) and of the phenolic acids, ferulic acid is most abundant (Žilić *et al*., 2012). The oil content of maize is about 6% with high polyunsaturated essential fatty acids (Graham *et al*., 1990) they lack niacin and have low protein content which is poor in lysine and tryptophan (Lopez *et al*., 1983). Maize forms a good substrate for lipolytic and proteolytic activities.

#### 2.1.1.2 Sorghum (*Sorghum vulgare*)

Sorghum originated from Africa and is an important cereal crop in the world. Global production of sorghum has ranged between 58–62 million tons (FAOSTAT, 2015) over the last ten years (Table 2.1). Sorghum is rich in vitamin B and minerals, such as phosphorus, but low in calcium. The starch concentration ranges from 50-75% in the whole grain and 72.2-82.5% in the honey and starchy endosperms (Wall and Blessin, 1970). Sorghum starch has low digestibility due to starch-protein interactions (Taylor and Belton, 2002). Sugars in sorghum include glucose, fructose, sucrose, maltose and raffinose and free sugars range from 0.3-2.5%. Sorghum fibre contains mostly cellulose, hemicellulose, lignin, pectin, and gums, most of which are insoluble (86.2%). Sorghum insoluble fibre increases after processing due to the increase in bound protein (kafirin) and enzyme-resistant starch. Sorghum is potentially a good substrate for microbial lipolysis and proteolysis having β - Kafirin which contains the monomeric or polymeric forms of the sulphur-containing amino acids, methionine and cysteine (Arendt and Zannini, 2013). Sorghum is however, low in lysine, arginine, histidine, glycine and methionine (Wall and Blessin, 1970). The oil content of sorghum is 3.5% and this is found in the pericarp, aleurone layer and germ with the germ containing about 70% of the total oil (Rooney, 1978)

### 2.1.2 Cereal grain quality and product challenges

Cereals have been associated with low nutritional value. However, they contribute to the major staples of several low income populations. Factors contributing to the restriction of nutrients in cereals include the swelling of the starch during heating and bioavailability of the micronutrients (Nout, 1993; Mouquet *et al*., 2008). Research interest in the improvement of the nutritional value and digestibility of traditional cereal-based products has increased recently (Chelule *et al*., 2010).

Cereal grains are prone to infestation by mould species capable of producing secondary toxic metabolites known as mycotoxins (Sweeney and Dobson, 1998) and

as such grains may contain a build-up of toxins prior to processing. Mould infestation can occur both in the field and during storage and is due to the high temperatures and relative humidities found in the tropics (Herrman and Trigo-Stockli, 2002). Mycotoxins are hazardous to both humans and animals. Some mycotoxins are neurotoxins while some affect protein synthesis (Sweeney and Dobson, 1998). They are mainly produced by species of *Aspergillus, Penicillium* (mostly present as contaminants) and *Fusarium* (present as a plant pathogen) and are characterized by various symptoms and subsequent disorders known as mycotoxicosis (Sweeney and Dobson, 1998; Herrman and Trigo-Stockli, 2002). Some of the different mycotoxins produced by these moulds include:

**(1) Aflatoxin:** This is produced by *Aspergillus flavus, Aspergillus nominus* and *Aspergillus parasiticus* and causes liver disorder. Naturally produced aflatoxins include $B_1$, $B_2$, $G_1$ and $G_2$. Aflatoxins M1 and M2 are derivatives of B1 and B2 (Frobish *et al*., 1986). The optimal temperature for mould growth was reported to be 32-33$^{o}$C and 12-40$^{o}$C for aflatoxin production (Koehler *et al*., 1985). Since toxin production depends on the metabolic activity of fungi (Scroeder and Hein, 1967), the rate of accumulation of aflatoxin would be greatest at 32-33$^{o}$C, thus under tropical conditions this toxin could be produced.

**(2) Ochratoxin:** This is implicated with grain infestation by *Aspergillus* or *Penicillum* species. Mould growth occurs at 12-31$^{o}$C and toxin is produced at 20$^{o}$C (ICMSF, 1996).

**(3) Fumonisin:** A carcinogenic toxin typical of *Fusarium verticilloides* but also produced by other species of *Fusarium* such as *F. moniliforme* and *F. proliforatum*. Fumonisin B1 is produced by *F. subglutinans* which grows at 5$^{o}$C. Toxin production is at 25$^{o}$C (Pitt and Hocking, 1997).

**(4) Trichothecenes:** Include zearalenone, deoxynivalenol and T-2 toxin.

* **Zearalenone**: Fusarium toxin which targets the reproductive system and is mostly produced by *Fusarium graminearum* and *Fusarium sporotrichiodes* with optimal growth temperature at 24-26$^{o}$C and 22.5-

35$^{o}$C, respectively. Other species include *F. equiseti (*Morrison *et al.,* 2002*).*

- **Deoxynivalenol**: This is produced by *Fusarium culmorum* and *Fusarium sporotrichiodes* with optimal growth temperatures at 21-31$^{o}$C and 22.5-35$^{o}$C respectively, and toxin production at 25$^{o}$C (Bottalico *et al*., 1982). This toxin is associated with vomiting and bloody diarrhoea.

The presence of mycotoxigenic moulds forms a major source of concern in food fermentations. Traces of toxins have been reported in some mould fermented foods (Ibeh *et al*., 1991; Jespersen *et al*., 1994). Interestingly significant reduction in mould count at the end of fermentations has been reported (Jespersen *et al*., 1994). Mycotoxin degradation has also been reported for certain bacterial and mould species (Table 2.2). This was mostly observed in fermentations with mixed cultures of mould and yeast. *Rhizopus oligospora*, used in fermentation of Indonesian *ontjom* and *tempe* respectively, have been found to reduce aflatoxin by 70% (Steinkraus, 2002). The mould *Neurospora sitopholia* was found to reduce total aflatoxin in groundnut cake by 80% (Fardiaz, 1991). *Lactobacillus plantarum* was reported to show higher reduction of aflatoxin (by up to 46%) than *Lactococcus lactis* which had 27% reduction (Sezer *et al*., 2013). However, the pathway and products of degradation of these toxins have not been fully established.

Again studies have shown that the level of toxin produced during fermentation can be lowered by preventing the growth of pathogens (Motarjimi and Nout, 1996). However, the extent and mechanism of reduction of toxins formed prior to processing by microbial activities, and what organisms are associated with anti-microbial activities in a particular natural fermentation of *ogi* is less well known. This reveals a gap in knowledge necessitating the need for further research to optimize the antimicrobial activities of selected strains from natural fermentation of *ogi*.

**Table 2.2** Microbial degradation of mycotoxin

| **Bacterial degradation** | | | |
|---|---|---|---|
| Mycotoxin | Bacteria | Product | †Reference |
| Aflatoxin $B_1$ | *Flavobaterium aurantiacum* | Water soluble products + $CO_2$ | (Line *et al*., 1994) |
| Ochratoxin A | *Acinetobacter calcoaceticus* | - | (Hwang and Draughton, 1994) |
| Trichothecene | *Butyrivubrio fibrisolvens*, *Lactobacillus* spp. | Deacylated 15-acetoxyscirpenol | (Matsushima *et al*., 1996) |
| Aflatoxin | *Bacillus subtilis* (FERM BP-3418), *Lactococcus lactis, Lactobacillus plantarum.* | -<br>- | (Kazuhiro, 1996)<br>(Sezer *et al*., 2013). |
| Ochratoxin A, Fumonisin B and $B_2$ | *Bacillus stearothermophilus* (87% reduction) | | (Faraj *et al*., 1993, Scott *et al*., 1995), |
| **Fungal degradation** | | | |
| Mycotoxin | Fungi | Product | †Reference |
| Aflatoxin | *Rhizopus* spp., *Aspergillus flavus* (through monoxygenase enzyme) | - | (Bol and Smith, 1990, Knol *et al*., 1990), |
| Ochratoxin A, Fumonisin B and $B_2$ | *Saccharomyces cerevisiae, A. flavus + A. niger* (80% reduction), *Rhizopus oryzae* (70% reduction) | - | (Faraj *et al*., 1993, Scott *et al*., 1995), |
| Zearalenone | Yeasts (*Candida tropicalis*, *Torulaspora delbruckii, Zygosaccharomyces rouxii*, *Saccharomyces* spp. | α- and β-ZEA | (Boswald *et al*., 1995) |

† Compiled from listed references

Various molecular techniques to identify pathogenic moulds and their mycotoxigenic potentials have been developed (Stępień *et al*., 2012). One such tool is the analysis of polymorphic regions of the biosynthetic gene pathways by PCR assays using specific gene markers (Kristensen *et al*., 2005; Stępień *et al*., 2011a)

### 2.2 Food fermentation: An overview

Fermentation is one of the oldest methods employed in food production (Chavan and Kadam, 1989). The earliest record on food fermentation technology dates back to 6000 BC and this was practiced without any knowledge of the microorganisms involved or the role they play (Blandino *et al*., 2003). Many fermented products with unique characteristics and for different uses ranging from main staples and beverages to condiments now exist. Fermentation involves biological activities of microorganisms or enzymes which result in biochemical changes with the production of compounds which confer health (such as antioxidants) or preservative (inhibiting activities of undesirable and pathogenic microorganisms) benefits (Ross *et al*., 2002). Microorganisms may be originally present on the raw material used as substrate or added as starters. All groups of microorganisms whether Gram positive or negative, aerobes or anaerobes, spore formers or non-spore formers may be involved in fermentation (Müller, 2001).

*Ogi* is processed by natural fermentation. Natural fermentation presents a complex system of microbiota and involves a wide range of substrates and end products (Table 2.3). During fermentation the substrate is oxidized and the intermediate derivatives reduced to the end product (Müller, 2001).

**Table 2.3:** Fermentation substrates, metabolites and products

| Type of fermentation | Substrate | Products | Microorganisms | †compounds | Reference |
|---|---|---|---|---|---|
| Lactic acid | cereal and milk | Fermented cereals and milk (Yoghurt, cheese) | Lactic acid bacteria (LAB) | Lactic and acetic acids, ethanol, carbon dioxide | (Chavan and Kadam, 1989, Hammes *et al.*, 2005, Kohajdová and Karovičová, 2007, Vegas *et al*., 2010) |
| Alcohol | Cereal, fruits, grapes | Alcoholic beverages (Wine, beers) | Yeast, heterofermentative LAB | ethanol | |
| Acetic acid | Seeds and vegetables | Wine-vinegar | *Acetobacter* | Acetic acid | |
| Propionic acid | Lactate | Propionic and acetic acids | *Propionibacterium* | Propionic acid, acetic acid, carbon dioxide | (Müller, 2001) |
| 2,3-butanediol | Glucose | Butanediol | *Enterobacter* | Ethanol, butanediol, lactic and formic acids, carbon dioxide, hydrogen. | |
| Butyrate | Starch, molasses | Acetone-butanol | *Clostridium* | Butyrate, carbon dioxide, hydrogen. | |

†Compounds associated with the fermentation process

Fermentable substrates include polymers (polysaccharides, proteins and lipids) and monomers (sugars such as pentoses, hexoses, and tetroses), organic acids, purines and pyrimidines (Müller, 2001, Moat *et al*., 2002f). Aromatic compounds (phloroglucinol, resorcinol), are also fermented especially by mixed cultures (Heider and Fuchs, 1997). Aromatic compounds are mainly of plant origin with high polymeric lignin as the most common source. Low molecular weight aromatics include amino acids (tyrosine, phenylalanine and tryptophan), quinones, flavonoids, phenolic compounds and tannins. These compounds are mostly degraded by bacteria and fungi (Heider and Fuchs, 1997).

Bacteria involved in food fermentation are grouped into three phyla (Hutkins, 2006), namely *Firmicutes* (LAB, *Brevibacterium* and *Bacillus*), *Actinobacteria* (*Bifidobacterium*, *Kocuria*, *Staphylococcus* and *Micrococcus*) and *Proteobacteria* (Gram negative bacteria such as *Acetobacter*). Bacteria of the genera *Enterobacter*, *Erwinia*, *Salmonella*, *Clostridium* and *Citrobacter* in addition to LAB have been identified in mixed fermentations similar to the natural fermentation of *ogi* (Müller, 2001). However, LAB are the most important group in food fermentation. LAB are Gram positive rods or cocci, catalase negative, acid-tolerant, fastidious, non-sporing, anaerobic but aerotolerant bacteria capable of undergoing fermentation (König and Fröhlich, 2009). They are classified based on their fermentation end products as homofermentative or heterofermentative species (Müller, 2001) producing lactic acid only or ethanol or acetic acid and carbon dioxide in addition to lactic acid, respectively.

Homofermentative LAB genera include *Lactobacillus* (*Lb. delbrukii* subspecies *bulgaricus* and *Lb. acidophilus*), facultative heterofermentative which are able to utilize a wide range of fermentable carbon sources (*Lb. plantarum*, *Lb. casei* and *Lb curvatus*) and *Pediococcus (P. pentosaceus, P. acidilactici, and P. claussenii)*. *Leuconostoc*, *Oenococcus* and *Weissella* constitute the heterofermentative group (König and Fröhlich, 2009). Other heterofermentative groups commonly present in natural fermentations but which are either not or hardly used as starters include *Enterococcus*, *Lactococcus* and *Aerococcus*. Lactic acid bacterial cells also vary in their morphological characteristics

ranging from rods, cocci, coccobacilli to irregular, spherical or lenticular cells in singles, pairs, or tetrads. Formation of different isomeric forms of lactic acid (D-, L- or DL-lactic acid) also differentiates these LAB genera from one another (König and Fröhlich, 2009).

Hexoses are the most common substrate for LAB. Apart from glucose other hexose sugars are fermented by many LAB species. These sugars through the glycolytic pathway are converted to pyruvate which is then reduced to lactate and probably other products (Fig. 2.1) depending on the fermenting species, pH and growth media (Müller, 2001; Moat *et al*., 2002c). Apart from carbohydrate fermentation, lactic acid bacteria are able to metabolise nitrogen through protein synthesis and ferment gluconate and citrate which generates flavour compounds such as diacetyl (Müller, 2001).

Food products are found to be modified by the activities of microbial enzymes which are enhanced during fermentation. In addition to promotion of activities of enzymes (which hydrolyse polysaccharides, proteins, phytates and lipids), LAB produce organic acids which lower the pH of the food and that impact preservation. Anti-nutrients such as tannin and phytic acid are reduced through microbial enzyme hydrolysis (Chelule *et al*., 2010) and the safety of the food is improved by inhibition of the spoilage and pathogenic organisms (Daeschel, 1989). Antimicrobial compounds are also produced such as hydrogen peroxide, carbon dioxide, acetaldehyde, amino acids (D-isomers), reutericyclin, ethanol and bacteriocins (Klaenhammer, 1988; Piard and Desmazeaud, 1991; Piard and Desmazeaud, 1992).

Moulds also play important roles in food fermentation. Although the presence of some moulds is undesirable due to the ability to produce toxins or spoil the food, they have been reported as active producers of hydrolytic enzymes such as amylases, lipases, proteases, esterases, phytase and tanninase contributing to the characteristic flavour of most fermented foods and breakdown of plant starch, fats, proteins, phenolics and antinutritional compounds (Evans, 2013; Owens *et al*., 2015).

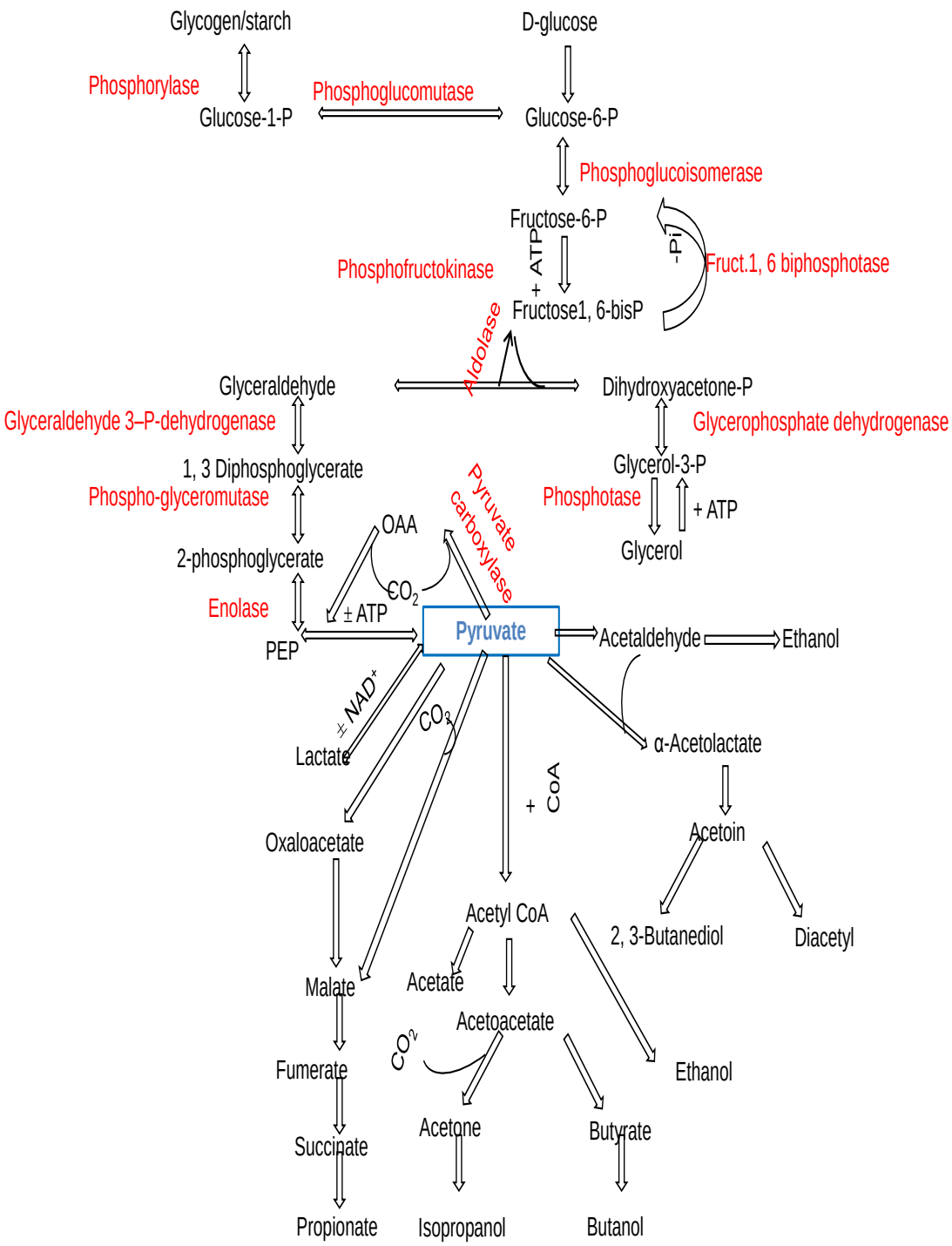



Figure. 2.1: Metabolic pathways of fermentation products. (Moat *et al*., 2002a; Moat *et al*., 2002c)

Mould genera: *Aspergillus*, *Rhizopus*, *Mucor*, *Actinomucor* and *Penicillum* are mostly found in food fermentations. Substrate and energy source varies in different mould species and include carboxylic acids, sugars, amino acids and fatty acids (Owens *et al*., 2015). Various flavour components have been identified in fermentations involving mycelial fungal (Basidiomycete) groups. These include groups of benzene derivative (coumaric and cinnamic acid derivatives), terpenes, lactones, ketones, alcohols, aldehydes and esters (Gallais *et al*., 1990).

Yeasts constitute one of the significant groups in food fermentations involving the production of alcohol and include ascomycetous yeasts (FAO, 2011). The roles of yeasts in fermentation have been reported to include increase in protein content of food, generation of aroma compounds, provision of essential metabolites for LAB, and possible inhibition of mycotoxigenic moulds and degradation of their metabolites, production of hydrolytic enzymes (cellulases and pectinases) and conferring probiotic properties (Jespersen, 2003). Metabolic activities of yeasts in cereal fermentation produce groups of aroma compounds consisting of esters, alcohols, fatty acids and aldehydes (Stam *et al*., 1998)

Microbial activities and products during fermentation may depend on growth conditions and ability to respond to stress. Hydrogen ion concentration (pH) is one of the factors that regulate the growth of microorganisms within an environment. Bacterial growth may occur at extreme ranges of pH 1 (acidophilic bacteria) or pH 11 (alkalophilic bacteria). However, most environmental bacteria grow between pH 5-9 but could withstand mild acid or alkaline conditions (Moat *et al*., 2002e). During fermentation moulds and yeasts could withstand pH ranges of 2.0-11.0 and 1.5 to-8.05, respectively (Mensah, 1997).

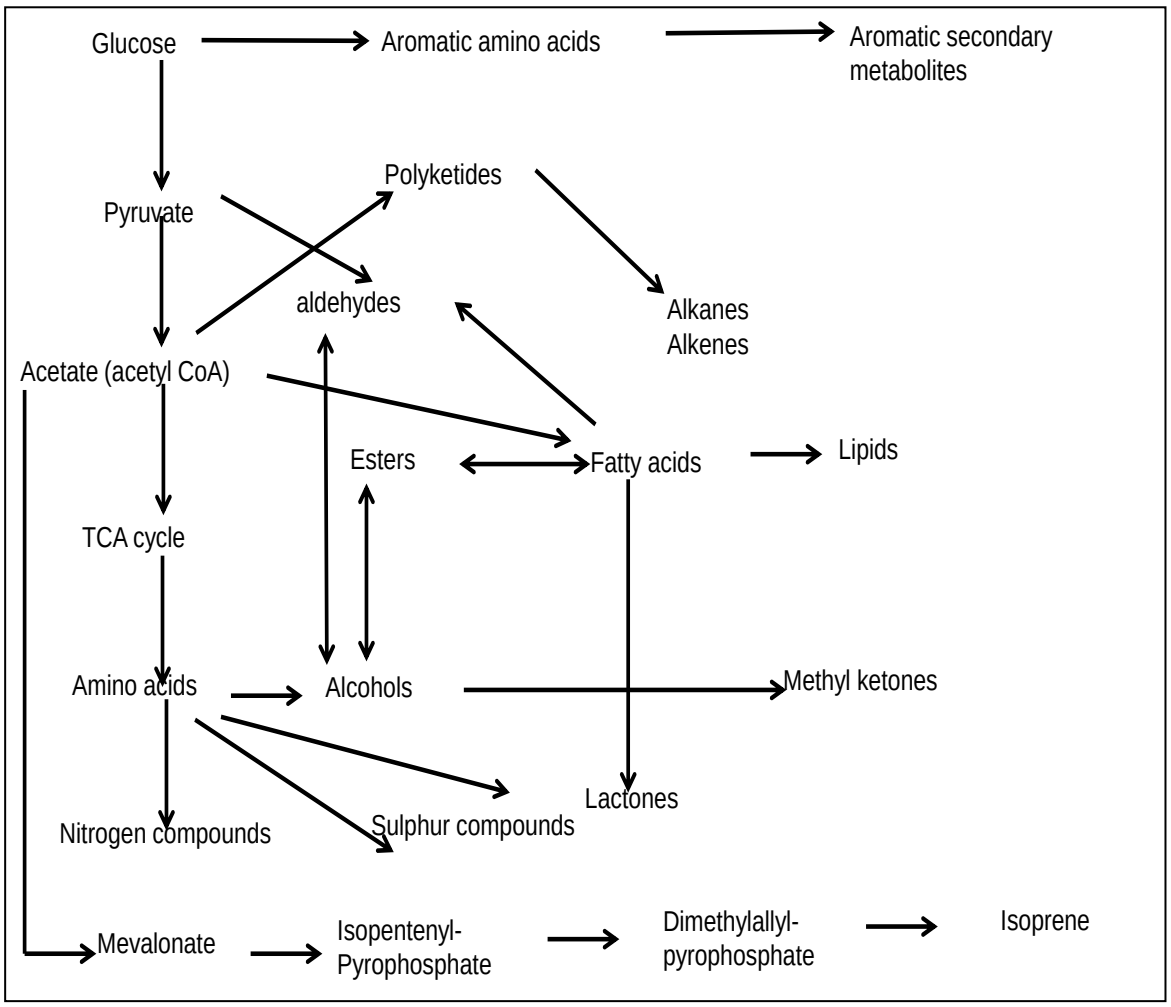
Microbial activities during fermentation could also depend on ability to withstand stress. Oxidation-reduction reactions and the passage and interaction of electrons with electron acceptors and donors generate the motive force required to move substances in and out of the cell (Müller, 2001, Moat *et al*., 2002b, Lolkema *et al.*, 1994) and as such microorganism are able to regulate the pH of their system. Thus the internal pH of microbial species depend on the cell buffer capacity, removal (through respiration or

ATP hydrolysis) and exchange of protons with certain cations such as $Na^+$ or $K^+$. In the case of acid or alkaline stress, microorganisms have been reported to produce enzymes which convert acids to neutral compounds or *vice versa* thus regulating the pH of the system. Such enzymes include glutamate decarboxylase, lysine decarboxylase and arginine decarboxylase as found in *E. coli* (Moat *et al*., 2002e). These enzymes through the process of pH homeostasis are able to trigger the $K^+/H^+$ and $Na^+/H^+$ antiporters based on pH levels and as such under acidic conditions the $K^+/H^+$ antiporter is activated adding $K^+$, removing a proton and increasing the pH to 7.5 whilst alkalinization triggers the $Na^+/H^+$ antiporter which removes $Na^+$ and adds a proton to restore acidity (Madshus, 1988, Moat *et al*., 2002b). The enzyme Na+/K+ ATPase enhances proton translocation and depends on $Mg^{+2}$ in regulation of microbial pH levels by generation of ATP needed to synthesize or move metabolites in and out of the cell as documented for *E. coli* and other microroganisms (Moat *et al*., 2002b). Apart from proton transfer microorganisms are able to induce an acid protection system (acid tolerance response) in which the metabolites produced by the induced genes protect the cell against damage (Moat *et al*., 2002b). However, population density/stress could also form a limiting factor to metabolic activities in addition to acid or alkaline stress especially in mixed fermentations. Gram positive bacteria such as LAB have NADH oxidase and are able to regenerate NAD+ during oxidative stress (Serrazanetti *et al*., 2013).

### 2.2.1 Fermentation of cereal grains

Quite a number of cereal foods undergo fermentation processes. However, cereal fermentation differs from all other fermentations as the food is passed through further processing, mostly cooking, prior to consumption. In cereal fermentation, microbial and enzymatic activities are both important (Hammes and Gänzle, 1998) and work together or individually to impact various product characteristics (Hammes *et al*., 2005). This fermentation, as in other fermentations, aims to affect sensory properties, enhance food safety (antimicrobial activities and removal of undesirable compounds such as toxins and flatulence-producing carbohydrates), improve nutrient availability by removing

antinutrients, extend shelf-life (Nout, 1994) and minimize the cooking energy (Steinkraus, 1994; Hammes *et al*., 2005). Prior to fermentation microbial and enzyme activities are activated by increasing water activity through steeping. Substrates are made available by hydrolytic activities (proteolysis, lipolysis, and amylolysis) and microbial physiological activities (Hammes and Gänzle, 1998). Fermentable substrate, nutrients, growth and growth inhibition factors depend on the type of cereal. Free sugars represents up to 3% of total sugars available in cereal grains with sucrose (>50%) as the major component. Numerous volatile compounds are generated (Fig. 2.2) and are present in natural cereal fermentations (Blandino *et al*., 2003) which contribute to the characteristic blend of flavours in the products (Table 2.4).




Source: (Korpi *et al.* 2009)

**Figure 2.2:** Metabolic pathways for production of microbial volatile organic compounds, TCA: tricarboxylic acid

Table 2.4: Volatile compounds identified in cereal fermentation

| Organic acids | | Alcohols | Aldehydes | Ketones |
|---|---|---|---|---|
| Propionic | Pyruvic | Ethanol | Acetaldehyde | 2-Methyl butanol |
| Lactic | Palmitic | n-propanol | Formaldehyde | Acetoin |
| Acetic | Itaconic | Isobutanol | Isovaleraldehyde | Diacetyl |
| n-Butyric | Lauric | Amyl alcohol | *n*-Valderaldehyde | Butanone |
| Heptanoic | Isocaprilic | Isoamyl alcohol | Isobutyraldehyde | Methyl ethyl ketone |
| Isovaleric | Benzylic | 2,3-Butanediol | Propionaldehyde | Acetone |
| Isobutyric | Myristic | β- Phenylethyl alcohol | *n*-Hexaldehyde | |
| Formic | Hydrocinnamic | | Furfural | |
| Succinic | Caproic | | Hydroxymethyl furfural | |
| Valeric | Capric | | 2-Methyl butanal | |
| | | | 3-Methyl butanal | |
| | | | Glyoxal | |
| | | | Methional | |

Source: Kohajdová and Karovičová, 2007

Products of cereal fermentation include fermented gruels such as *ogi*, alcoholic (beer, sake) and acid fermented drinks (boza, mahewu), vinegar and colourants e.g red colour of Angkak from fermented rice (Hammes *et al*., 2005).

### 2.2.2 *Ogi*

*Ogi* is a fermented breakfast food generally consumed in Nigeria, West Africa. It is an important complementary weaning food for infants and a major meal for convalescents. The microbiology of *ogi* following natural fermentations has been studied over the years and found to vary from different batches of the same grain. This inconsistency of different fermentations forms a major challenge to efforts put in place to achieve large scale production of *ogi*.

The microbiological changes during the traditional production of *ogi baba* (sorghum *ogi*) revealed variations in composition at different fermentation stages (Odunfa and Adeyele, 1985). Ashiru *et al*. (2012) reported the presence of starch-degrading *Bacillus megaterium* and *Bacillus subtilis* from *ogi* after seven days of fermentation which produced simple sugars enhancing the growth of non-starch degrading yeast and *Brevibacterium* spp.(Teniola and Odunfa, 2002) associated with spoilage in *ogi*. Lactic acid bacteria, yeasts and moulds ferment *ogi* and metabolic processes occurring during natural fermentation of *ogi* classifies it as a mixed fermentation. Other bacterial genera such as *Corynebacterium* known to hydrolyze corn starch and *Enterobacter cloacae* have also been identified during fermentation of *ogi* (Akinrele, 1970; Caplice and Fitzgerald, 1999). Odunfa and Adeyele (1985) reported the presence of moulds (*Aspergillus* and *Penicillum*), yeast (*Debaryomyces hansenii*) and bacteria of the following genera, *Bacillus, Lactobacillus, Lactococcus, Leuconostoc,* in natural fermentations of *ogi baba* (sorghum *ogi*) with *Lactobacillus plantarum* and *Lactococcus lactis* predominating. Twenty-five LAB isolates from indigenous fermented maize gruel and five fungal isolates from spoilt stiff gel were detected by Dike and Sanni (2010) some of which included *Lb. plantarum, Lb. casei, Lb. brevis,*

*Lb. acidophilus, Lb. fermentum, Lb. delbruecki, Pediococcus acidilactici, Aspergillus flavus, A. fumigatus, Rhizopus* and *Penicillum* spp.

The association of yeast with LAB during fermentation has been reported to increase growth and activities of LAB in a fermenting medium since the nutrients (vitamins) required for the bacteria to grow are obtained directly from yeast cells or indirectly from yeast-synthesized nutrients (Narendranath *et al*., 1997; Querol and Fleet, 2006). Yeast on the other hand grows well in acidic medium and benefits from lactic acid produced by LAB as an energy source for its metabolic activities. Microbial activities and product quality of fermentation involving yeast and LAB will depend on their concentrations in the medium. Yeast may be present in the original grains or arise as part of the fermentation. Higher concentrations of yeast may lead to high pH which promotes proteolytic activities with production of substances (e.g ammonia) and subsequent growth of spoilage bacteria (Querol and Fleet, 2006).

Several microbial species have been identified during natural fermentation of *ogi* but the significance of the individual species is less known. The use of starter cultures in *ogi* fermentation has recently received attention. Vitamin enrichment of *ogi* was studied with *Lb. plantarum* and *Enterobacter cloacae* as starters (Akinrele, 1970). Mould species, usually present as contaminants in *ogi,* cause spoilage and could be eliminated by bacteriocin producing LAB. Influence of starter cultures of LAB on spoilage of stiff gel (*agidi*) was studied by Dike and Sanni (2010) who reported highest and least antagonistic activities of *Lb. plantarum* and *Lb. brevis,* respectively to *Aspergillus.*

The presence of *Enterobacteriaceae* in natural fermentation of *ogi* may be quite significant but has received little attention. These are mostly pathogenic and are not used for controlled fermentations. Nevertheless, some species have been identified with the production of organic acids (citrate, lactate, acetate, and formate) and volatile compounds mostly ketones (acetone, 2-butanone, 2-heptanone, 2-nonanone, 3-hydroxy-2- butanone, 2,3-butanedione), alcohol (ethanol, 2,3 butanediol and 1-pentanol (Chaves-Lo´pez *et al*., 2006). Species identified in Italian ewe's cheese

include: *Escherichia coli*, *Enterobacter sakazakii*, *Kluyvera* spp, *Serratia odorifera*, *Citrobacter braakii*, and *Salmonella enterica* ssp *arizonae.* However, *Enterobacter*, *Serratia*, and *Erwinia*, are known to produce acetoin, 2, 3-butanediol or diacetyl (Moat *et al*., 2002d; Chaves-Lo´pez *et al*., 2006)

Comparative studies on proximate composition of raw maize and *ogi* showed 22.7% decrease in protein and 7.0% decrease in carbohydrate content of *ogi* (Aminigo and Akingbala, 2004). Low protein content in *ogi* has been attributed to certain microorganisms in the natural fermentation such as *Enterobacter cloacae* and *Lactobacillus plantarum* which use some of the amino acids for growth, and the elimination of germ (high in protein) from the maize kernels (FAO, 2011). Lysine excreting mutant produce higher concentrations of amino acids as against the usual regulated concntrations (Sands and Hankin, 1974). Sands and Hankin (1974, 1976) and Newman and Sands (1984) reported the use of lysine-excreting mutants of *Lactobacillus* to fortify various foods to improve their nutritional value for human and animal consumption.

The ability of certain heterofermentative LAB to degrade acids and alcohols has been reported. Although previously identified in corn silage, *Lb. buchneri* has been shown to degrade lactate to acetate, ethanol, and 1,2-propanediol (Oude-Elferink *et al*., 2001). The knowledge of the interactions of microbial species during fermentation and optimization of their positive contributions will significantly contribute to good process control and improve quality of *ogi*.

### 2.3 Other fermented cereal products

A number of other fermented cereal products are known with specific local differences in composition and method of preparation. These products include Nigerian *massa* (snack from fermented maize dough), South and East African *amahewu/mahewu* (sour maize/millet/sorghum-based gruel) and *incwancwa* (non-alcoholic fermented maize beverage), Ghanaian & Botswanian *kenkey, banku* and *bogbe* (dough from maize/sorghum/millet*)*, Kenyan *uji,* Tanzanian *togwa* (maize-sorghum gruel),

Sudanese *kisra* and *hussuwa* (fermented sorghum dough), Zambian *munkoyo* (fermented maize gruel with root extract), Saudi Arabian *Sobia* (malt and wheat), and Ethiopia *injera (*sorghum Dough) (Zulu *et al*., 1997; Nago *et al*., 1998; Mugula *et al*., 2001; Ali and Mustafa, 2009; Yousif *et al*.,2010; Chelule *et al*., 2010) and Fowoyo *et al*. (2010). The optimal pH from the start to the end of all the fermentations are similar and range from 7.0-6.5 to 4.7-3.7 (Kohajdová and Karovičová, 2007; Nout, 2009). These fermented products have characteristic flavours and some commonly identified volatile compounds include: 2, 3-butanediol, 3-methylbutanoate, 2-phenylethanol, propanoic, lactic and butanoic acids (Nout, 2009, Nyanzi and Jooste, 2012). Microorganisms isolated from these products are similar to those classically found in *ogi* and include: *Lactobacillus delbrukii*, *Lactobacillus bulgaricus*, *Lactococcus lactis* (*mahewu*); *Lactobacillus* spp and yeasts (*kenkey, Munkoyo, Bogbe and injera*); *Lactobacillus bulgaricus* and yeasts (*Kisra*); *Lactobacillus* spp. and moulds (*Banku, Tempeh, and angkak*) ( Blandino *et al*., 2003; Nout, 2009; Osungbaro, 2009; Nyanzi and Jooste, 2012).

Molecular techniques have been employed for profiling of microbial communities in fermented products to complement the cultural methods in species characterization. These include profiling of 16S rRNA PCR denaturing gradient gel electrophoresis (DGGE), (Obodai and Dodd 2006; Oguntoyinbo *et al*., 2011; Van Hijum *et al*., 2013), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (TRFLP) (Ercolini, 2004; Coppola *et al*., 2006; Giraffa and Carminati, 2008; Cocolin *et al*., 2013). The use of DGGE has been able to reduce the limitations of cultural microbiological methods in revealing hidden species which may be important in fermentations (Cocolin *et al*., 2013).

## 2.4 Cereal phytochemicals

### 2.4.1 Phytate

Phytate or phytic acid (myo-inositol hexakisphosphate - $IP_6$) is barely soluble in the complex forms with zinc, iron and calcium (Lopez *et al*., 1983; Grela *et al*., 2011). Research has shown that phytate is not well digested in monogastric animals such as humans, pigs, and poultry resulting in a high loss of phosphorus as waste product. Poor phytate digestion has also been reported to have a negative effect on protein and mineral digestion since it forms complexes with positively charged ions having multiple valences (Maenz, 2001; Kaya *et al*., 2009). Again proteolytic digestion has been reported to be reduced in phytate–protein complexes (O'Dell and De Boland, 1976). Ravindra *et al*. (1995) reported the formation of complexes when the mineral content is higher than phytate under neutral or basic pH. Phytate is therefore regarded as an anti-nutritional factor for humans and animals and can be degraded by the enzyme, phytase, in most grains (intrinsic and activated during digestion) or isolated from microorganisms such as fungi (Ullah and Gibson, 1987; Mullaney *et al*., 2000), bacteria (Kim *et al*., 1998; In *et al*., 2004; Oh and Lee, 2007) and yeast (Quan *et al*., 2002; Kaur *et al*., 2007). Intrinsic phytase concentration and activity varies amongst grain species (Düngelhoef *et al*., 1994; Czech and Grela, 2004; Grela *et al*., 2011) and is reported to be poor in maize (Düngelhoef *et al*., 1994; Selle *et al*., 2000). In animal nutrition microbial phytase is usually added to diets fed to the animals to increase the availability and digestibility of phosphorus and organic nutrients, respectively (Johnston *et al*., 2004; Hanczakowska *et al*., 2009; Grela *et al*., 2011).

Factors affecting the efficiency of phytase include the physical reaction conditions, content and its location in the plant source (Eeckhout and De Paepe, 1994).The efficiency of phytate hydrolysis by phytase has also been reported to be reduced by minerals and this effect could be minimized by reducing the pH of the reaction (Maenz, 2001). Processes such as germination, steeping and fermentation have been reported to increase phytase activity, reduce phytate content and increase free organic phosphate, soluble zinc, calcium and iron (Lu *et al*., 1987; Skoglund *et al.,*

1997; Blandino *et al*., 2003; Nout and Ngoddy, 1997). Fermentation presents the optimal pH needed for phytate degradation.

### 2.4.2 Phenolic compounds

Phenolic compounds (polyphenols) are synthesized in plants (Mattila *et al*., 2005). These include phenolic acids, flavonoids and condensed tannins (Dykes and Rooney, 2006) found in the outer layers of plant tissues that usually protect their inner parts. Phenolic acids are grouped into two major classes: derivatives of benzoic acids and derivatives of cinnamic acids (Manach *et al*., 2004). These can be found as free, soluble conjugate or insoluble bound compounds. Free phenolics are found in pericarp, testa, and aleurone layers while the bound phenolics exist in cell walls (Hahn *et al*., 1983; Troszyńska and Ciska, 2002). Examples of phenolic acids are gallic, p-hydroxybenzoic, vanillic, gentisic, salicylic, and syringic acids (hydroxybenzoic acids) and ferulic, caffeic, p-coumaric, cinnamic and sinapic acids (hydrocinnamic acids). Various authors have reported ferulic and p-coumaric acids as the major phenolic acids found in cereal grains (Hahn *et al*., 1983; Zhou *et al*., 2004; Mattila *et al*., 2005; Holtekjolen *et al*., 2006). The dominant phenolic acid in maize is ferulic acid (Mattila *et al*., 2005), while gallic (Hahn *et al*., 1983; Subba Rao and Muralikrishna, 2002) vanillic acid, p-hydroxybenzoic (Hahn *et al*., 1983; McDonough *et al*., 1986), gentisic (McDonough *et al*., 1986; Waniska *et al*., 1989), salicylic (Waniska *et al*., 1989) and syringic acids (McDonough *et al*., 1986, Waniska *et al*., 1989;) were detected in sorghum. Changes in the level of phenolics in plants occur in the course of development; this includes incorporation into lignin and other components of the cell wall making a significant proportion of these compounds present in the bound state.

Phenolics have biological potential as antioxidants, anti-inflammatory, anti-artherosclerosis, anti-carcinogen and angiogenesis inhibitors due, in part, to their reducing ability (Hodzic *et al*., 2009). Studies have shown that phenolic compounds in plant foods affect their sensory (taste, appearance, odour) and chemical (oxidative stability) properties (Hodzic *et al*., 2009) and have also been associated with inhibition

of mould and microbial growth in grains (Waniska *et al*., 1989). Phenolic acids occur naturally as esters in hemicelluloses and pectin, linked with polysaccharides containing L-arabinofuranose (Topakas *et al*., 2007). Phenolic hydrolysis has been noted to occur in the gastrointestinal tract (Guglielmetti *et al*., 2008; Szwajgier and Dmowska, 2010). The breakdown of phenolics in ingested foods takes place in the large intestine by bacteria which exhibit esterase activity while the free phenolics are well absorbed in the stomach and small intestine (Guglielmetti *et al*., 2008). However, bioavailability of polyphenols varies among the different classes of food (Manach *et al*., 2004) and digestibility would depend on their release from the food matrix in the small intestines (Szwajgier and Dmowska, 2010). Knowledge of the bioavailability of phenolic compounds in *ogi* fermentation would help to establish the potential of *ogi* to confer protective (antioxidant) health benefits.

**2.5 Objectives of study**

2.6.1 To study the diversity and changes in microbial populations of maize and sorghum grains as well as naturally fermented *ogi*.

2.6.2 To monitor the impact of processing and fermentation on phytate and phenolic contents.

2.6.3 To identify organisms associated with key changes in phytate, phenolic and flavour volatiles of *ogi.*

2.6.4 To assess the selected organisms as potential starter cultures for *ogi* production.

# CHAPTER 3

# GENERAL MATERIAL AND METHODS

## 3.1 Sample preparation

### 3.1.1 Flour preparation

Yellow maize (*Zea mays*) and brown sorghum (*Sorghum vulgare*) grains used for this study were purchased in duplicates from northern (maize, M1 and sorghum, S1) and eastern (maize, M2 and sorghum, S2) regions of Nigeria, West Africa. Two hundred grams of each grain was dry milled in a sterilized stainless steel Warring commercial blender for 10 min to obtain a whole meal flour of about 2 mm particle size. Part of the sample was immediately taken for microbial enumeration and isolation while the remaining was aseptically packaged in 30 ml universal air tight plastic containers and frozen at -20$^{o}$C for phenolic and phytate analysis.

### 3.1.2 *Ogi* preparation

*Ogi* was fermented in the laboratory mimicking the traditional fermentation conditions, but was processed aseptically to minimize environmental contamination so as to assess the impact of the original grain microbiota on fermentation. Two hundred grams of grain was soaked in 400 ml sterile tap water in 1000 ml sterile flask, covered with aluminium foil and fermented at 30$^{o}$C for 72 h. Two independent fermentations were set up for each grain. Duplicate samples of steep water (10 ml) were collected at 6, 12, 24, 30, 36, 48, 54, 60, 72 h, one for microbial enumeration and the other for pH, phytate, phenolic acid, organic acid and volatile organic compound analyse.

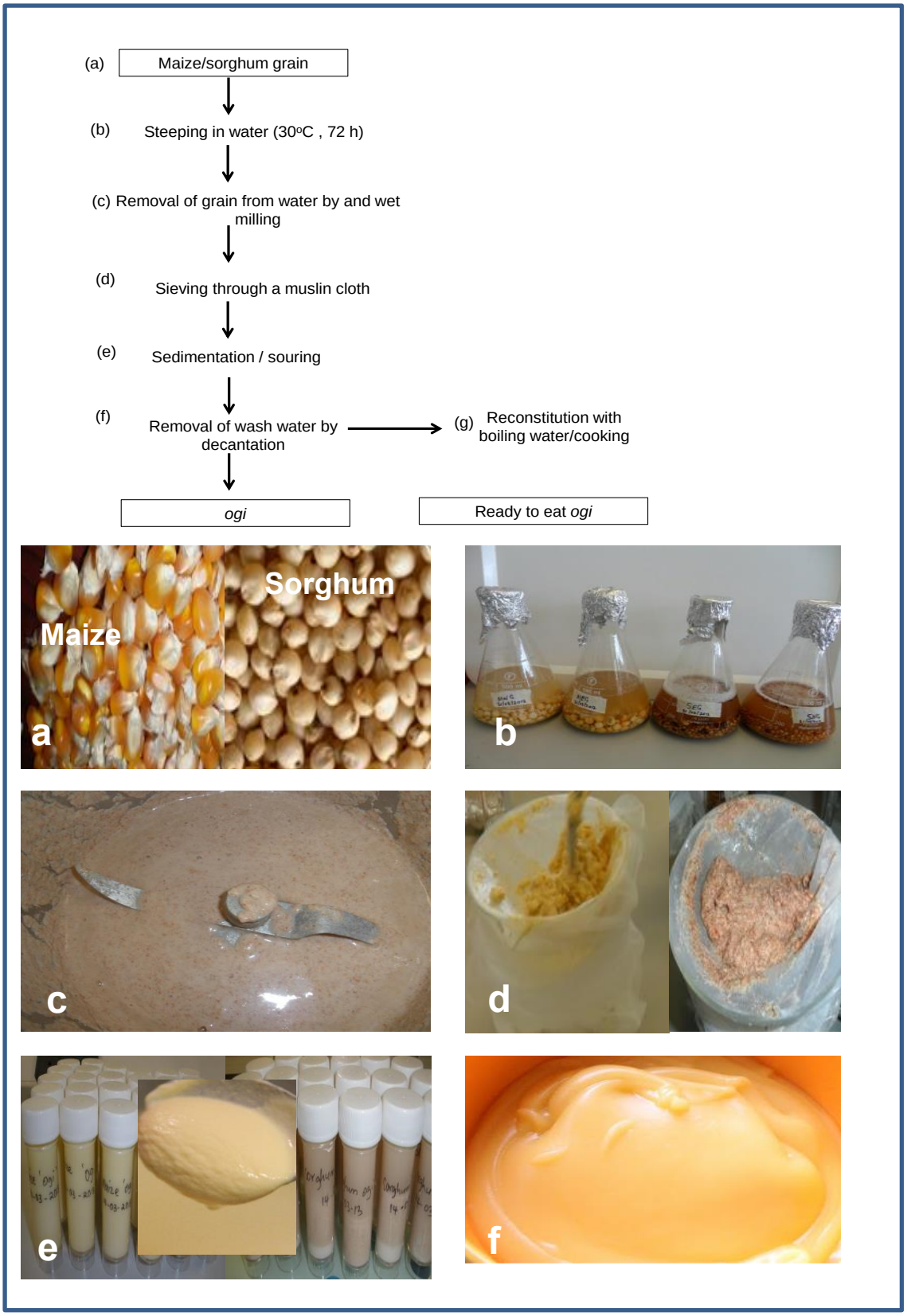



**Figure 3.1:** Processing steps of *ogi*, a: grains, b: fermentation, c: milling, d: wet sieving, e: sedimentation/souring, f: ready to eat *ogi*.

The pH of the fermentation medium was measured using a Hanna pH212 meter (Hanna Instruments, Japan). Fermented grain was collected using a silver sieve and milled in a Warring blender at high speed for 10 min with addition of 180 ml sterile tap water to facilitate milling. The slurry obtained was sieved through a muslin cloth with gradual addition of 500 ml of sterile tap water until the starch (*ogi*) was completely washed off. *Ogi* and water were kept at $4^{o}$C overnight to settle before decanting the wash water. *Ogi* was the stored in aliquots of 25g in 30 ml capacity sterile polystyrene tubes and residue and wash water were retained for phenolic analysis. Samples for microbial enumeration were analysed immediately and others were stored in1 ml aliquots at $-80^{o}$C until analysed.

### 3.2 Isolation and characterization of microorganisms in *ogi*

#### 3.2.1 Microbial enumeration, collection of pure isolates and bulk cells

Ten grams of each sample was mixed with 90 ml of sterile quarter strength Ringer's solution (Oxoid, Basingstoke, Hampshire, England) in a stomacher bag and blended using a Stomacher laboratory blender (Stomacher® 400 circulator, Seward Ltd., UK) for 2 min at 230 rpm (Kramer and Gilbert, 1978). Serial decimal dilutions were made up to $10^{-10}$ using the same diluent. Individual dilutions (0.1ml) were spread plated in duplicate on five different media (M17 agar (Oxoid) for *Lactococcus*, MacConkey agar (Oxoid) for coliforms, MRS (Oxoid) for total lactic acid bacteria, nutrient agar (Acumedia, Lansing, USA) for aerobic plate count, and Rose Bengal chloramphenicol agar (Oxoid) for yeasts and moulds. Culture media were prepared as described in Table 3.1 and incubated at durations and temperatures specific to target organisms (Table 3.2). Plates having 30 – 300 colonies were selected, counted and expressed as means of duplicate determinations of the number of colony forming units in 1g of sample (cfu/g). Colonies on different media plates were randomly selected based on their physical appearance and sub-cultured to obtain pure isolates which were stored in microbank$^{Tm}$ tubes (Pro-Lab diagnostics, UK) at – $80^{o}$C after microscopic examination until needed for further examination and characterization. All colonies on $10^{-1}$ serial dilution plates were suspended in 1.8 ml quarter strength Ringer's solution

in a 2 ml microfuge, harvested with a sterile pipette and stored at – 20 $^{o}$C (Ercolini *et al.*, 2004) for molecular characterization ('bulk cells').

**Table 3.1**: Recipes and preparations of culture media

| Media | Composition | gm/litre | Preparation |
|---|---|---|---|
| **MRS agar (Oxoid)** | Peptone | 10.0 | MRS agar powder (31 g) was weighed |
| | `Lab-Lemco' powder | 8.0 | into in a bottle, suspended in 500 ml of |
| | Yeast extract | 4.0 | RO (Reverse osmosis) water, dissolved |
| | Glucose | 20.0 | and sterilized by autoclaving at 121$^{o}$C for |
| | Sorbitan mono-oleate | 1ml | 15 min. |
| | Dipotassium hydrogen phosphate | 2.0 | |
| | Sodium acetate $3H_2O$ | 5.0 | |
| | Triammonium citrate | 2.0 | |
| | Magnesium sulphate $7H_2O$ | 0.2 | |
| | Manganese sulphate $4H_2O$ | 0.05 | |
| | Agar | 10.0 | |
| | pH 6.2 ± 0.2 @ 25°C | | |
| **M17 agar (Oxoid)** | Tryptone | 5.0 | M17 agar powder (24.13 g) was |
| | Soya peptone | 5.0 | suspended in 475 ml of RO water, |
| | Meat digest | 5.0 | dissolved and autoclaved at 121$^{o}$C for |
| | Yeast extract | 2.5 | 15 min. Sterile lactose solution (10% |
| | Ascorbic acid | 0.5 | w/v) was added to the boiled media |
| | Magnesium sulphate | 0.25 | cooling to 50$^{o}$C. |
| | Di-sodium-glycerophosphate | 19.0 | |
| | Agar | 11.0 | |
| | pH 6.9 ± 0.2 @ 25°C | | |
| **MacConkey agar** | Peptone | 20.0 | The agar powder (26g) was weighed into |
| **(Oxoid)** | Lactose | 10.0 | a bottle, suspended in 500 ml of RO |
| | Bile salts | 5.0 | water and boiled to dissolve. The media |
| | Sodium chloride | 5.0 | was sterilized by autoclaving at 121$^{o}$C |
| | Neutral red | 0.075 | for 15min. |
| | Agar | 12.0 | |
| | pH 7.4 ± 0.2 | | |
| **Rose Bengal** | Mycological peptone | 5.0 | RBCA base powder (16 g) was weighed |
| **Chloramphenicol** | Glucose | 10.0 | into a bottle and suspended in 500 ml of |
| **agar – RBCA** | Dipotassium phosphate | 1.0 | RO water. The suspension was boiled to |
| **(Oxoid)** | Magnesium sulphate | 0.5 | dissolve completely and autoclaved at |
| | Rose-Bengal | 0.05 | 121$^{o}$C for 5 min. The media was allowed |
| | Agar | 15.5 | to cool to 50$^{o}$C before mixing with 50 mg |
| | pH 7.2 ± 0.2 @ 25°C | | of chloramphenicol supplement |
| | | | (SR0078E) reconstituted in 2 ml ethanol. |
| **Nutrient agar** | Enzymatic digest of gelatin | 5.0 | The agar powder (11.5 g) was weighed |
| **(Acumedia)** | Beef extract | 3.0 | into a bottle and suspended into 500 ml |
| | Agar | 15 g | of RO water and boiled for 1 min to |
| | pH 6.8 ± 0.2 @ 25°C | | dissolve before autoclaving at 121$^{o}$C for |
| | | | 15 min. |

**Table 3.2:** Incubation conditions for microbial enumerations on different media

| Agar | Incubation Conditions | Duration |
|---|---|---|
| M17 | 30°C (Aerobic) | 48 h |
| MacConkey | 37 °C (Aerobic) | 24 h |
| MRS | 30°C and 42°C (Anaerobic, by placing 1 sachet of Anaerogen (AN0035, Oxoid) in an air tight 3.5 L plactic jar) | 48 h |
| Nutrient | 37 °C (Aerobic) | 24 h |
| Rose Bengal Chloramphenicol | 25 °C (Aerobic) | 5 days |

#### 3.2.1.1 Assessment of the presence of acetic acid bacteria (AAB) in *ogi*

The presence of acetic acid bacteria in *ogi* was investigated on three different media. Samples were serially diluted in ¼ strength Ringer's solution up to $10^{-10}$ The different dilutions (0.1ml) were spread plated in duplicate on solid GYC medium (1% yeast extract (Oxiod), 3% $CaCO_3$ (Fisher Scientific), 10% glucose (Fisher Scientific), and 4% agar (Oxoid) and aerobically incubated at 30°C for 72 h. Six well isolated colonies showing differences in morphology were selected randomly from the plates for each sample and further subcultured on solid GYC medium as previously described to obtain pure isolates for morphological characterization and biochemical tests. Gram stain, motility, catalase and oxidase tests were carried out as described in sub section 3.2.2. All selected isolates were then subcultured in duplicates on Frateur medium (1% yeast extract, 2% $CaCO_3$, 2% ethanol and 2% agar) and incubated for 72 h at 30°C. Isolates showing clear halo (indication of acid production from $CaCO_3$) were distinguished to be able to differentiate potential Acetobacter and Gluconobacter species. These were further subcultured.in duplicates on Carr medium (3% yeast extract, 2% agar, 0.002% bromocresol green, and 2% ethanol) and incubated at 30°C for 48 h initially to inspect for any colour change to yellow on the media and then further incubated for 24 h to check for any reversion of media colour to green. This procedure was to further distinguish *Acetobacter* from *Gluconobacter* using bromocresol green as *Acetobacter* turns the medium from green to yellow and then back to green with further incubation while *Gluconobacter* only turns it yellow (Sharafi *et al*., 2010).

### 3.2.1.2 Isolation of Phytate hydrolysing fungi (PHF)

Phytate hydrolysing fungi were isolated using the method described by Hosseinkhani *et al*. (2009). Briefly samples (0.2 g) were suspended in 10 ml of 0.9 % saline solution and incubated at 25 $^{o}$C for 2 h with shaking at 150 rpm. One millilitre of each sample (unfermented) suspension was inoculated into 100 ml of phytate specific medium (PSM) (1.5% glucose, 0.5% $(NH4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.H_2O$ and 0.5% filter sterilized phytic acid sodium salt hydrate (Sigma Aldrich), added to the medium after autoclaving).The inoculated medium was incubated at 30 $^{o}$C while shaking at 150 rpm for 10 days and then re-inoculated in fresh 100 ml PSM liquid medium (PSML) and incubated at 30 $^{o}$C for 7 days at 165 rpm. The fungal culture was inoculated on PSM solid medium (PSMS) (containing the entire classic PSM ingredients with addition of 0.5% calcium phytate and 1.5% (w/v) agar bacteriological (Oxoid)) and incubated for 5 days at 30 $^{o}$C. Fungal colonies showing zones of clearing were isolated and further sub-cultured on fresh PSM plates. Fungal colonies were counterstained with 2% (w/v) cobalt chloride (Fisher Scientific) and equal volumes of 6.25 % (w/v) ammonium molybdate (Sigma) and 0.42% (w/v) ammonium vanadate (Sigma) as described by (Bae *et al*., 1999).

### 3.2.1.3 Microscopic identification of moulds

Moulds were identified morphologically with appearance, hyphae and spore characteristics by microscopic examination. This was performed following the lactophenol cotton blue technique Mould colonies were isolated from Rose Bengal chloramphenicol agar (Oxoid) as previously described in § 3.2.1. One drop of mounting medium lactophenol cotton blue, (Fluka Analytical) was placed on a microscopic slide. A small portion of the colony close to the margin was collected by pressing a mould collection cellophane tape lightly on the colony and sticking the tape on the mounting medium and viewed at 1000x magnification with oil immersion objective

#### 3.2.1.4 Screening of moulds in *ogi* for mycotoxigenic potentials

Moulds detected in *ogi* were screened for mycotoxin production potential by PCR. Each mould (*Aspergillus oryza, Penicillium biourgeianum, Fusarium oxysporum, Cladosporum cladosporioides*, *Cladosporum macrocarpum* and *Aspergillus tubingensis*) was subcultured on potato dextrose agar (PDA, Oxoid) at 25$^{o}$C for 7 days to obtain pure colonies. Mycelia from pure isolate was inoculated in 100 ml flask containing 20 ml of Sabouraud liquid medium and incubated at 25$^{o}$C for 6 days under static condition. Mycelia was harvested by filtration through sterile Whatman paper no 1 into a sterile conical flask (Patiño *et al*., 2005). Fungal DNA was extracted from identified moulds as described in §3.2.3.3. The DNA was tested for PCR suitability by amplifying the D1 domain of 26S fungal gene with NL1 and LS2 primers (Table 3.3).

To monitor the major mycotoxin biosynthetic pathway genes in these moulds, polyketide synthase, pks1, pksA, pks4 and pks13 for zearalenone, norsolonic acid-1 (nor-1) for aflatoxin, and ochratoxin (OCRA 1: OCRA 2, and CAR 1: CAR 2) gene primers (Table 3.4) were used for PCR amplification of the target regions of the structural genes following the conditions outlined on Table 3.5.

### 3.2.2 Phenotypic, morphological and biochemical identification of bacterial isolates

#### 3.2.2.1 Gram Stain

Colonies observed with different morphologies from each plate were isolated and further sub-cultured twice on BHI and incubated for 18-24h under appropriate conditions for each isolate (Table 3.2). A loop-full of sterile maximum recovery diluent (MRD) was dropped on a clean glass slide and a small section of a colony picked with a sterile loop was mixed with the liquid on the slide to make a thin smear. The slide was allowed to dry before heat-fixing by passing it briefly (5 times) through a Bunsen flame. The slide was then dipped into crystal violet (PL 8000 Pro Lab, UK) for 1 min and gently rinsed off with water. It was treated with Lugol's iodine solution (PL 8010 Pro Lab, UK) for 30 s, decolourized with absolute ethanol (Fisher Scientific Ltd.) for 1

min, and finally counterstained with carbol fuchsin (PL 8004 Pro Lab, UK) for 30 s. The slide was properly rinsed in water after each treatment and removing excess water to minimize dilution of subsequent stain solutions. The stained slide was allowed to air dry at room temperature and examined at 1000x total magnification on a bright-field microscope with the oil immersion objective. Gram positive bacteria stained purple while Gram negative bacteria stained pink (Bartholomew and Mittwer, 1952). *Enterococcus faecalis* (NCTC 775) and *Pseudomonas flourescens* (Sm06 type strain) collected from food microbiology laboratory of University of Nottingham were used as Gram positive and negative controls, respectively.

#### 3.2.2.2 Catalase activity

Pure colonies (18-24 h old) of isolated organisms on BHI agar (Oxoid) were used for this test. A small amount of the organism was placed on the base of a petri dish using a sterile inoculating loop. A drop of 3% (v/v) hydrogen peroxide (Fisher scientific, UK) was then placed onto it using a Pasteur pipette. The petri dish was covered and observed against a dark background (to enhance readability) for the formation of bubbles (immediate effervescence) indicating a positive reaction (Karen, 2010). *Pseudomonas flourescens* (Sm06 type strain) and *Enterococcus faecalis* (NCTC 775) obtained from University of Nottingham food microbiology laboratory culture collection (UNFMCC) were used as catalase positive and negative controls, respectively.

#### 3.2.2.3 Oxidase test

Cytochrome oxidase test which involves the oxidation of cytochrome c and reduction of oxygen to form water, catalysed by the enzyme cytochrome oxidase during bacterial respiration was investigated using oxidase detection strip (MB0266 Oxoid) according to manufacturer's procedure. A well isolated pure colony of 18-24 h incubated on BHI agar at 30-37$^{o}$C was touched with an oxidase detection strip and observed for 5 s for a deep blue /violet colour indicating a positive reaction.

*Pseudomonas flourescens* (Sm06 type strain) and *E.coli* (1075 strain) collected from UNFMCC were used as positive and negative controls, respectively.

#### 3.2.2.4 Motility test

Motility test was carried out by a wet mount technique. Bacterial isolates were cultured in BHI broth for 18-24 h at 30$^{o}$C. Broth culture (20 µl) was placed in the middle of a glass slide and carefully covered with a cover slip avoiding air bubbles. The wet mount preparation was then observed under a microscope starting with 40x and the 100x magnification to identify the motile strains (Shields and Cathcart, 2011).

#### 3.2.2.5 Coagulase test

The Staphytect plus a latex slide agglutination kit (DR0850 Oxoid) which differentiates between coagulase positive and coagulase negative *staphylococci* by detecting bound coagulase on the bacterial cell wall was used to perform the coagulase test on all presumptively identified *Staphylococci* strains (Gram positive cocci, catalase positive), following the manufacturer's procedure. The organisms were freshly cultured for 18-24 h 37$^{o}$C on sheep blood agar (CM0854; SR0051, Oxoid). The latex reagents were brought to room temperature and mixed vigorously by shaking. One drop of the test latex (DR0851 Oxoid) and was placed onto one circle on the reaction card while a drop of the control latex (DR0852 Oxoid) was placed on another circle. An equivalent of 5 average colonies was collected from the culture plate using a sterile loop and smeared on the control latex circle. A separate sterile loop was again used to pick up and smear the colonies on the test latex. The reaction card was then rocked for 20 s and observed for agglutination (positive) reactions under normal light condition (Oxoid, 2005). *Staphylococcus aureus* (NCTC 1803) and *Staphylococcus epidermidis* (NCTC 12100) collected from UNFMCC were used as positive and negative controls, respectively.

#### 3.2.2.6 Lysostaphin test

Lysostaphin test was performed using the MAST ID ™ Lysostaphin discs following the manufacturer's procedure (Mast Laboratories Ltd., Bootle, England) to differentiate between *Staphylococcus* and *Micrococcus* presumptive isolates through enzyme (endopeptidase) biochemical activities (lysis). Pure colonies of young (18-24 h) test organism incubated at 30 - 37$^{o}$C on BHI plate were picked up using a sterile loop and dispensed in 2 ml phosphate–buffered saline (PBS) pH7.4 in a test tube to obtain a bacterial suspension equivalent to McFarland standard 1. The suspension (1 ml) was transferred into a second test tube and a lysostaphin disc was added to only the first test tube and mixed vigorously while the second remained as a control. Both suspensions were incubated at 37$^{o}$C for 2 h in a water bath. The turbidity of bacterial suspension in both tubes was compared to ascertain a positive result. Complete clearing of the suspension with lysostaphin indicated a positive result while a negative result showed identical turbidity of both bacterial suspensions. *Staphylococcus aureus* (NCTC 1803) obtained from UNFMCC was used as a positive control.

#### 3.2.2.7 Streptococcal grouping test (Lancefield)

Lancefield test was performed to classify pathogenic Streptococci using streptococcal grouping kit (DR0585, Oxoid) and following the manufacturer's procedure. Isolates were cultured overnight in sheep blood agar (Oxoid). All reagents were allowed to equilibrate to room temperature. Extraction enzyme solution (0.4 ml; DR0594, Oxoid) was dispensed in an eppendorf tube and 2-5 distinct colonies were emulsified in the enzyme. The suspension was then incubated at 37$^{o}$C for 10 min with mixing within the first 5 min. One drop of the test suspension was placed on each of the six circles on the test card and mixed with latex grouping reagent (A, B, C, D, F, G; DR0586-91, Oxoid). The card was rocked for 1 min before reading the result. Polyvalent positive control (DR0592) was included in the test.

#### 3.2.2.8 Indole test

Four millilitres of Tryptone water (Oxoid) was dispensed into bijou bottles and autoclaved at 121$^{o}$C for 15 min. The broth was inoculated with small amount of pure culture was incubated at 37$^{o}$C for 24 h. Kovács reagent (1-2 drops) containing isoamyl alcohol (150ml), *p*-dimethylaminobezaldehyde (10 g) and concentrated HCl (50 ml) was added to each tube. A pink to red colour indicates a positive result and yellow is negative. *E.coli* (1075 strain collected from UNFMCC) was used as positive control (MacWilliams, 2009).

#### 3.2.2.9 Acid tolerance

Isolates were grown overnight in MRS broth at 37$^{o}$C. One millilitre of culture was centrifuged at 7500 xg for 15 min at 4$^{o}$C. Harvested cells were washed in sterile phosphate buffered saline (PBS, pH 7.0), resuspended in 1 ml PBS and adjusted to OD 1.0 at 600 nm. Cell suspension (1%) was inoculated in duplicate in MRS broth adjusted to pH 2.0 and 3.0 with concentrated HCl (Fisher Scientific), and incubated at 37 $^{o}$C for 2, 3 and 24 h. A control was set up for each isolate without pH adjustment. Growth was monitored by absorbance measurement at 600 nm (Jin *et al*., 1998).

#### 3.2.2.10 Glucose fermentation

Ten millilitres of base broth (1% Proteose peptone (BD Diagnostics, USA), 0.5% NaCl, 0.25% bromothymol blue, 1 % sugars, 500 ml RO $H_2O$) was dispensed into test tubes containing inverted Durham tubes (to detect gas production) and autoclaved at 116$^{o}$C for 15 min. The cooled broth was inoculated with one drop of overnight culture (incubated at 30$^{o}$C in BHI broth) and incubated at 30$^{o}$C for 24 h. Change of broth colour to yellow and presence of bubbles in the Durham tubes indicates acid and gas production, respectively.(Karen, 2012).

#### 3.2.2.11 Amylolytic test

Starch agar (pH 7.5) was prepared with 1.5 g beef extract (LAB Lemco powder, Oxoid), 5.0 g soluble starch (Fisher Scientific) and 6.0 g agar bacteriological (Oxoid) in

500 ml of RO water, autoclaved at 121$^{o}$C for 15 min. Agar was allowed to cool down to approx. 50$^{o}$C before dispensed into petri dishes to solidify. Single colony of each test isolate was inoculated on the agar plates and incubated for 48 h at 37$^{o}$C. Culture plates were flooded with Lugol's iodine (1:100 dilutions) and results read immediately as colour may fade. Clearing around the colony indicating production of amylase (an exo-enzyme which breaks starch into di- and monosaccharides (simple sugars) which are then be catabolized by the cells) is a positive result while a blue black colour indicating the presence of starch in the medium is negative. *Bacillus subtilis* (UNFMCC strain) and *E.coli* (1075 strain collected from UNFMCC) were used as positive and negative controls, respectively (Lal and Cheeptham, 2012).

**3.2.2.12 API 20E**

Bacterial strains identified as Gram negative and catalase positive rods (Presumptive *Enterobacteriaceae*) were further identified using Analytical Profile Index identification system, API 20E (BioMérieux, France) following the manufacturer's instruction. The incubation box was labelled with the appropriate strain code and prepared by distributing 5 ml of distilled water into the wells of the tray to create a humid atmosphere. The API 20E strip was removed from its pack and placed in the incubation box. The inoculum was then prepared in normal saline (0.85% NaCl medium) by collecting a single well-isolated colony from young culture (18-24 h incubation at 30-37$^{o}$C) on BHI plate using a sterile loop and emulsifying the medium to obtain a homogenous bacterial suspension. A sterile Pasteur pipette was used to fill both the tubes and cupules of the strip with the bacterial suspension for tests CIT, VP and GEL while only the tubes were filled for all other tests. Anaerobic condition was further created in tests ADH, LDC, ODC, $H_2S$ and URE by filling the cupule with mineral oil. The incubation box was covered with its lid and incubated at 37$^{o}$C for 18-24h. The strip was read after the incubation period by referring to API 20 E reading table (BioMérieux, France). Spontaneous reactions were recorded for a strip in which three or more tests were positive with GLU test having positive or negative reaction.

Tests requiring additional reagents were revealed and 1 drop each of TDA, JAMES and VP reagents were added to TDA, IND and VP tests respectively. The reaction was observed after 10 min and reddish brown, pink or red and pink colours were recorded as positive reactions for TDA, VP and IND tests, respectively. Oxidase test was performed as described in §3.2.2.3 and recorded appropriately on the result sheet. The result was interpreted with the API identification software /database (Version 4.0) inserting the 7-digit numerical profile determined from the record sheet (BioMérieux, France).

#### 3.2.2.13 API Staph

API Staph identification system (BioMérieux, France) was used for further identification of the Gram positive and catalase positive cocci strains. The organism was sub-cultured on sheep blood agar plate (CM0854; SR0051, Oxoid) and incubated at 37$^{o}$C for 18-24h to obtain pure colonies. API staph incubation box tray wells were filled with 5 ml of distilled water to increase humidity and the strip removed (from the pack) was placed in the incubation box. Pure colonies from the agar plate were suspended in API staph medium to achieve a homogenous suspension of turbidity equivalent to McFarland standard 0.5. The microtubes were carefully under-filled (to form a convex meniscus) with the inoculated API Staph medium using a sterile Pasteur pipette by tilting the strip forward and placing the tip of the pipette against the side of the cupules to prevent air bubbles. Tests ADH and URE were further slightly filled with mineral oil to ensure anaerobic condition. The box was then covered with its lid and incubated at 37$^{o}$C for 18-24h. After incubation, 1 drop each of VP 1 and VP 2, NIT 1 and NIT 2, and ZYM A and ZYM B reagents were added to VP, NIT, and PAL tests respectively. The reactions were observed for 10 min and recorded on the result sheet. Lysostaphin test was performed as described in §3.2.2.6 and recorded. Organisms were identified using API identification software §3.2.2.7 (BioMérieux, France).

#### 3.2.2.14 API 20 strep

Further classification of Gram positive, catalase negative, oxidase negative cocci identified as members of *Streptococcaceae* was performed using the API 20 strep kit (BioMérieux, France) following the manufacturer's procedure. Pure culture suspension was prepared in 0.3 ml sterile water and used to flood Columbia sheep agar plate. The plate was incubated for 18-24 h at 37$^{o}$C in anaerobic condition generated by placing 2.5L AnaeroGen™ sachet (AG0025A, Oxoid) in a 2.5 litre plastic box. API 20 strep incubation box tray honey combed wells were filled with 5 ml of distilled water to increase humidity. The strip removed was placed in the labelled incubation tray. Pure colonies were harvested from the agar plate and suspended in API suspension medium to achieve a dense homogenous suspension of turbidity equivalent to McFarland standard 4. The suspension was carefully distributed into the 1$^{st}$ half of the strip (VP-ADH), avoiding air bubbles by tilting the strip forward and placing the tip of the pipette against the side of the cupule and using a sterile Pasteur pipette. Tests VP - LAP were filled with 100 µl of suspension. The rest of the suspension was transferred into API GP medium and mixed thoroughly. This suspension was distributed into the tubes RIB – GLYG. Cupules of ADH – GLYG were filled with mineral oil and incubated in aerobic condition at 37$^{o}$C. First reading was taken after 4 h before further incubation for 24 h. One drop of VP1 and VP2 to VP test, 2 drops of NIN to HIP test and 1 drop of ZYM A and ZYM B, each into PYRA, α GAL, β GUR, βGAL, PAL, and LAP tests. Results were recorded after 10 min.

#### 3.2.2.15 API 50 CH

Identification of Gram positive, catalase negative, anaerobic bacteria (*Lactobacillus*) was performed by carbohydrate metabolism using API 50CH. Incubation box tray wells were filled with 10 ml of distilled water to increase humidity and the strip removed (from the pack) was placed in the box. Pure colonies cultured anaerobically for 24 h at 30$^{o}$C were harvested from MRS agar using a swab to make a heavy suspension in API suspension medium (2ml). Then another suspension was prepared

to turbidity equivalent to 2 McFarland by adding a number of drops from the previous suspension. This suspension (twice the number of drop used in preparation) was then transferred to API 50 CHL medium. Bacterial suspension was inoculated on the prepared strips, filling the tubes only and covering with mineral oil to create anaerobic condition. The strips were incubated at 30$^{o}$C for 48 h. Yellow colour indicates a positive result for all the tube except the esculin test which is black. Biochemical profile obtained was identified using the API identification software §3.2.2.7 (BioMérieux, France)

### 3.2.3 Molecular characterization

#### 3.2.3.1 Isolation of bacterial DNA from *ogi*

Bacterial genomic DNA was extracted directly from the samples and bulk cells harvested from $10^{-1}$ serial dilution plates using PowerFood microbial DNA isolation kit - 21000-50 (MO BIO Laboratories, USA) following the manufacturer's procedure. The PowerFood microbial DNA kit was designed to produce high quality DNA from food cultures through optimized lysis and removal of PCR inhibitors such polysaccharides and lipids and was found to be more efficient in direct microbial DNA isolation from maize and sorghum grains and *ogi*. Harvested 'bulk cells' (§ 3.2) 1.8 ml, was centrifuged at 13000 xg for 1 min at room temperature and the supernatant was decanted. It was further centrifuged at same conditions as above and the remaining supernatant was removed with a pipette tip. The cell pellet was re-suspended in 450µl of previously warmed (at 55$^{o}$C for 5-10 min) solution PF 1 (MO BIO 21000-50-1) and transferred into a micro-bead tube (MO BIO, 21000-50-BT). The cell suspension in the micro-bead tube was heated at 65$^{o}$C for 10 min in a water bath to increase yield. This was vortexed to mix at maximum speed for 10 min and centrifuged at 13000 xg for 1 min at room temperature. The supernatant was then transferred into a clean 2ml collection tube (MO BIO, 21000-50-T). Hundred microliters of solution PF 2 (MO BIO, 21000-50-2) was added to the supernatant and briefly mixed by vortex. This was incubated at 4$^{o}$C for 5 min and centrifuged at 13000 xg for 1 min at room temperature.

Two millilitres of the supernatant was then mixed with 900 µl of warm (55$^{o}$C) solution PF 3 (MO BIO, 21000-50-3) in a clean 2 ml collection tube. The mixture (650 µl) was loaded onto a spin filter (MO BIO, 21000-50-SF) and centrifuged at 13000 x g for 1 min at room temperature. The flow through was discarded and the step was repeated until all the supernatant was loaded. The spin filter basket was then transferred into a clean 2 ml collection tube and 650 µl of solution PF 4 (MO BIO, 21000-50-4) was added and centrifuged at 13000 x g for 1 min at room temperature. The flow through was again discarded and the cells were then washed twice with 650 µl of solution PF 5 (MO BIO, 21000-50-5), centrifuging each time at 13000 xg for 1 min and discarding flow through. The tube was further centrifuged at 13000 x g for 2 min to remove residual wash solution and the spin filter was placed into another clean 2 ml collection tube. Hundred microliters of solution PF 6 (MO BIO, 21000-50-6) was and centrifuged at 13000 x g for 1 min to elute the DNA (MO BIO Laboratories, 2010).

A rapid DNA extraction method was used for pure bacterial isolates as described by (Schmalenberger *et al*., 2001). A single well isolated colony was suspended in 50 µl of lysis buffer (0.05M NaOH, 0.25% sodium dodecyl sulfate (Fisher Scientific) in a 1.5 ml sterile tube (Eppendorf, Hamburg Germany) using a sterile pipette tip. The cell suspension was heated (lysed) for 15 min at 95$^{o}$C and diluted with 450 µl of sterile milli-Q water. The DNA solution 10-20 ng/µl (2 µl) was used as template for PCR.

#### 3.2.3.2 Genomic DNA extraction by GES method

GES reagent containing 5M Guanidine thiocyanate (Sigma Aldrich), 0.1 M EDTA (Fisher Scientific) and 0.5% sarkosyl (Sigma Aldrich) was prepared by mixing Guanidine thiocyanate (60g), 20 ml of 0.5 M EDTA at pH 8.0, and deionized, heated at 65$^{o}$C to dissolve and cooled before adding 5 ml of 10% (w/v) sarkosyl. One millilitre of bacterial culture grown overnight in BHI broth at 30$^{o}$C was transferred into a sterile eppendorf tube and centrifuged at 13000 xg for 1 min. The supernatant was removed and the pellet was suspended in 1ml ice cold lysis buffer (25mM Tris HCl pH 8.0, 10mM EDTA, and 50mM sucrose) and centrifuged at 13000 x g for 1 min. The

supernatant was discarded and the pellet was further resuspended in 100 µl of lysis buffer supplemented with 50 mg/ml lysozyme (Sigma Aldrich) only for Gram positive bacteria, and incubated at 37$^{o}$C for 30 min. The cell suspension was mixed with 0.5 ml of GES reagent and allowed to incubate at room temperature for 10 min. The lysate was cooled on ice for 2 min before adding 0.25 ml of ice cold 7.5 M ammonium acetate (Fisher Scientific). The lysate was again incubated on ice for 10 min and then mixed with 0.5 ml of chloroform and isoamyl alcohol mixture (24:1). The cell mixture was then centrifuged at 13,000 x g for 10 min and 800 µl of the aqueous phase was transferred into a clean eppendorf tube. An ice cold isopropanol 0.54 volume was added and mixed by inverting the tube for 1 min. The DNA solution was centrifuged at 6500 x g for 20 s. The supernatant was discarded and DNA pellet was wash in 70% ethanol for five times (Pitcher *et al*., 1989). DNA was allowed to air dry, resuspended in 50 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and stored at -20 $^{o}$C until needed.

#### 3.2.3.3 Isolation of fungal DNA

A rapid mini-preparation procedure of fungal DNA for PCR as described by Liu *et al*., 2000 was employed. A representative small lump of mycelia on Rose Bengal chloramphenicol (Table 3.2) culture plate was transferred into a sterile 1.5 ml Eppendorf tube containing 500 µl of lysis buffer (400 mM Tris-HCl (pH 8.0), 60 mM EDTA (pH 8.0), 150 mM NaCl, and 1% sodium dodecyl sulfate) using a sterile toothpick. The tube was left at room temperature for 10 min after which 150 µl of potassium acetate solution pH 4.8 (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml distilled water) was added to the suspension. The tube was vortexed briefly, centrifuged at 10,000 x g and the supernatant was transferred into a clean 1.5 ml Eppendorf tube. Equal volume of isopropyl alcohol was added to the tube and mixed by inversion. The tube was then centrifuged at 10,000 x g for 2 min and the supernatant discarded. The resulting DNA pellet was washed by adding 300 µl of 70% ethanol, centrifuging at 10,000 x g for 1 minute and discarding the supernatant. The

DNA pellet was air dried, resuspended in 50 µl of 1 x Tris-EDTA and stored at – 20 $^{o}$C until used. One microlitre of the DNA solution was used for PCR.

#### 3.2.3.4 Quantification of DNA concentration

The concentration and purity of DNA was verified using the nano drop system (Nano Drop ND-1000 spectrophotometer, Labtech. UK). Briefly, the lower and upper pedestals of the measurement panel were properly cleaned with lens tissue. Nuclease free water (1 µl) was loaded onto the lower pedestal, covered with the sampling arm and the spectra initialized through the operating software. Blank sample (DNA eluent) was measured first before each test sample and both the upper and lower pedestals were cleaned after each measurement to reduce sample carryover. The DNA samples were stored at -20$^{o}$C until analysed.

#### 3.2.3.5 Polymerase chain reaction (PCR)

##### 3.2.3.5.1 Amplification of 16S and 26S rRNA gene fragments

The V3, and V6-V8 variable regions of 16S rRNA bacterial genes and the D1 domain of 26S rRNA fungal genes were PCR amplified and analysed by gel electrophoresis § 3.2.3.6. All primers used in this study are listed in Table 3.3 and 3.4. Amplification of bacterial DNA was performed using two different universal primer pairs, the V3f and V3r , and U968f - GC and L1401r targeting the V3 and V6 – V8 regions of the 16S rRNA genes, respectively. The 16S A 39 bp GC clamp sequence was added to the 5' end of V6-V8 forward primer and amplicons were analysed by denaturing gradient gel electrophoresis (DGGE).

PCR for V3 primers was performed on pure isolates with 2X Dream Taq™ DNA polymerase master mixes (Fermentas Life Scientific, UK) in 0.2 ml sterile PCR tubes. The PCR mixture (50 µl) contained 25 µl of 2X Dream Taq™ Green PCR master mix (green buffer, 0.4mM each of dATP, dCTP, dGTP and dTTP and 4mM $MgCl_2$,

Fermentas Life Scientific, UK), 0.2 µM (1µl of 10pmol) each of V3f and V3r primers (Eurofins MWG, Ebersberg, Germany), 2 µl of DNA lysate and nuclease free water to make up the final volume. Amplification of DNA over 35 cycles was performed under the following conditions: initial denaturation (95$^{o}$C; 5min), 33 cycles of denaturation (94$^{o}$C; 1 min), annealing (66$^{o}$C; 1 min), extension (72$^{o}$C; 3 min) and final extension (72$^{o}$C; 10 min).

DNA extracted from bulk cells and directly from the *ogi* samples was amplified with V6- V8 primer. PCR was performed with Taq DNA polymerase kit with thermopol$^{Tm}$ buffer (BioLabs, UK). The PCR mixture contained 2.5 µl of 10X thermopol reaction buffer (20 mM Tris – HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton$^{®}$ X-100, pH 8.8), 0.5 µl of 10mM dNTPs (Sigma Aldrich), 0.5 µl each of 10 µM V6 – V8 forward (with GC clamp) and reverse primers (Eurofins MWG, Ebersberg, Germany), 1.25 units (0.125 µl ) of Taq DNA polymerase (BioLabs, Uk), 20 ng DNA template and nuclease free water (Fermentas Life Scientific, UK) to make up to 50 µl volume. The initial denaturation temperature was 95$^{o}$C for 30 s, 30 cycles of denaturation at 95$^{o}$C for 30 s, annealing at 55$^{o}$C for 30 s, extension at 68$^{o}$C for 1 minute and final extension at 68$^{o}$C for 5 min.

Amplification of the conserved regions of 16S rRNA gene sequence was performed using 16Sd and 16Sr primers following the conditions; Initial denaturation at 94$^{o}$C for 5 min, and then 30 cycles for 1 min, annealing at 60$^{o}$C for 45 s, extension at 72$^{o}$C for 2 min and then final extension for 10 min.

Amplification of 26S rRNA fungal gene was performed using primers NL1f with and without GC clamp and LS2r with 2X Dream Taq green PCR master mix in a reaction mixture as described above for V3 primers. The PCR conditions were as follows: initial denaturation (95$^{o}$C, 5 min), 30 cycles of denaturation (95$^{o}$C, 60 s), annealing (52$^{o}$C, 45 s), extension (72$^{o}$C, 60 s), and final extension (72$^{o}$C, 7 min) PCR amplifications were performed in C1000 thermal cycler (Bio Rad, Laboratories, UK).

#### 3.2.3.5.2 Amplification of mycotoxin coding gene

The presence of structural genes for aflatoxin (polyketide synthase acetate (PKSA) and norsolonic acid-1 (nor-1)) and ochratoxin in *Aspergillus* and *Penicillum* isolates as well as polyketide synthase of zearalenone (PKS13 and PKS4) biosynthetic pathways in *Fusarium* were evaluated by polymerase chain reaction with biosynthetic gene primers (Table 3.4). Suitability for PCR amplification of all mould genomic DNA was confirmed with 26S rRNA fungal genes primers.

#### 3.2.3.5.3 Enterobacterial repetitive intergenic consensus (ERIC) PCR

Bacterial DNA was extracted by heat lysis method (Englen and Kelley, 2000). Pure single colony was emulsified in 100 µl of sterile nuclease free water (Fisher Scientific) dispensed In 1.5 ml eppendorf tube. The cell suspension was boiled in an eppendorf thermomixer® (Eppendorf, UK) at 95$^{o}$C for 10 min. This was then cooled on ice for 2 min and centrifuged at 15000 xg for 10 min. The supernatant (1-2 µl) was then used as template for PCR using ERIC 1 and ERIC 2 primers (Table 3.3). Bands were resolved on 2% agarose gel in 1X TAE buffer §3.2.3.6.1. Molecular marker, 1kb plus invitrogen™ ranging from 100-12,000 bp was used. The quality of DNA was verified by nano drop system (Nano Drop ND-1000 spectrophotometer, Labtech. UK). Band images were visualised on gel doc imager and processed using FP Quest software §3.2.3.7 (Bio-Rad).

**Table 3.3:** Universal primers used in this study

| Primer | Sequence | Target region | †Position | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| U968-f-GC (forward)<br>U968-f (forward)<br>L1401-r (reverse) | 5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGC ACGGGGGAACGCGAAGAACCTTC3'<br>5'AACGCGAAGAACCTTAC3'<br>5'CGGTGTGTACAAGACCC3' | V6-V8 of 16S rRNA bacterial gene | 968-1401 | 489 | (Nübel *et al*., 1996) |
| F357 (forward)<br>R518 (reverse) | 5'CCTACGGGAGGCAGCAG'<br>5'ATTACCGCGGCTGCTGG3' | V3 of 16S rRNA bacterial gene | 341-534 | 217 | (Muyzer *et al*., 1993) |
| 16Sd (forward)<br>16Sr (reverse) | 5'AGAGTTTGATCCTGGCTCAG3'<br>5'ACGGCTACCTTGTTACGACCT3' | 16S rRNA bacterial gene | Almost complete | 1500 | (Sharafi *et al*., 2010) |
| ERIC 1<br>ERIC 2 | 5'ATGTAAGCTCCTTGGGGATTCAC3'<br>5'AAGTAAGTGACTGGGGTGAGCG3' | *MetE-metR* region *of E. coli* gene sequence | - | - | (Versalovic *et al*., 1991) |
| NL1-f-GC (forward)<br>NL1-f (forward)<br>LS2-r (reverse) | 5'GCGGGCCGCGCGACCGCCGGGACGCGCGAGCC GGCGGCGGGCCATATCAATAAGCGGAGGAAAAG3'<br>5'GCCATATCAATAAGCGGAAAAG3'<br>5'ATTCCCAAACAACTCGACTC3' | D1/D2 domain of 26S rRNA of fungal gene | 266-285 | 265 | (Cocolin *et al*., 2000) |

† Position based on *E. coli* 16SrRNA gene sequence

**Table 3.4:** Biosynthetic fungal gene primers used in this study

| Primer | Sequence | Target region | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| OCRA1-(forward)<br>OCRA2-(reverse) | 5’ CTTCCTTAGGGGTGGCACAGC 3’<br>5’ GTTGCTTTTCAGCGTCGGCC 3’ | - | 400 | (Patiño *et al*., 2005) |
| CAR1-(forward)<br>CAR2-(reverse) | 5’GCATCTCTGCCCTCGG 3’<br>5’ GGTTGGAGTTGTCGGCAG 3’ | - | | (Patiño *et al*., 2005) |
| PksA -f (forward)<br>PksA -r (reverse) | 5’ TTCTCATGGGTTCCTTGGC 3’<br>5’ CCATTGTGGGCCGGTAAACA 3’ | - | 395 | (Leema *et al*., 2011) |
| Pks4-f (forward)<br>Pks4-r(reverse) | 5’ AGACGGCGCAACAAGGGCTG 3’<br>5’ GCAGTTGCCCGTGTCGGACA 3’ | Translation elongation factor 1α (tef-1α) | 355 | (Kroken *et al*., 2003) |
| Pks13-1 (forward)<br>Pks13 -2(reverse) | 5’ CCCAGCCAAGCCCAGTACGC 3’<br>5’ ACAGCGGCTGACCTGGGTCA 3’ | - | 532 | (Kim *et al*., 2005) |
| Nor1-f (forward)<br>Nor1-r (reverse) | 5’GGGTAGACCGCCTGAGGAG 3’<br>CTTCAGCGACGGTTAGTGCC 3’ | - | 168 | (Leema *et al*., 2011) |

**Table 3.5**: PCR conditions for biosynthetic fungal gene primers used in this study

| Primers | Denaturation | Annealing | Extension | References |
|---|---|---|---|---|
| OCRA1-(forward)<br>OCRA2-(reverse) | 1 cycle; 270s at 95$^{o}$C<br>30 cycles 30s at 95$^{o}$C | 30s at 63$^{o}$C | 1 min at 72$^{o}$C<br>1 cycle, 3 min at 72$^{o}$C | (Patiño *et al*., 2005) |
| CAR1-(forward)<br>CAR2-(reverse) | 1 cycle; 270s at 95$^{o}$C<br>25 cycles 30s at 95$^{o}$C | 25s at 59$^{o}$C | 40s at 72$^{o}$C<br>1 cycle, 5 min at 72$^{o}$C | (Patiño *et al*., 2005) |
| PksA-f (forward)<br>PksA-r (reverse) | 1 cycle; 5 min at 95$^{o}$C<br>30 cycles 1 min at 95$^{o}$C | 1 min at 59$^{o}$C | 1 min at 72$^{o}$C<br>1 cycle, 5 min at 72$^{o}$C | (Leema *et al*., 2011) |
| Pks4-f (forward)<br>Pks4-r(reverse) | 1 cycle; 5 min at 95$^{o}$C<br>35 cycles 1 min at 94$^{o}$C | 30s at 63$^{o}$C | 1 min at 72$^{o}$C<br>1 cycle, 10 min at 72$^{o}$C | (Kroken *et al*., 2003) |
| Pks13 -1(forward)<br>Pks13 -2(reverse) | 1 cycle; 5 min at 95$^{o}$C<br>35 cycles 1 min at 94$^{o}$C | 30s at 63$^{o}$C | 1 min at 72$^{o}$C<br>1 cycle, 10 min at 72$^{o}$C | (Kim *et al*., 2005) |
| nor1-f(forward)<br>nor1-r(reverse) | 1 cycle; 5 min at 95$^{o}$C<br>30 cycles 1 min at 95$^{o}$C | 1 min at 60$^{o}$C | 1 min at 72$^{o}$C<br>1 cycle, 10 min at 72$^{o}$C | (Leema *et al*., 2011) |

#### 3.2.3.6 Gel electrophoresis

##### 3.2.3.6.1 Agarose gel electrophoresis

Agarose (Melford Laboratories, UK) gels were prepared by mixing 1 or 2g with 100 or 200 ml of 1x TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA) into a conical flask, as required depending on the expected size of DNA (1 % or 2 % (w/v).for 400-10,000 bp and 50-2000 bp, respectively). The mixture was heated in a microwave oven and swirling at intervals until completely dissolved. The gel was allowed to cool down before adding ethidium bromide to 0.2 µg/ml concentration. The DNA amplicons were separated by running alongside with molecular markers on the agarose gel in 1x TAE buffer at 70 V for 1h 30 min (until the dye reached 70-80% down the gel) and visualized and taken under a UV light in a molecular imager gel doc XR system (Bio-Rad Laboratories, USA).

##### 3.2.3.6.2 Denaturing gradient gel electrophoresis (DGGE)

The V6–V8 amplicons (400 bp) and NL1; LS2 amplicons (256 bp) from PCR of bulk cells §3.2.3.5 were analysed by DGGE on a Bio-Rad Dcode system (Bio-Rad, Hercules, USA). This was performed by running the samples in 8% (w/v) polyacrylamide gel in 1 X TAE buffer at 60$^{o}$C with the gels containing 20 to 60% urea-formamide denaturing gradient. Gel was prepared with low and high gradient denaturing solutions of 0% containing 20 ml 40% acrylamide/bisacrylamide (Bio-Rad), 2 ml 50x TAE (242g Tris base, Sigma Aldrich; 57.1 ml Glacial acetic acid, Fisher Scientific; and 100 ml 0.5 M EDTA) and 78 ml deionized water, and 100% containing 20 ml 40% acrylamide/bisacrylamide, 40 ml deionized formamide (Bio-Rad), 2 ml 50x TAE, 42g pure urea. Low gradient solution (20%) was prepared by adding 12 ml of 0% and 3 ml of 100% denaturing solutions. High gradient (60%) was prepared with 6 ml of 0% and 9 ml of 100% solutions. The gel electrophoresis analysis was performed at 20 V for 10 min to slowly start band migration and confirm amperage and then increased to a constant voltage of 50 V for 17 h. The gel was stained with GelStar$^{TM}$ (Lonza, UK)

solution (5 µl of gel star in 50 ml 1 X TAE buffer) for 30 min and image was visualised as described in § 3.2.3.6.1.

#### 3.2.3.6.3 Pulsed field gel electrophoresis

One colony from each isolate was inoculated in 10 ml BHI broth and incubated for 24 h at 37$^{o}$C with gentle shaking (100 rpm). The cells were harvested by centrifugation of 1 ml of broth culture at 13000 xg for 10 min. Bacterial cells were resuspended in equal volume (1 ml) of PIV buffer (10 mM Tris-HCl, pH 7.6; 1 M NaCl) and diluted to obtain OD 1.0 at 600 nm. Cell suspension (200 µl) was mixed with warm 200 µl of 1% (w/v) low melting agarose (Certified™ megabase agarose, Bio-Rad, CA) prepared by mixing 1g of agarose in 100 ml PIV buffer and heating to completely dissolve. Quickly 100 µl of the suspension mix was dispensed into perpex plug moulds (Bio-Rad) and allowed to solidify. Each plug was placed in a bijou bottle containing 5 ml of fresh lysis buffer (6 mM Tris-HCl, pH 7.6; 1 M NaCl, 0.1 mM EDTA, 1% N-lauroyl-sarcosine sodium salt, Sigma) with 25 µl of 20 mg/ml lysozyme (Sigma) per plug. The plugs were incubated at 37$^{o}$C with shaking at 100 rpm for 16 h. After incubation, lysis buffer was removed and plugs were washed three times in 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, PH 7.6). Five millilitres of proteinase k (Sigma) solution (0.5 M EDTA, 1% N-lauroyl-sarcosine sodium salt, 25 µl of 20 mg/ml of proteinase k per plug) was added to the plugs and incubated at 55$^{o}$C with shaking for 24 h. The plugs were again washed two times with warm (50$^{o}$C) sterile milli-Q water and four times with warm 1X TE buffer and then incubated in 200 µl of restriction buffer J (Promega, UK) solution (180 µl nuclease free $H_2O$ + 20 µl restriction buffer J) at 25$^{o}$C for 10 min. Plugs were cut (2-3 mm) and digested in Sma 1 restriction endonuclease (Promega, UK) solution (87 µl nuclease free $H_2O$ + 10 µl of 10X Sma 1), incubating at 25 $^{o}$C for 2 h.

Agarose gel (1% w/v) was prepared by melting 1g of certified megabase agarose in 100 ml 0.5x TBE buffer in a microwave. The gel was allowed to cool to 55-60$^{o}$C, poured into the gel tray with 10-well capacity comb and allowed to solidify for ~30 min. Digested plugs were loaded onto the wells of the solidified gel with low-range (130 – 194,000 bp)

and mid-range (15,000 – 291,000 bp) molecular size markers ( New England BioLabs, UK) on both ends. The wells were then sealed with 5 ml of molten 1% (w/v) megabase agarose gel. DNA bands were separated by running the gel in chilled 0.5X TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid (Sigma) and 12.5 mM EDTA pH 8.0) on clamped homogenous electric field (CHEF) dynamic regulation cell (CHEF- DRII®, Bio-Rad) at 6 V/cm and 1-20 s ramp switch time for 17 h maintaining the temperature at 14$^{o}$C. Gel was stained in ethidium bromide solution (200 ml deionized $H_2O$ and 20 µl of ethidium bromide) for 30 min and destained for 20 min twice. The gel was visualised as described in described in §3.2.3.6.1 and gel images were processed using the FP Quest software (Bio-Rad).

#### 3.2.3.6.4 Restriction fragment length polymorphism

Amplicons from 16S rRNA PCR amplification were used as DNA template. Briefly the restriction mix was prepared by adding 16.3 µl of nuclease free water, 2 µl of 10X restriction buffer, 0.2 µl acetylated BSA (10 µg/ µl) and 1 µl DNA template. This was mixed by pipetting before adding 0.5 µl of restriction endonuclease (EcoRI, XbaI (Promega) at 10u/ µl). The solution was mixed again by pipetting and incubating at 37$^{o}$C for 3 h. Loading buffer (Promega) was added at 1X concentration and bands were detected on 2% agarose by gel electrophoresis §3.2.3.6.1 (Sharafi *et al*., 2010).

### 3.2.3.7 Cluster Analysis

Fingerprint patterns of PFGE and ERIC PCR were analysed using the FP Quest software (Bio-Rad) following the approach described by (Tenover *et al*., 1995) based on band similarities. The gel image was uploaded, edited and normalized. Cluster analysis was calculated by pairwise similarities with the Unweighted Pair Group Method using Arithmetic averages (UPMGA) based on band dice similarity coefficient.

### 3.2.3.8 Purification of DNA band amplimers and Sequencing

DNA band amplimers were cut with sterile scalpel and transferred into 1.5 ml sterile eppendorf tubes. For DGGE, band amplimers were eluted in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) and 2 µl of eluted DNA was used as template for PCR using the same primer without GC clamp, following conditions as described in §3.2.3.5. Re-amplified amplicons were separated on 1% agarose gel before purification. All DNA band amplimers were purified with Zymoclean™ Gel DNA Recovery Kit (D4001) (Zymo Research, USA). Sequencing was performed by Eurofins MWG (Eurofins, Ebersberg, Germany). The closest related sequences for partial 16S rRNA gene were obtained using NCBI Blast program. (http://blast.ncbi.nlm.nih.gov/).

## 3.3 Starch and non-starch polysaccharides

### 3.3.1 Starch analysis

**i) Starch solubilization:**

One gram of sample (flour or *ogi*) was weighed into a 250ml flask containing 20ml of DMSO (Dimethylsulphoxide) and 5ml of 8M HCl. The flask was covered with aluminium foil and incubated at 60$^{o}$C for 30min in a water bath. Fifty millilitres of deionized water was added to the flask and pH was adjusted to between 4-5 with 5 N NaOH. The solution was cooled to room temperature and then diluted to 100 ml with deionized water.

**ii) Starch assay:**

Four test tubes marked A (starch assay reagent blank), B (sample blank), C (Glucose assay blank) and D (test) were filled with solutions as follows:

A 1 ml of starch assay reagent + 1 ml of deionized water

B 1 ml of solubilized sample + 1 ml of deionized water.

C 2 ml of deionized water.

D 1 ml of starch assay reagent +1 ml of solubilized sample.

The tubes were mixed by vortexing and incubated at 60$^{o}$C for 15 min in a water bath. The tubes were removed from the water bath and cooled to room temperature.

**iii) Glucose assay:**

Another four test tubes marked A (starch assay reagent blank), B (sample blank), C (Glucose assay blank) and D (test) were filled with:

A 1 ml of glucose assay reagent + 20 µl starch assay reagent blank

B 1 ml of glucose assay reagent + 20 µl of sample blank

C 1 ml of glucose assay reagent + 20 µl of glucose assay reagent blank

D 1ml of glucose assay reagent + 20 µl of test (1 ml of starch assay reagent +1 ml of solubilized sample)

The tubes were mixed by vortexing and incubated for 15 min at room temperature (18-25$^{o}$C). Absorbance (A) was then recorded at 340 nm.

Starch concentration (Sc) was calculated as follows:

$$Sc = \frac{(\Delta A)(TVSA/SVSA)(TVGA/SVGA)(\text{starch Mw})(F)}{(\varepsilon)(d)(1000)}$$

$$Sc\ (mg/ml) = \frac{(Sc)(25)}{\text{Sample weight}}$$

Where:

$\Delta A = A_{test} - A_{total\ blank}$

$A_{Total\ blank} = (A_{sample\ blank} - A_{glucose\ assay\ reagent\ blank}) + A_{starch\ assay\ reagent\ blank}$

TVSA = Total assay volume from starch assay

SVSA = Sample volume from starch assay

TVGA = Total assay volume from glucose assay

SVGA = Sample volume from glucose assay

Starch Mw = 162.1g/mol.

F = dilution factor

ε = Millimolar extinction coefficient for NADH at 340 nm (6.3 /cm)

d =light path (1 cm)

### 3.3.2 Analysis of non-starch polysaccharide

Non-starch polysaccharide content in grains and *ogi* was determined following the method described by (Dubois *et al*., 1956) Dry samples (0.2 mg) or wet samples (1 g) were used for the assay as recommended in the procedure.

**Step 1: Dispersion and hydrolysis**

The required amount of sample in each case (0.2 mg flour or 1 g *ogi*/pomace) were placed in two glass tubes each containing 2 ml of dimethyl sulfuroxide (DMSO) . The suspensions were mixed for 2 min on a magnetic stirrer and placed in a beaker of boiling water for 1 h. The tubes were removed and 8 ml of sodium acetate buffer pH 5.2 (pre-equilibrated at 50$^{o}$C) was added to the suspensions immediately and without cooling. After mixing by vortexing the tubes were incubated at 40$^{o}$C for 2-3 min and then 0.5 ml of pancreatin suspension (containing 700 µg pancreatin in 9 ml distilled water) was added. The tubes were again incubated at 42$^{o}$C for 1 h before mixing. The samples were then left for 16 h (overnight) at 42$^{o}$C before adding 40 ml of absolute ethanol. Tubes were mixed by inversion and left at room temperature for 1 h, and then centrifuged at 1500 x g for 10 min. Supernatant was gradually removed without disturbing the residue. The residue was washed twice with 50ml 85% ethanol. Acetone (40 ml) was added to the washed residue, stirred for 5 min and centrifuged at 1500 x g for 10 min. Supernatant was removed as before and the tubes were placed in a water bath at 65$^{o}$C and left open to allow the acetone to evaporate.

**Step 2: Acid hydrolysis**

Two millilitres of 12 M $H_2SO_4$ was added to the tubes and vortexed to mix. The suspension was left at 35$^{o}$C for 1 h and mixed at intervals. Water (22 ml) was added rapidly and mixed by vortexing before placing in a boiling water bath to boil for 2 h.

**Step 3: Phenol-sulphuric acid assay**

Hydrolysed samples (0.1 ml) were placed in test tubes with 1 ml of 2.5% phenol (Sigma). One millilitre of concentrated sulphuric acid (2.5%) was rapidly added by

means of a dispenser. The suspension was allowed to cool and absorbance was determined at 415 nm. Glucose standards were prepared alongside the test samples up to 1 mg/ml (0.2, 0.4, 0.6, 0.8 and 1.0). Non starch polysaccharide (NSP) concentrations were calculated based on a standard plot of $A_{415nm}$ against concentration.

### 3.3.3 Sugar analysis by HPLC

In order to access the types of polymers present in the NSP apart from starch which was removed by DMSO (§3.3.2), HPLC analysis of sugars was performed on the NSP extract after acid hydrolysis (§ 5.2.2 step3) under isocratic conditions using a JASCO HPLC system (JASCO AS-2055 autosampler, JASCO PU-1580 pump and JASCO RI-2031 plus Intelligent RI detector) (JASCO Essex, UK). Carbohydrate polymers were separated on a Rezex™ ROA-Organic Acid $H^+$ column of 5 µm particle size and 300 x 7.8 mm dimension (Phenomenex, UK) with 0.005 N $H_2SO_4$ as mobile phase with injection volume of 20 µl. Flow rate was 0.5 ml/min and. signal was detected by a refractive index (RI) detector (VG Lab., UK) at 220 nm wavelength. Data were obtained and processed using Azur software (Azur, France). Glucose (Fisher), xylose (X1500, Sigma-Aldrich), arabinose (A3131, Sigma-Aldrich), galactose (G0750, Sigma-Aldrich), mannose (M2069, Sigma-Aldrich), cellobiose (C7252, Sigma-Aldrich) fructose (F0127, Sigma-Aldrich) and rhamnose (83650, FLUKA) were used as standards. These were prepared by serial decimal dilutions with concentrations ranging from 20.0-0.6 g/l and were analysed with the samples. Peak areas were plotted against concentration to obtain a standard calibration plot used for quantification of the sugars (Figure 3.2).

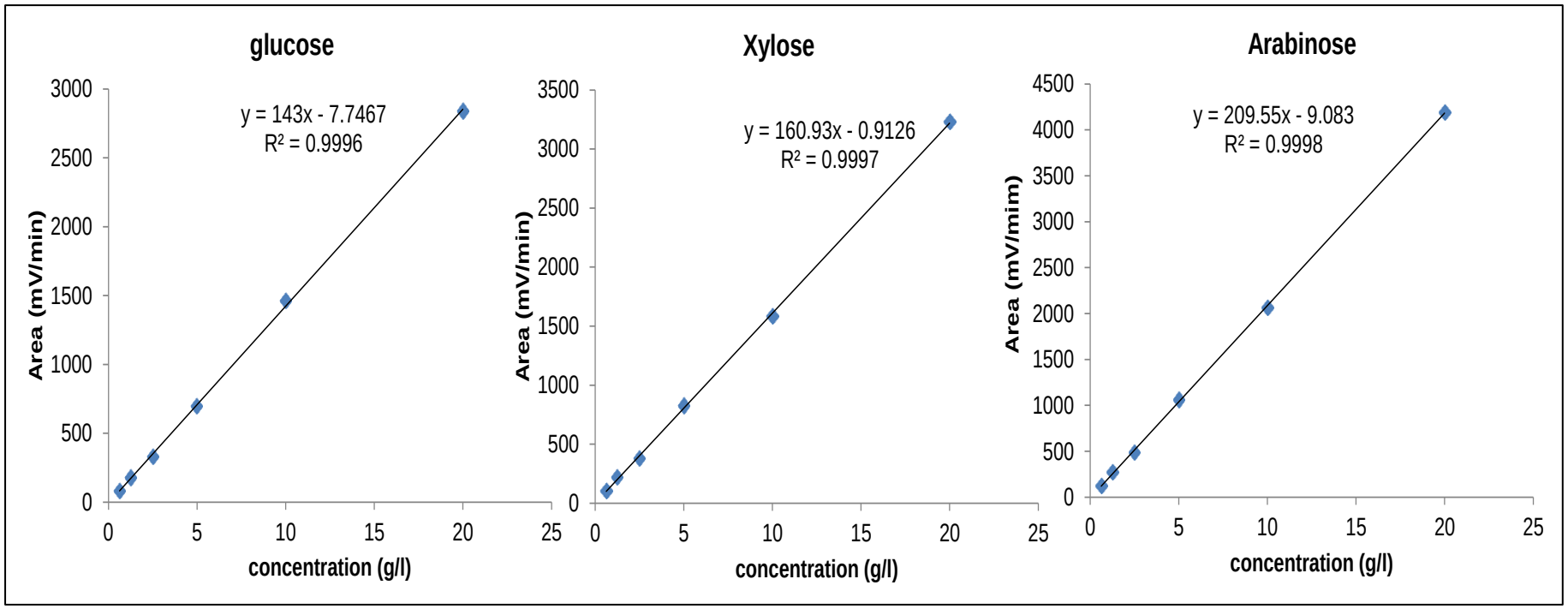



**Figure 3.2:** Calibration plots of sugar standard solutions

### 3.4 Phenolic acid assay

#### 3.4.1 Total phenolics

Total phenolic acids were extracted directly from the wholemeal flour and *ogi* samples (Singleton and Rossi, 1965). One gram of sample was homogenized in 20 ml of 0.5 M NaOH and left for 24 h at room temperature. The homogenate was transferred into a polystyrene universal tube and centrifuged for 10 min at 2060 x g. The supernatant was then transferred into a clean universal glass bottle and adjusted to pH 7.0 (neutralization) with concentrated HCl and the volume made up to 22 ml with distilled water. The neutralised sample was then used directly for phenolic acid estimation.

#### 3.4.2 Soluble phenolics

Sample (1 g) was extracted in 20 ml 70% (v/v) methanol (Singleton and Rossi, 1965) using a high efficiency homogenizer (Polytron® PZ 100 homogenizer) at room temperature. Homogenized sample was centrifuged at 2060 x g for 10 min and the supernatant was used directly for analysis.

#### 3.4.3 Bound phenolics

Bound phenolic acids were extracted from the residue obtained from the methanol extraction. Samples were treated as described for total phenolics and the supernatant was used directly for analysis (Singleton and Rossi, 1965).

#### 3.4.4 Preparation of gallic acid standard curve

Phenolic acid content was determined using the Folin-Ciocalteu reagent colorimetric method (Singleton *et al*., 1999).

Gallic acid (G7384, Sigma Aldrich) was used as standard for the calibration plot. Gallic acid standard stock solution was prepared by dissolving 10 mg of gallic acid in 2 ml of 100% ethanol and making volume up to 10 ml with distilled water to obtain a

concentration of 1000 µg/ml. One millilitre of gallic acid standard solutions with concentrations ranging from 200–1000 µg/ml was prepared from the stock solution. Standards were analysed by colorimetric procedure §3.4.1.5. Absorbance values obtained were plotted against gallic acid standard concentrations to obtain the calibration curve (Singleton and Rossi, 1965, Singleton *et al*., 1999).

### 3.4.5 Colorimetric determination of phenolic acid

Samples (0.1, 0.5 or 1mL) or standard (0.1 ml) were transferred into test tubes containing 6.0 ml of distilled water. Undiluted Folin-Ciocalteu reagent (0.5 ml) (F-9252, Sigma) and 1.5 ml (200g/l) of saturated sodium carbonate (Sigma, Aldrich) were added one at a time to the mixture. The total volume was made up to 10 ml with distilled water and vortexed. The solutions were incubated at 40$^{o}$C for 2 h in a water bath. Absorbance was determined at 765 nm using Ultrospec 2000 - spectrophotometer (Pharmacia Biotech.UK). A blank was included for each assay. Phenolic acid contents were estimated as µg gallic acid equivalent per gram of sample. Data from triplicates analyses of the three different dilutions of each sample were normalized to same dilution with the standard (0.1 ml) and expressed as means of three replicate determinations.

## 3.5 Phytate assay

### 3.5.1 Determination of phytate

Total phosphorus and phytic acid content were estimated using phytic acid/total phosphorus assay kit, K-PHYT, (Megazyme International, Ireland) according to the manufacturer's procedure. One gram of sample was extracted overnight with 20 ml of 0.66 M HCl in a 100 ml beaker with vigorous stirring on SM8 magnetic stirrer (Stuart Scientific, UK). One millilitre of the extract was transferred into a 1.5 ml Eppendorf tube and centrifuged at 13,000 x g for 10 min. The supernatant (500 µl) was then transferred into a clean Eppendorf tube and adjusted to pH 7.0 with dropwise addition

of 0.75M NaOH (Sigma-Aldrich, USA). The neutralised sample extract (50 µl) was then used for the colorimetric determination. Absorbance was measured at 655 nm using the microplate reader (model 680 XR, Bio-Rad) and corrected for the blank measurement. Total phosphorus was quantified from a phosphorus calibration curve generated over a range of 0.5–7.5 µg of phosphorus (figure 3.3) and expressed as gram of phosphorus per 100g of sample. The amount of phosphorus released was then converted to phytic acid on the assumption that phosphorus comprises 28.2% of phytic acid (Megazyme International, Ireland).

**Table 3.6:** Phosphorus standard dilutions used in the calibration plot

| standards | Concentration (µg/ml) | Distilled water | Phosphorus standard | Total volume |
|---|---|---|---|---|
| ‡STD0 | 0.0 | 5.00 | 0.00 | 5.00 |
| STD1 | 0.5 | 4.95 | 0.05 | 5.00 |
| STD2 | 2.5 | 4.75 | 0.25 | 5.00 |
| STD3 | 5.0 | 4.50 | 0.50 | 5.00 |
| STD4 | 7. 5 | 4.25 | 0.75 | 5.00 |

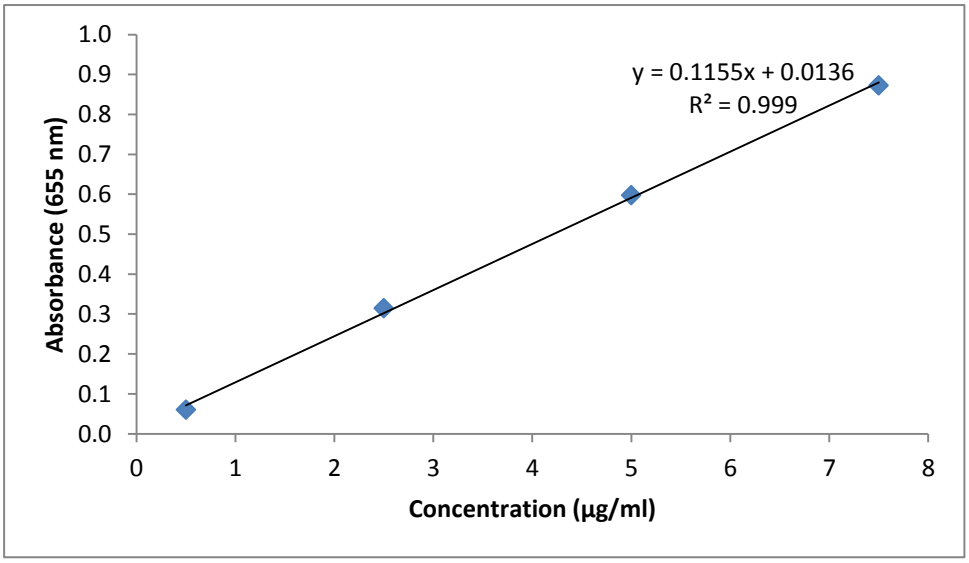



**Figure 3.3:** Calibration plot of phosphorus standard solutions

### 3.5.2 Determination of Phytase activity

Phytate hydrolysing fungi (PHF) were assessed for enzyme activity. Isolates were cultured on PSM solid medium for 5 days to obtain pure colonies which were then inoculated into 20 ml PSM liquid medium (PSML) and incubated at 30$^{o}$C for 10 days. Spores were harvested from the liquid culture by centrifugation (4000 xg for 10 min) and washed with 20 ml of sterile purified water into sterilized 250 ml beaker through sterile cheesecloth to remove the filament. Spore suspension was inoculated in 100 ml PSML (at approximately 1 x $10^{7}$/ml) containing 0.5% sodium phytate in 500 ml flask and incubated at 30$^{o}$C, shaking at 150 rpm for 14 days. Two millilitres of culture was centrifuged at 13000 xg and supernatant was used for analysis. One millilitre of substrate solution (10 mM sodium phytate as substrate (0.462 g in 50 ml $dH_2O$) and 0.2 M sodium acetate buffer pH 5.5 (0.1 M glacial acetic acid, 0.1 M sodium acetate) and 1 ml of culture filtrate was incubated at 37 $^{o}$C for 1 h. Colour reagent was prepared by mixing 125 ml of ammonium heptamolybdate (10% ammonium molybdate in 0.25% ammonia solution), 125 ml ammonium vanadate solution (0.235 g in 100 ml of 2% nitric acid (v/v)) and 82.5 ml of 65% nitric acid in 500 ml final volume. One millilitre of colour reagent was added to stop the reaction and absorbance was measured at 415 nm. Deionized water was used for blank test and potassium dihydrogen phosphate (KH2PO4) was used for standard plot with concentration ranging from 0-1000 µmol (Engelen *et al*., 1994).

## 3.6 Organic acids and volatile compounds

### 3.6.1 HPLC analysis of organic acids

Analysis of organic acids was performed by High performance liquid chromatography (HPLC) using a JASCO HPLC system (JASCO AS-2055 autosampler, JASCO PU-1580 pump and JASCO RI-2031 plus Intelligent RI detector) (JASCO Essex, UK). Lactic, acetic and malic acids were separated on a Rezex™ ROA-Organic Acid $H^{+}$ column of 5 µm particle size and 300 x 7.8 mm dimension (Phenomenex, UK) with 0.005 N $H_2SO_4$ as mobile phase.

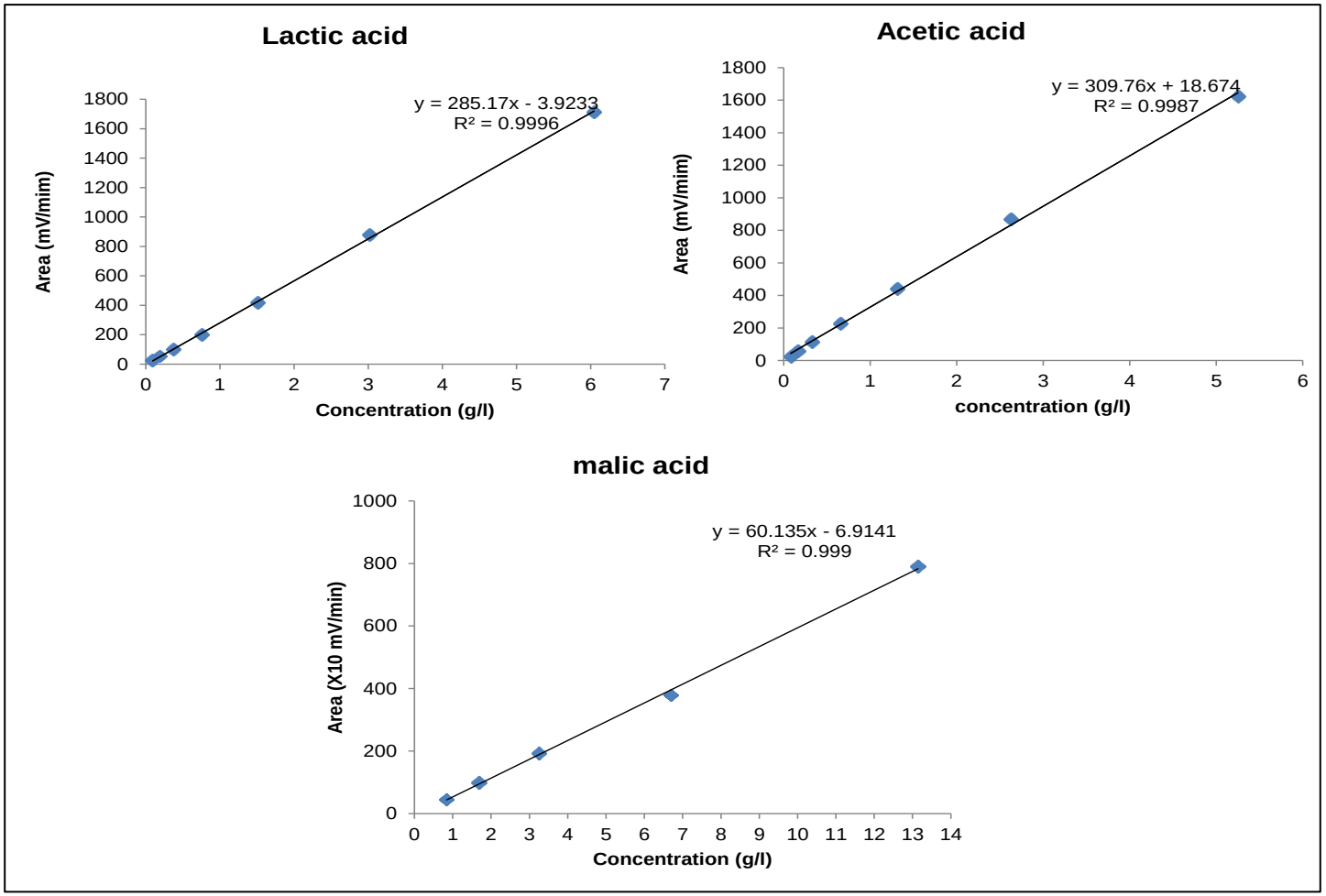



**Figure 3.4:** Calibration plots of organic acid standard solutions

Flow rate was 0.5 ml/min and. signal was detected by a UV detector (VG Lab., UK) at 220 nm wavelength. Data were processed and obtained using Azur software (Azur, France).

DL-Lactic acid ~90% (69785 Fluka-Sigma Aldrich), Acetic acid (320099 Sigma-Aldrich) and DL-malic acid (27606 Sigma) were used as standards and prepared by serial decimal dilutions with concentrations ranging from 13.0-0.1 g/l, these were analysed with the samples. Peak areas were plotted against concentrations to obtain a standard calibration plot used for quantification of the acids (Figure 3.4).

### 3.6.2 Headspace analysis by GC-MS

A headspace solid phase microextraction (SPME) method by gas chromatography-mass spectrometry was used to evaluate the volatile components. Samples (5 g) were placed in 20 ml vials (22.5mm x 75.5mm, Grace Alltech, UK) and sealed with PTFE-Silicone magnetic caps (20mm x 5mm, Grace Alltech, UK). Headspace analysis was performed with a 1 cm StableFlex™ fibre (SUPLECO Bellefonte PA, US) coated with 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane. The fibre was inserted into the injection port and conditioned for 40 min at 220$^{o}$C before use. The analysis was performed using a GC-MS system consisting of a TRACE™ GC Ultra gas chromatography and a DSQ mass spectrometer (Thermo Scientific, Massachusetts, USA). Samples were initially heated at 40$^{o}$C for 2 min and extracted at 220$^{o}$C for 20 min with splitless time 1 1/2 min and ramp 6$^{o}$C/min through a Zebron™ ZB-WAX capillary GC column (30m x 0.25mm x 1µm film thickness) (Phenomenex, USA). The desorption time was 1 min at 220$^{o}$C, pressure 18 psi and mass range was set at 50-650 m/z. The carrier gas was Helium flowing at 1ml/min. Signal detection was expressed as percentage relative abundance by peak areas based on total ion current (TIC). Data were processed using the Xcalibur™ software (Thermo Scientific) and compounds were identified through a library search by comparison with the mass spectra and retention times in the database.

### 3.7 Statistical analysis

All samples were run in three replicates and significant differences between samples were tested by ANOVA Duncan's multiple range tests with SPSS statistical software (version 22, IBM SPSS, UK). The relationship of volatile compounds detected by GC-MS SPME analysis with the different fermentations was evaluated by principal component analysis (PCA) using Unscrambler® software v 9.0 (CAMO, Norway) and values were based on four replicate samples from two independent fermentations (n=4) and expressed as means of peak areas and standard deviation. Analysis of molecular variance (AMOVA) in GenALEx 6.502 software was used to test for the significance of clustering of the DNA fingerprinting profiles.

# CHAPTER 4

# MICROBIAL DIVERSITY OF GRAINS AND POPULATION DYNAMICS DURING FERMENTATION OF *OGI*

## 4.1 Introduction

Foods of plant origin such as grains are known to have microbial consortia consisting of mostly environmental species. These natural organisms are thought to initiate a mixed fermentation in *ogi* production (Odunfa and Adeyele, 1985) and could facilitate the proliferation of other groups of species through production of energy sources and changes in pH which enhance their growth. While the microbial diversity during *ogi* fermentation is receiving greater attention, very little information is available on the initial grain microbiota. Diversity of the microbial population in *ogi* has been studied by both cultural (Banigo and Muller, 1972; Teniola *et al*., 2005; Omemu, 2011; Ashiru *et al*., 2012) and culture-independent techniques (Ampe and Miambi, 2000; Oguntoyinbo *et al*., 2011) with limited information on the latter which was focused only on characterization of bacterial species. Studies on the population diversity of the traditionally fermented products revealed significant variations in the organisms present and the quality of product (Boswald *et al*., 1995; Anthony and Chandra, 1997; Vogel, 1997; Omar and Ampe, 2000; Hammes *et al*., 2005; Giraffa and Carminati, 2008; Shanna and Ye, 2014) as discussed in §2.2. It has been established that *ogi* fermentation involves bacterial (mostly LAB), mould and yeast species (Teniola *et al*., 2005) but with inconsistency in the specific species present suggesting different populations in different *ogi*. Therefore microbial analysis of both fungal and bacterial species in *ogi* production using both cultural and molecular methods is important to better understand the species mostly common, their interaction and their importance.

The aim of this study was to isolate, characterize and assess the organisms originally on the grains (maize and sorghum) and in *ogi,* using both culture and culture–independent techniques*,* in order to understand the population diversity and dynamics

of predominant species during fermentation and select suitable species for starter culture application.

## 4.2 Results

### 4.2.1 Microbial enumeration and identification

#### 4.2.1.1 Grains

The initial microbiota of maize and sorghum grains from duplicate samples from two different sources (northern (1) and eastern (2) geographical regions of Nigeria) were studied in order to identify which organisms initiate the fermentation and how they change during processing of *ogi*.

##### 4.2.1.1.1 Viable count

Microbial counts were obtained by serial dilution of flour made from the grains and plating on five different culture media (§3.2.1). Initial microbial counts varied significantly ($p>0.05$) between the same grain from different sources and was observed to be higher in eastern grains (M2 and S2) for all groups of organisms investigated (Table 4.1). Maize from both regions gave higher counts than sorghum and ranged between 3.72–7.54 log cfu/g. Sorghum from the northern region (S1) had the least microbial population with counts for the dilutions being based on less than 30 colonies per plate due to low population levels.

Yeast and moulds were the predominant population group for M1 (4.11 log cfu/g) and S2 (5.17 log cfu/g) while M2 was dominated by M17 organisms. Total vable plate count was highest in M2 (5.89 log cfu/g) and least in M1 and S1 (3.48 log cfu/g) and was found to be at the same level with counts on M17 in maize (M1) and sorghum (S1 and S2). This result shows variations in the group of microorganisms present in each grain. The differences observed in the same grain from the different sources (M1 and M2) may suggest a regional effect. Higher counts on M17 suggest that *Lactococcus* may be the major population in M2. Total plate counts on M1, S1 and S2 could also represent the *Lactococcus* population recorded on the respective M17 plates.

**Table 4.1**: Fermentation pH‡ and microbial counts on maize (M) and sorghum (S) grains from different regions and their respective *ogi* (MO, SO)

| **Media** | **Viable cell count (log cfu/g)†** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M1* | M2 | S1 | S2 | MO1 | MO2 | SO1 | SO2 |
| M17 | 3.72 (0.11)$^{1c}$ | 7.54 (0.08)$^{3c}$ | 3.41 (0.41)$^{1b}$ | 4.33 (0.07)$^{2b}$ | 7.60 (0.14)$^{3b}$ | 7.79 (0.01)$^{3c}$ | 6.57 (0.40)$^{3c}$ | 7.71 (0.16)$^{4b}$ |
| MRS | 3.04 (0.08)$^{2b}$ | 4.23 (0.17)$^{3a}$ | 2.30 (0.00)$^{1a}$ | 2.54 (0.13)$^{1,2a}$ | 7.52 (0.57)$^{5b}$ | 7.57 (0.08)$^{5c}$ | 7.57 (0.41)$^{4d}$ | 6.69 (0.23)$^{5a}$ |
| MacConkey | 2.30 (0.00)$^{1a}$ | 4.75 (0.21)$^{2a}$ | 2.45 (0.35)$^{1a}$ | 3.98 (0.44)$^{2b}$ | 5.73 (0.23)$^{3a}$ | 4.70 (0.49)$^{3b}$ | 4.71 (0.16)$^{4b}$ | 6.97 (0.24)$^{5a}$ |
| Nutrient agar | 3.48 (0.09)$^{1c}$ | 5.89 (0.35)$^{3b}$ | 3.48 (0.42)$^{1b}$ | 4.46 (0.007)$^{2b}$ | 7.68 (0.11)$^{5b}$ | 7.75 (0.05)$^{5c}$ | 6.61 (0.07)$^{4c}$ | 7.77 (0.09)$^{5b}$ |
| RBCA | 4.11 (0.23)$^{2d}$ | 6.40 (0.49)$^{4b}$ | 2.30 (0.25)$^{1a}$ | 5.17 (0.38)$^{3c}$ | 5.63 (0.10)$^{3a}$ | 3.82 (0.17)$^{2a}$ | 3.82 (0.11)$^{2a}$ | 6.78 (0.17)$^{4a}$ |
| ‡pH | - | - | - | - | 4.80(0.07)$^{1}$ | 5.04 (0.07)$^{1}$ | 4.80 (0.15)$^{1}$ | 4.82 (0.03)$^{1}$ |

*Two different sources of grains: 1 northern and 2 eastern
‡End of fermentation pH measurement.
†Data are means of duplicate counts of two independent fermentations (n=4) with standard deviation in parentheses.
Means in the same row with same superscripts (red or green numbers) are not significantly different (p>0.05)
Means in the same column with same superscripts (letters) are not significantly different (p>0.05)

#### 4.2.1.1.2 Phenotypic and biochemical analyses

Four groups of organisms were investigated based on the selected media. Preliminary screening by examination of microscopic, biochemical and colony characteristics of 82 microbial isolates from grain revealed the presence of:

A. Oxidase positive or negative, catalase positive, non-motile, Gram positive cocci (presumptive *Micrococcus* or *Staphylococcus* sp. (*Micrococcaceae*) from M17 agar.
B. Oxidase negative, catalase positive, motile, Gram negative rods (presumptive Enterobacteriaceae) from MacConkey and M17 agar.
C. Oxidase negative, catalase positive, motile, Gram positive rods (presumptive *Bacillus* sp.) from M17 and nutrient agar.
D. Yeast and mould from Rose Bengal chloramphenicol and MRS agar.

Cultural identification of microbes originally present in the grains showed the presence of similar microbial groups in both maize and sorghum (Table 4.2). Surprising LAB were not part of the group identified in maize grains on MRS agar from single colonies.

Group A isolates (presumptive *Micrococcus* or *Staphylococcus* spp.) showed cream medium or tiny yellowish circular, convex colonies with entire margin on BHI agar (Fig. 4.1a-A). These were identified in both maize (M1, M2) and in only one sorghum (S1) sample.

*Staphylococcus* is one of the commonly existing organisms. Highly pathogenic *Staphylococcus aureus* is of key interest and is mostly present in humans, animals and plants through contamination. In order to further classify and investigate the presence of *S. aureus* all microbial isolates in this group were cultivated on Baird Parker agar (Oxoid), a selective and diagnostic medium for isolation of *S. aureus*, which is identified by grey-black shiny colonies (due to reduction of tellurite) surrounded by zone of clearing due to proteolysis. All cultivated isolates grew well on Baird Parker agar but

colonies formed were not diagnostic of *S. aureus*, therefore this result presumptively eliminated the presence of *S. aureus* in M1, M2 and S1 and suggests that other *Staphylococcus* species or related organisms may be present in these samples..

*Staphylococcus aureus* is a coagulase positive organism and so to confirm the result observed on Baird Parker agar, the microbial isolates were then tested for coagulase production which differentiates the highly pathogenic *Staphylococcus aureus* from less pathogenic species. All isolates showed negative results confirming the absence of *S. aureus* in these samples. However identification by Analytical Profile index kit (API Staph, BioMérieux, France) revealed the presence of *Micrococcus* sp (ID=99.9%; t=0.88) in both maize samples (M1, M2) and *Staphylococcus sp* (ID 86.4%; t=0.43) in maize (M2) and sorghum (S2).

**Table 4.2:** ‡Microbial groups in maize and sorghum grains

| Grains | A (*Micrococcaceae*) | B (Enterobacteriacae) | E (*Bacillus*) | F (yeast & mould) |
|---|---|---|---|---|
| M1 | 3 | 9 | 4 | 4, 4 |
| M2 | 4 | 9 | 2 | 2, 5 |
| S1 | 2 | 8 | 2 | 1, 2 |
| S2 | 1 | 13 | 3 | 2, 3 |

*Two different sources of grains: 1 northern and 2 eastern; maize (M) and sorghum (S).
‡Number of randomly isolated microbes and numbers in red represents the number of moulds.

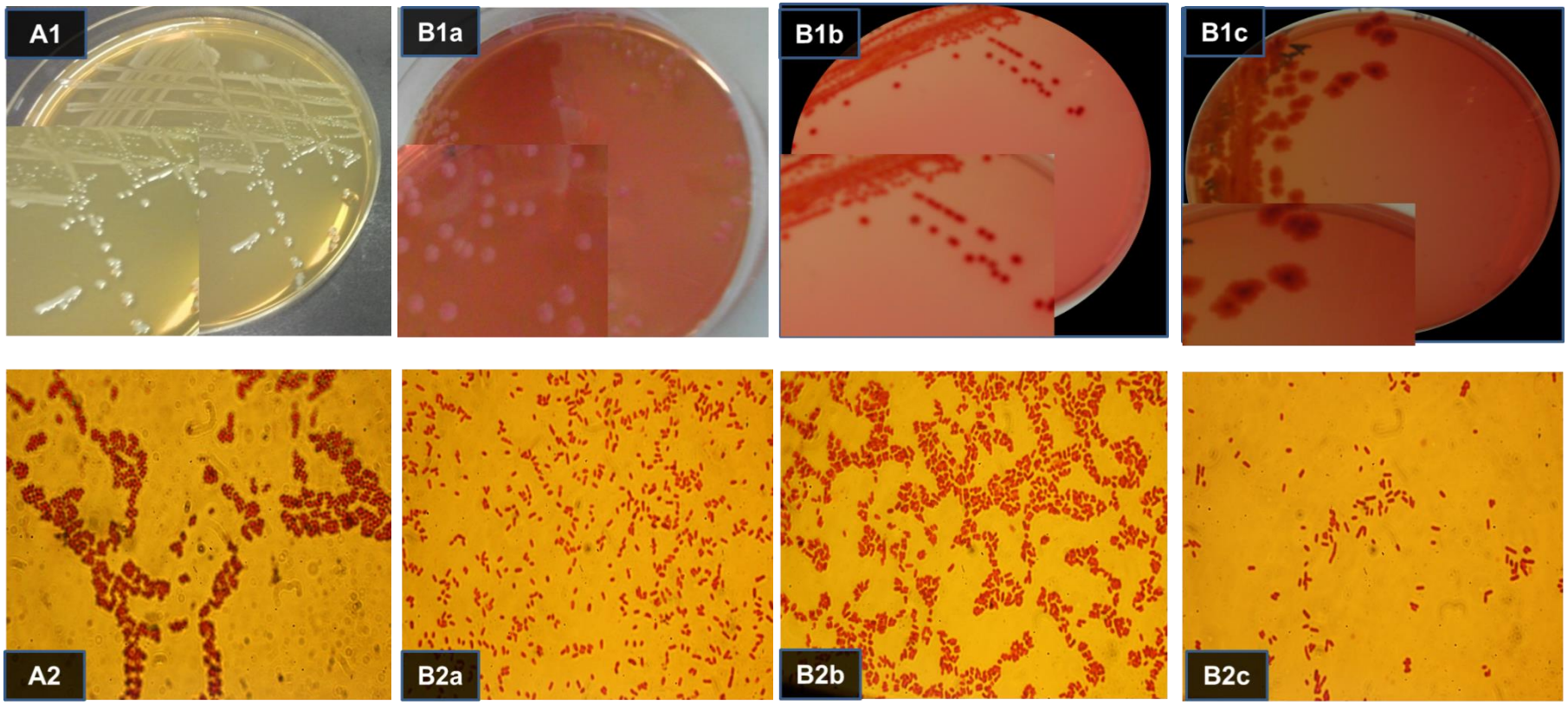



**Figure 4.1a:** Bacterial groups identified from maize and sorghum grains and *ogi;* 1: Plate image, 2: Microscopy (bright-field at x1000 magnification). A: *Staphylococcus* (BHI agar), B: Enterobacteriaceae (MacConkey agar)

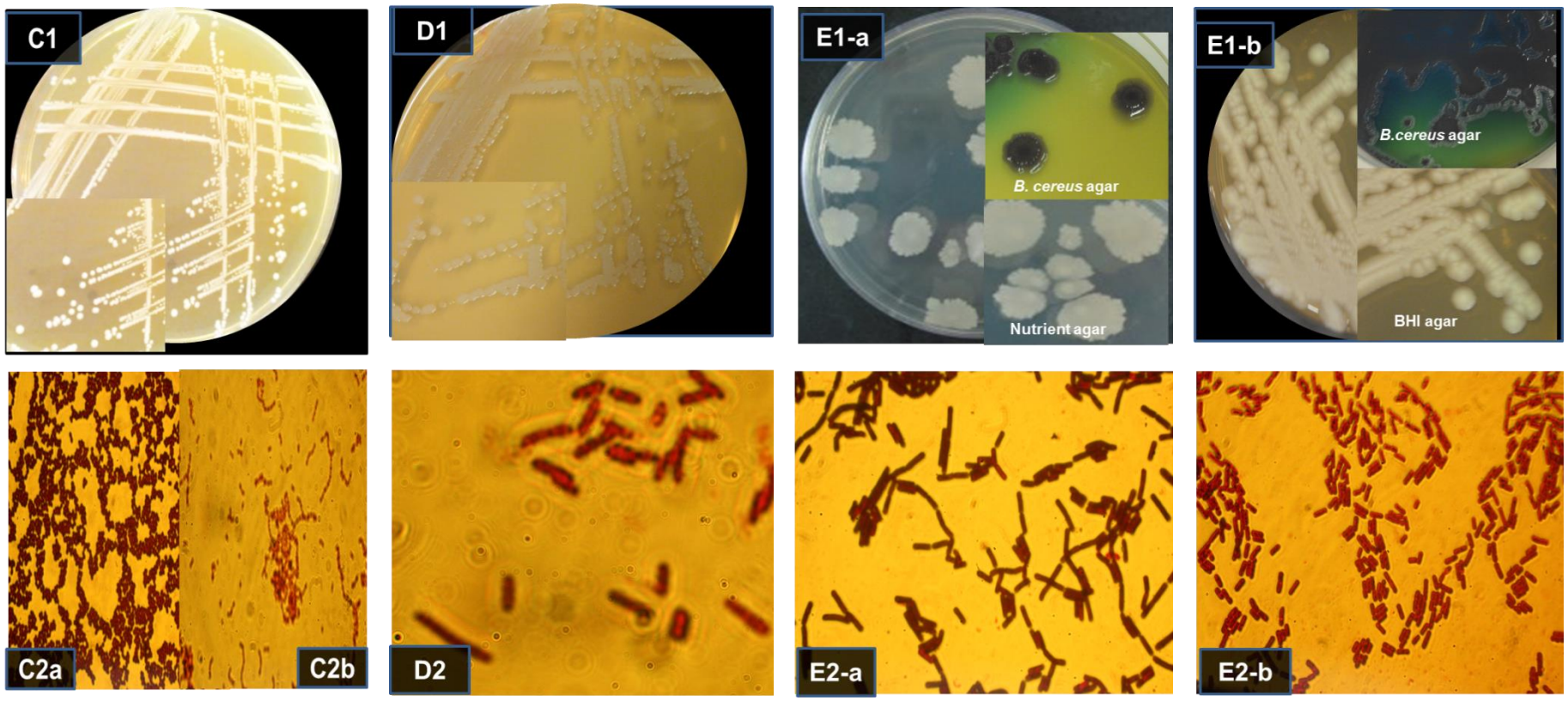



**Figure 4.1b:** Bacterial groups identified from maize and sorghum grains and *ogi;* 1: Plate image, 2: Microscopy (bright-field at x1000 magnification). C: *Lactococcus* (M17 agar), D: Lactic acid bacteria (MRS agar), E: *Bacillus* sp.

Group B microbial isolates from the grains were presumptively identified as Enterobacteriaceae (Fig. 4.1a-B), based on their ability to tolerate bile salts and grow on MacConkey agar in addition to the biochemical characteristics (§4.2.1.1.2); these were classified (Table 4.3) as:

(a) Lactose fermenters/coliforms with deep red/pink colonies due to assimilation of neutral red colour which develops as the pH of the medium drops when the organism ferments sugar (lactose) and produces acid.

(b) Non-lactose fermenters characterized by colourless colonies due to the inability of the organism to ferment lactose and produce acid.

Identification of these microbial isolates in each subgroup from the samples based on carbohydrate fermentation and enzymatic activities by Analytical Profile index test (API 20E, BioMérieux, France) revealed the presence of Enterobacteriaceae of the genera *Ewingella* and *Cronobacter* in maize and *Rahnella* in sorghum in addition to *Pantoea*, and *Enterobacter* which were the dominant organisms in all the grain samples with identification similarities (% ID) ranging from 59.4 to 99.4% (Table 4.4). Biochemical tests of pure isolates mostly identified species of *Pantoea* and *Enterobacter* in both maize and sorghum. At least one isolate from each subgroup was tested and apart from these12 isolates presented, all others showed doubtful identifications.

**Table 4.3**: Enterobacteriaceae in maize and sorghum grains.

| Grains | †(a) | | (b) | | Total no of isolates |
|---|---|---|---|---|---|
| | i | ii | i | ii | |
| M1 | 3 | 0 | 6 | 0 | 9 |
| M2 | 3 | 0 | 2 | 4 | 9 |
| S1 | 0 | 2 | 5 | 1 | 8 |
| S2 | 3 | 0 | 8 | 2 | 13 |

*Two different sources of grains: 1 northern and 2 eastern; maize (M) and sorghum (S). Colony types†: . (ai): Pink, tiny convex, shiny, colonies with entire margin; (aii): Red, 1-3mm colonies with deep red centre and irregular margin, (bi): colourless tiny convex colonies; (bii): colourless, flat, 1-2mm colonies.

**Table 4.4**: Identification of Enterobacteriaceae by Analytical profile index system (API 20E)

| Grains | Isolates | Gram Stain | Catalase | Oxidase | ONPG | Arginine Di hydrolase Lysine Decarboxylase | Ornithine Decarboxylasese Citrate | H2S | Urease | Tryptophan Deaminase Indole | Voges Proskauer | Gelatinase | Glucose | Mannitol | Inositol | Sorbitol | Rhamnose | Saccharose | Melibiose | Amygdalin | Arabinose | % ID | Presumptive identities |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M1 | M121 | – | + | – | + | – | – | – | + | – | – | – | – | + | – | + | + | + | – | + | + | – | – | + | 99.4 | *Pantoea sp.3* |
| | M122 | – | + | – | – | – | – | – | + | – | – | – | – | + | – | – | + | – | – | – | – | – | + | – | 98.5 | *Ewingella americana* |
| | M123 | – | + | – | + | + | – | + | + | – | – | – | – | + | – | + | + | + | + | + | + | + | + | + | 59.4 | *Enterobacter cloacae* |
| M2 | M221 | – | + | – | + | | – | | + | – | – | – | – | + | – | + | + | + | – | + | + | - | – | + | 90.5 | *Pantoea sp.3* |
| | M222 | – | + | – | – | – | – | – | – | – | – | – | – | + | – | | + | – | – | – | – | – | + | – | 88.7 | *Ewingella americana* |
| | M224 | – | + | – | + | + | – | – | + | – | – | – | + | + | – | + | + | – | – | + | + | + | – | + | 92.4 | *Cronobacter sakazakii* |
| S1 | S121 | – | + | – | + | – | – | – | – | – | – | – | + | + | – | + | + | – | + | + | + | + | + | + | 64.0 | *Pantoea sp.* |
| | S124 | – | + | – | + | + | – | – | – | – | – | | + | + | – | + | + | + | – | + | + | + | + | + | 99.0 | *Cronobacter sakazakii* |
| | S125 | – | + | – | + | + | – | + | – | – | – | – | – | + | – | + | + | – | + | + | – | + | + | + | 98.3 | *Enterobacter amnigenus 2* |
| | S126 | – | + | – | + | – | – | – | – | – | – | – | – | + | – | + | + | – | + | + | + | + | + | + | 98.9 | *Rahnella aquatilis* |
| S2 | S221 | – | + | – | + | – | – | – | + | – | – | – | – | + | – | + | + | + | – | + | + | – | – | + | 99.4 | *Pantoea sp.3* |
| | S222 | - | + | - | + | - | - | - | + | - | - | - | + | + | - | + | + | - | - | + | + | + | + | + | 96.2 | *Pantoea sp.2* |

*Two different sources of grains: 1 northern and 2 eastern; maize (M) and sorghum (S).

Group C microbial isolates presumptively identified with spores as *Bacillus* sp (Fig 4.1b-E) showed two different characteristic colonies of (a) 5-7 mm with irregular margins on nutrient agar (Fig. 4.1E-a) and (b) 2-3mm, raised and undulate colonies (Fig. 4.1E-b) suggesting two different strains may be present in maize and sorghum. However strain-a, was identified in M1, M2, and S2 while strain-b was identified in M1, M2 and S1. For further classification these isolates were cultivated on a selective and diagnostic medium (*Bacillus cereus* agar) for isolation of *Bacillus cereus.* Strain-a isolates had good growth on this medium with straw coloured colonies (Fig. 4.1E-a) not classical of *B. cereus* but suggestive of the presence of other species of *Bacillus*. Strain–b isolates also showed good growth with diagnostic colonies (peacock blue colour with egg yolk precipitate) of *B. cereus* (Fig. 4.1E-b) suggesting the presence of this species in the respective grain samples.

Yeasts and moulds (Group D) were also present in all grain samples. However, only the moulds were classified based on microscopic identification (Fig. 4.2a-c). Moulds present in the grain samples were of four genera namely, *Aspergillus:* characterized by white, brown to black or green conidiophores, uniseriate or biseriate phialides and one-celled conidia with rough divergent or aggregate hyalines; *Cladosporium*: with yellowish green to brown suede-like floccose, conidiophores distinct from vegetative hyphae and pigmented, 1-4-celled echinulate and pigmented conidia in chains, with distinct dark hilum; *Fusarium*: with white aerial mycelium having lateral monophialides, short single conidiophores with fusiform, slightly curved, non-septate and pointed macroconidia, and *Penicillium*: with green or white conidiophores, single-celled, cylindrical conidia in chains, flask-shaped phialides and generally has brush-like appearance (Gross and Robbins, 2000; Lhani *et al*., 2006). *Aspergillus* and *Fusarium* species were identified only in maize (M1 and M2) while *Cladosporium* species was found in both maize (M2) and sorghum (S2). *Penicillum* was present in all grain samples.

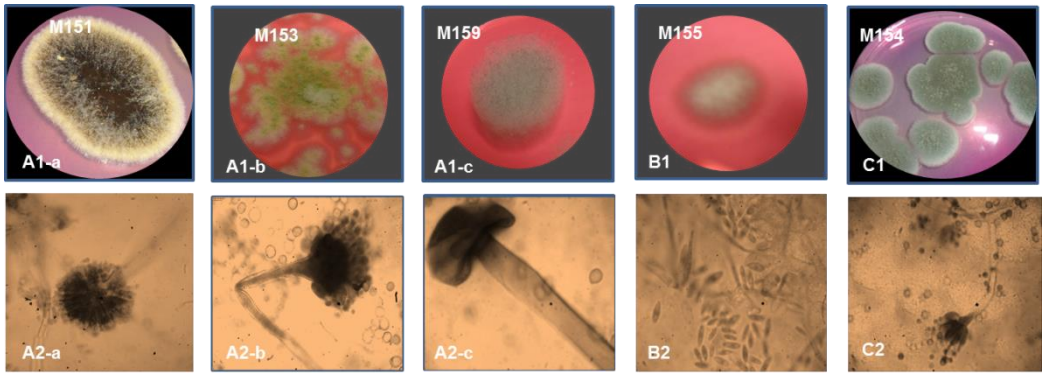



**Figure 4.2a:** Moulds identified from maize grain (M1)*;* 1: Plate image, 2: Microscopy. Isolates were cultivated on Rose Bengal Chloramphenicol agar and identified by morphological characteristics through wet mount in lactophenol cotton blue under a microscope (bright-field at x1000 magnification). A: *Aspergillus,* B: *Fusarium,* C: *Penicillum.*

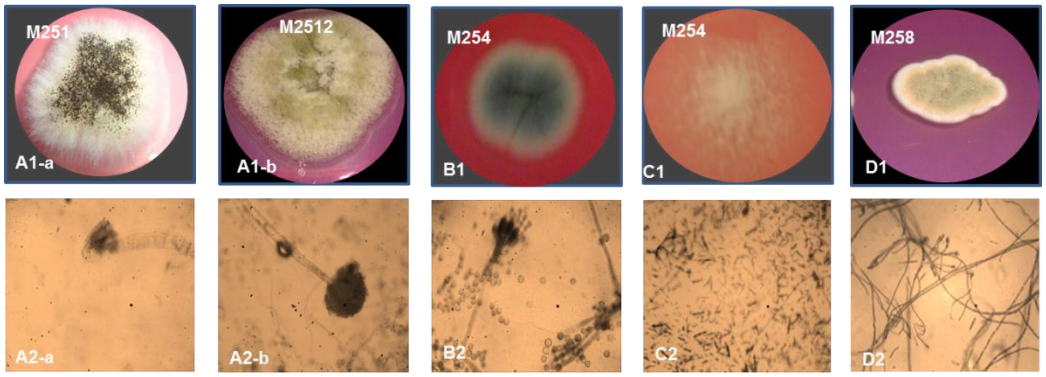



**Figure 4.2b:** Moulds identified from maize grain (M2)*;* 1: Plate image, 2: Microscopy. Isolates were cultivated on Rose Bengal Chloramphenicol and identified by morphological characteristics through wet mount in lactophenol cotton blue under a microscope (bright-field at x1000 magnification). A: *Aspergillus* sp., B: *Penicillum* sp.*,* C *Fusarium* sp., D: *Cladosporium* sp.

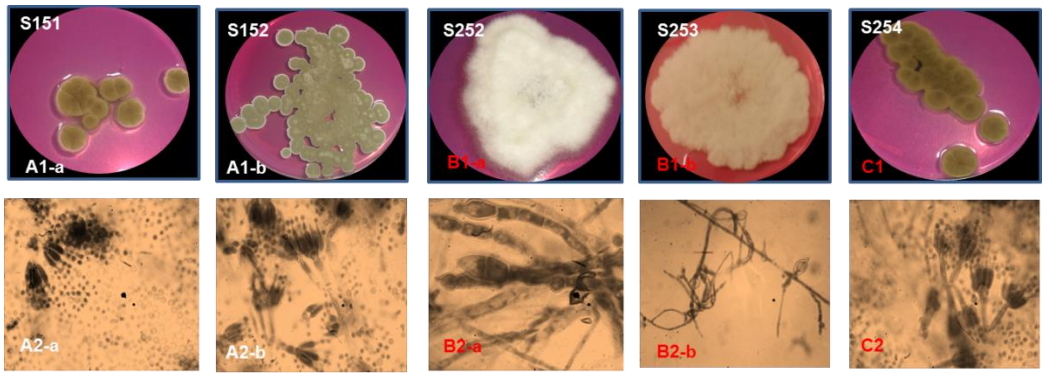



**Figure 4.2c:** Moulds identified from sorghum grains (S1: A and S2: B-C)*;* 1: Plate image, 2: Microscopy. Isolates were cultivated on Rose Bengal Chloramphenicol and identified by morphological characteristics by wet mount in lactophenol cotton blue under a microscope (bright-field at x1000 magnification). A and C: *Penicillum* sp., B: *Cladosporium* sp.

#### 4.2.1.1.3 Molecular identification

##### 4.2.1.1.3.1 Identification by PCR amplification of 16S rRNA and 26S rRNA genes and sequencing

Further identification of bacterial groups from grains was performed by DNA amplification and sequencing. Group A and B isolates which failed or gave doubtful results with the API identification were analysed by this method. The V3 regions of the 16 rRNA genes of pure isolates were amplified by PCR and purified DNA amplimers with required concentrations were sequenced and analysed by NCBI nucleotide blast.

Group A bacterial species identified in maize (M2) as *Micrococcus* sp. by the API method were further analysed by sequencing to confirm their identity as these may be significant in the fermentation. *Micrococcus luteus* was identified with 100% ID as shown in Table 4.5 for strains M211, M212, and M213 with one isolate (M214) not clearly identified. The query coverage for all analysis was above 96% with reasonably low Expect (E) values ranging from 1e-65-2e-11. Alignment of sequences of the *Micrococcus* species with the unidentified strain on clustalw2 multiple alignment tool revealed only a few mismatches with numerous gaps or deletions compared to identical regions of the 16S rRNA gene (Appendix 4.1).

Group B bacterial isolates were of the Enterobacteriaceae family as shown by the sequence results in Table 4.5. Identified microbial species in this group revealed the presence of *Enterobacter cloacae* in maize (M1) and *Enterobacter aerogenes* in sorghum (S2). Species of *Pantoea* also identified in sorghum (S2) were closest relatives to *Pantoea stewartii* and *Pantoea dispersa.*

Group C sequence results of representative isolates identified closest relatives to *Bacillus tequilensis* in maize (M1) and *Bacillus cereus* in sorghum (S1).

**Table 4.5:** Identities of 16S rDNA sequence of V3 PCR amplimers of pure isolates from grains

| *Source | Primers | Gene Accession number | Length of sequence (bp) | E-value | % identity | Closest relative |
|---|---|---|---|---|---|---|
| **M17 isolates 30°C** | | | | | | |
| M111 | V3 | KC565672 | 157 | 6e-72 | 100 | *Bacillus tequilensis* strain 25BN12U-4 |
| M112 | V3 | GU459207 | 158 | 4e-76 | 100 | *Enterobacter cloacae* |
| M211 | V3 | FJ22946 | 141 | 1e-64 | 100 | *Micrococcus luteus* strain KLE 1011 |
| M212 | V3 | FJ22946 | 146 | 1e-65 | 100 | *Micrococcus luteus* strain KLE 1011 |
| M213 | V3 | FJ22946 | 136 | 2e-64 | 100 | *Micrococcus luteus* strain KLE 1011. |
| M214 | V3 | EU134634 | 46 | 2e-11 | 100 | Uncultured bacterium clone FFCH1845 |
| S111 | V3 | KC540842 | 159 | 7e-74 | 100 | *Bacillus cereus* strain BcTNAU6 |
| S211 | V3 | KF835742 | 159 | 0.0 | 100 | *Enterobacter aerogenes* strain Z34 |
| S212 | V3 | JQ311963 | 162 | 2e-64 | 96 | *Pantoea stewartii* subsp *stewartii* |
| S213 | V3 | LC015550 | 148 | 0.0 | 100 | *Pantoea dispersa* |
| S218 | V3 | HM103364 | 161 | 1e-75 | 100 | *Enterobacter* spp AJAR-A1. |
| **MacConkey isolates 37°C** | | | | | | |
| S222 | V3 | GU459207 | 161 | 1e-75 | 100 | *Enterobacter cloacae* strain LH-S11 |
| S223 | V3 | GU143909 | 163 | 1e-76 | 100 | *Pantoea agglomerans* strain Z3-06 |
| S224 | V3 | JQ924075 | 146 | 2e-63 | 97 | *Enterobacter soli* strain KNUC5013 |
| S225 | V3 | JQ659939 | 144 | 2e-62 | 99 | *Pantoea dispersa* strain R8-396 |

*Red numbers represent two different sources of grains: 1 northern and 2 eastern; maize (M) and sorghum (S).

#### 4.2.1.1.3.2 Analysis of the microbial community in grains by DGGE

Identification of microbes by PCR analysis and sequencing of the 16S rRNA gene may be limited by direct extraction of DNA from the samples due to possible low cell numbers. It is also possible that some microbes which may not be cultivable on the media may also be present in the samples. Again cultivable cells may be present in low numbers in the grains and not picked up as single colonies following dilutions. However these cells may be present on the confluent plates. Therefore in order to

accommodate the low count cells, achieve a broad identification, and further support the result of 16S rDNA sequence of pure isolates previously obtained, fingerprinting (using V6-V8 primers for amplification of bacterial rRNA genes and NL1, LS2 for fungal genes) of the microbial community with DNA extracted directly from the grains and from cultivated bulk cells of $10^{-1}$ dilutions (incubated at temperatures ranging from $25^{o}C$–$42^{o}C$) were analysed by denaturing gradient gel electrophoresis (DGGE).

The profile of microbes detected by direct DNA extraction and bulk cell analysis by PCR-DGGE is shown in Tables 4.6, 4.7 and 4.8. Similar organisms were found as previously identified by cultural methods (§4.2.1.1.2) and PCR analysis of pure bacterial isolates (§4.2.1.1.3.1). The DGGE profile image of direct DNA extraction of microbial isolates from grains showed fewer bands (0-3) compared to the bulk cells with 1-8 bands (Appendix 4.5a). Only a few bacterial species were identified by direct DNA extraction from both maize and sorghum.

From maize, *Aeribacillus* sp was identified in M2 and *Bacillus* sp in M1 by direct DNA extraction. Analysis of bulk cells indicated the presence of microbe detected by direct microbial DNA extraction (Table 4.6) in M1. Other identified microbes in maize were *Enterococcus casseliflavus*, *Kytococcus sedentarius, Bacillus sp.,* and *Staphylococcus xylosus*. Overall similar genera were present in both maize samples except for *Amycolatopsis, Methanoculleus* and *Erwinia* which were detected only in M1 and *Schizosaccharomyces* in M2. In sorghum, identification by direct microbial DNA extraction from the grains showed different organisms in S1 and S2 except for *Aeribacillus pallidus* which was detected in S2 by both methods (Table 4.8). Bulk cell analysis indicated different organisms from those identified by direct DNA extraction. However from the microbial profile only *Enterobacter* was detected in both S1 and S2.

**Table 4.6**: Identities of amplimers obtained from PCR-DGGE analysis of bulk cells and directly extracted microbial DNA from maize grain (M1)

| Source | Primers | Accession number | E-value | % identity | Closest relative |
|---|---|---|---|---|---|
| | | **Direct extraction** | | | |
| ‡M1 | V6-V8 | JQ808132 | 1e-133 | 99 | *Bacillus* sp. |
| | | **Bulk cells** | | | |
| **M17 isolates** | | | | | |
| M1;MI7 37$^{O}$C | V6-V8 | CP001686 | 8.5 | 100 | *Kytococcus sedentarius* |
| M1; MI7 30$^{O}$C | V6-V8 | KC565672 | 6e-49 | 100 | *Bacillus tequilensis* |
| M1; MI7 30$^{O}$C | V6-V8 | HQ425655 | 0.0 | 100 | *Enterobacter cloacae* |
| M1; MI7 30$^{O}$C | V6-V8 | JQ396609 | 3e-51 | 99 | *Bacillus* sp. |
| M1; MI7 30$^{O}$C | V6-V8 | JQ409333 | 2e-65 | 96 | *Bacillus subtilis subsp. subtilis* |
| **MacConkey isolates** | | | | | |
| M1; Mac. 37$^{O}$C | V6-V8 | KC153127 | 9e-35 | 98 | *Pantoea cypripedii* |
| M1; Mac. 37$^{O}$C | V6-V8 | JN224975 | 0.0 | 99 | *Erwinia* sp. |
| M1; Mac. 37$^{O}$C | V6-V8 | JQ660138 | 0.0 | 99 | *Pantoea dispersa* |
| M1; Mac. 37$^{O}$C | V6-V8 | JN224975 | 9e-35 | 99 | *Erwinia* sp. |
| M1; Mac. 37$^{O}$C | V6-V8 | FJ976591 | 0.0 | 99 | *Enterobacter ludwigii* |
| **MRS isolates** | | | | | |
| M1; MRS 25$^{O}$C | V6-V8 | KC111450 | 4e-102 | 100 | *Meyerozyma caribbica* |
| M1; MRS 25$^{O}$C | V6-V8 | KC192660 | 8e-99 | 99 | *Hyphopichia burtonii* |
| M1; MRS 25$^{O}$C | V6-V8 | GQ222349 | 8e-99 | 100 | *Pichia burtonii* |
| M1; MRS 42$^{O}$C | V6-V8 | CP003729 | 7e-2 | 100 | *Amycolatopsis mediterranei* |
| M1; MRS 37$^{O}$C | V6-V8 | HE964772 | 4.5 | 100 | *Methanoculleus bourgensis* |
| **Nutrient agar** | | | | | |
| M1; NA 37$^{O}$C | V6-V8 | JQ795145 | 0.0 | 100 | *Bacillus subtilis* strain 131 |
| M1; NA 37$^{O}$C | V6-V8 | EU624423 | 5e-132 | 99 | *Bacillus subtilis* strain S8-18 |
| M1; NA 37$^{O}$C | V6-V8 | JQ905092 | 6e-49 | 97 | *Bacillus tequilensis* |
| **RBCA** | | | | | |
| M1; RBCA 25$^{O}$C | NL1;LS2 | JX999490 | 3e-67 | 97 | *Aspergillus brasiliensis* |
| M1; RBCA 25$^{O}$C | NL1;LS2 | JN089772 | 8e-36 | 95 | *Eupenicillium ochrosalmoneum* |
| M1; RBCA 25$^{O}$C | NL1;LS2 | KC469697 | 2e-63 | 98 | *Aspergillus fumigatus* |
| M1; RBCA 25$^{O}$C | NL1;LS2 | AF454150 | 1e-68 | 99 | *Aspergillus chevalieri* |
| M1; RBCA 25$^{O}$C | NL1;LS2 | FJ195795 | 2e-82 | 98 | *Aspergillus oryzae* |
| M1; RBCA 25$^{O}$C | NL1;LS2 | KC341936 | 3e-67 | 97 | *Aspergillus niger* |

‡M1: northern maize. PCR amplicons were analysed on 8% acrylamide gel with denaturing gradient range of 20-60% at 50V for 17h (bacteria) and at 170V for 4h (fungi). RBCA: Rose Bengal chloramphenicol agar.

**Table 4.7:** Identities of amplimers obtained from PCR-DGGE analysis of bulk cells and directly extracted microbial DNA from maize grain (M2)

| | Primers | Accession number | E-value | % identity | Closest relative |
|---|---|---|---|---|---|
| | | **Direct extraction** | | | |
| ‡M2 | V6-V8 | KC333049 | 0.0 | 99 | *Aeribacillus pallidus* |
| | | **Bulk cells** | | | |
| **M17 isolates** | | | | | |
| M2;MI7 37$^{O}$C | V6-V8 | JX035942 | 0.0 | 100 | *Staphylococcus sp.* |
| **MacConkey isolates** | | | | | |
| M2; Mac. 37$^{O}$C | V6-V8 | KC153127 | 0.0 | 99 | *Pantoea cypripedii* |
| M2; Mac. 37$^{O}$C | V6-V8 | JQ660138 | 0.0 | 99 | *Pantoea dispersa* |
| **MRS isolates** | | | | | |
| M2; MRS 30$^{O}$C | V6-V8 | KC192660 | 0.0 | 99 | *Hyphopichia burtonii* |
| M2; MRS 30$^{O}$C | V6-V8 | GQ222349 | 0.0 | 100 | *Pichia burtonii* |
| M2; MRS 30$^{O}$C | V6-V8 | NM001020959 | 0.0 | 100 | *Schizosaccharomyces pombe* |
| **Nutrient agar** | | | | | |
| M2; NA 37$^{O}$C | V6-V8 | JQ660138 | 0.0 | 99 | *Pantoea dispersa* |
| M2; NA 37$^{O}$C | V6-V8 | JX035942 | 0.0 | 99 | *Staphylococcus xylosus* |
| M2; NA 37$^{O}$C | V6-V8 | AB726090 | 0.0 | 100 | *Staphylococcus gallinarum* |
| **RBCA** | | | | | |
| M2; RBCA 25$^{O}$C | NL1;LS2 | HM469428 | 0.0 | 99 | *Penicillium citrinum* |
| M2; RBCA 25$^{O}$C | NL1;LS2 | JF812068 | 0.0 | 100 | *Penicillium oxalicum* |
| M2; RBCA 25$^{O}$C | NL1;LS2 | KC192660 | 0.0 | 100 | *Hyphopichia burtonii* |
| M2; RBCA 25$^{O}$C | NL1;LS2 | JN938944 | 0.0 | 100 | *Aspergillus wentii* |

‡M2: eastern maize. RBCA: Rose Bengal chloramphenicol agar.

**Table 4.8** Identities of amplimers obtained from PCR-DGGE analysis of bulk cells and directly extracted microbial DNA from sorghum grains (S1 and S2).

| Source | Primers | Accession number | E-value | % identity | Closest relative |
|---|---|---|---|---|---|
| | | **Direct extraction** | | | |
| ‡S1 | V6-V8 | JQ660138 | 0.0 | 99 | *Pantoea dispersa* |
| | V6-V8 | JN568091 | 2e-30 | 96 | *Cyanobacterium sp* |
| S2 | V6-V8 | KC333049 | 1e-163 | 99 | *Aeribacillus pallidus* |
| | | **Bulk cells** | | | |
| **M17 isolates** | | | | | |
| S1;MI7 37$^{O}$C | V6-V8 | AB647204 | 0.0 | 99 | *Acinetobacter calcoaceticus* |
| S2;MI7 37$^{O}$C | V6-V8 | KC333049 | 1e-163 | 99 | *Aeribacillus pallidus* |
| **MacConkey isolates** | | | | | |
| S1; Mac. 37$^{O}$C | V6-V8 | GQ890355 | 4e-179 | 99 | *Enterobacter aerogenes* |
| **MRS isolates** | | | | | |
| S1; MRS 30$^{O}$C | V6-V8 | KC150018 | 0.0 | 99 | *Enterococcus casseliflavus* |
| S1; MRS 30$^{O}$C | V6-V8 | JQ219685 | 0.0 | 99 | *Enterococcus gallinarum* |
| **Nutrient agar** | | | | | |
| S1; NA 37$^{O}$C | V6-V8 | HE651926 | 1e-55 | 99 | *Acinetobacter gerneri* |
| S2; NA 37$^{O}$C | V6-V8 | JQ832565 | 2e-48 | 97 | *Enterobacter cloacae* |
| **RBCA** | | | | | |
| S2; RBCA 25$^{o}$C | NL1;LS2 | EF363781 | 8e-94 | 99 | *Fusarium oxysporum* |
| S2; RBCA 25$^{o}$C | NL1;LS2 | FJ527683 | 1e-97 | 100 | *Fusarium annulatum* |

‡S1: northern sorghum, S2; eastern sorghum. RBCA: Rose Bengal chloramphenicol agar.

#### 4.2.1.2 *Ogi*

##### 4.2.1.2.1 Viable count

Microbial populations of maize and sorghum *ogi* increased by 1-4 log cfu/g compared to the grain populations and were of a similar order on MRS (6.69–7.57 log cfu/g) and nutrient agar (6.61–7.77 log cfu/g). Counts on M17 were similar to those of MRS in both maize *ogi* but differed significantly ($p<0.05$) in sorghum. Yeast and mould populations decreased significantly in only one maize *ogi* (MO2) from 6.40-3.82 log cfu/g but increased in other samples (Table 4.1). Overall both maize *ogi* (MO1 and MO2) had similar counts while both sorghum *ogi* (SO1 and SO2) differed significantly.

This result gave an indication that the microbial population dynamics in M1, S1 and S2 fermentations were more consistent with increased population in the *ogi* for all target microbial groups than in the M2. It is also notable that, with the exception of yeast and mould counts, the final counts in maize *ogi* were similar despite the significant difference in the initial grain counts. Thus the fermentation process produced similar flora composition in maize regardless of the starting counts and this was not the case in sorghum.

#### 4.2.1.2.2 Phenotypic, biochemical and physiological characterization

A total of 127 isolates were obtained from both maize (49) and sorghum (78) *ogi* among the groups of organisms investigated (Table 4.9). Preliminary screening of these isolates by morphological and biochemical characteristics presumptively revealed the presence of:

A. Oxidase negative, catalase positive, Gram negative rods (presumptive Enterobacteriaceae) from MacConkey and M17 agar.

B. Oxidase negative, catalase positive, Gram positive rods (Presumptive *Bacillus* sp.) from M17 and nutrient agar.

C. Oxidase negative, catalase negative, Gram positive cocci in clusters or in chains (presumptive *Lactococcus* or *Enterococcus* spp) from M17 agar.

D. Oxidase negative, catalase negative, Gram positive rods (presumptive *Lactobacillus*) from MRS agar.

E. Yeast and mould from Rose Bengal chloramphenicol agar.

Group A microbes identified as Enterobacteriaceae were isolated from both maize (MO1, MO2) and sorghum (SO1, SO2) *ogi* (Table 4.9). Further classification of this group into different Gram negative genera using API 20E also indicated *Enterobacter* and *Pantoea* as dominant genera in all *ogi* samples.

Group B isolates were detected in all *ogi* samples and showed good growth with diagnostic colonies of *B. cereus* or *B. thuringiensis* (peacock blue colour with egg yolk precipitate).

Group C were identified in MO1, MO2, SO1 and SO2. Two different cell arrangements were observed (Fig 4.1b: C2a and C2b). These isolates were examined as presumptive *Enterococcus* by cultivation on KF streptococcus agar) and identified by red colonies and confirmed by the Lancefield test. All isolates in this group, except MO113, MO1318, MO231, SO132, SO133 and SO137, gave red colonies on KF agar and showed agglutination with Lancefield Group D and therefore were identified as *Enterococcus* sp. Representative isolates from this group were further tested with API 20 Strep kit for species identification by enzymatic activity and sugar fermentation. Results obtained identified all of the isolates as *Enterococcus* sp with ID>99% and t=0.95. Species mostly identified were *E. faecium*, *E. casseliflavus* and *E. durans.*

**Table 4.9:** ‡Microbial groups identified in maize and sorghum *ogi.*

| ***Ogi*** | **A** | **B** | **C** | **D** | **E** |
|---|---|---|---|---|---|
| | **Enterobacteriacae** | ***Bacillus*** | ***Lactococcus*** | **Total lactic acid bacteria** | **Yeast & mould** |
| **MO1** | 10 | 3 | 2 | 2 | 1, 3 |
| **MO2** | 2 | 11 | 7 | 8 | 3, 6 |
| **SO1** | 3 | 24 | 14 | 14 | 2, 2 |
| **SO2** | 6 | 18 | 3 | 4 | 3, 2 |

*Two different sources of grains: 1 northern and 2 eastern; maize *ogi* (MO) and sorghum *ogi* (SO).
‡Number of randomly isolated microbes and those in red represents the number of moulds.

Group D isolates presumptively identified as lactic acid bacteria (*Lactobacillus*) were detected only in sorghum *ogi*. Identification by API 50CHL showed the presence of *Lactobacillus delbrueckii* (SO213).with %ID = 90.9% and t=0.3.

Group E isolates classified as yeast and moulds were present in both maize and sorghum *ogi*, with yeast as the dominant group in sorghum *ogi*. Moulds presumptively identified in sorghum *ogi* were: *Penicillium* sp (SO156) and *Cladosporium* sp (SO151) in SO1 with *Cladosporium* sp dominant in SO2. In maize *ogi* (MO1) were species of *Aspergillus* (MO151), *Penicillium* (MO152) and *Fusarium* (MO153), while *Penicillium* (MO251), *Cladosporium* (MO253), and *Aspergillus* (MO257) were mostly present in MO2 (Fig.4.3a-b).

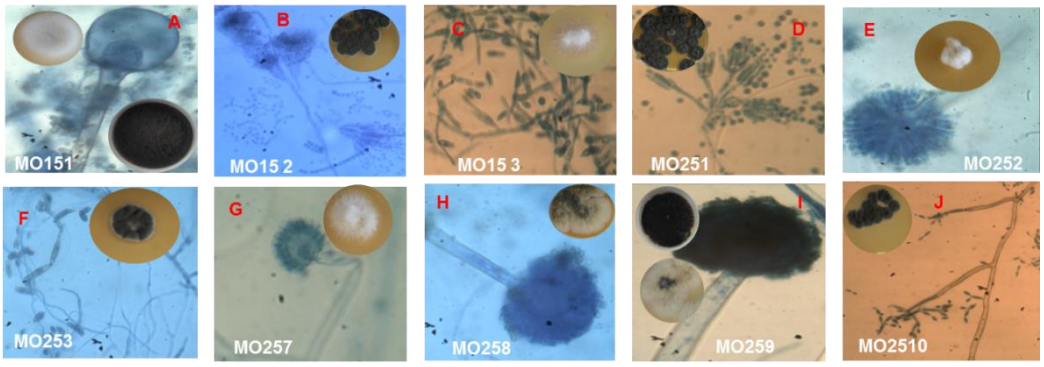



**Figure 4.3a**: Moulds identified in maize *ogi.* Isolates were identified by morphological characteristics by wet mount in lactophenol cotton blue under a microscope (bright-field at x1000 magnification). A, E, G, H and I: *Aspergillus* sp., B and D *Penicillium* sp., C: *Fusarium* sp., F and J: *Cladosporum* sp.

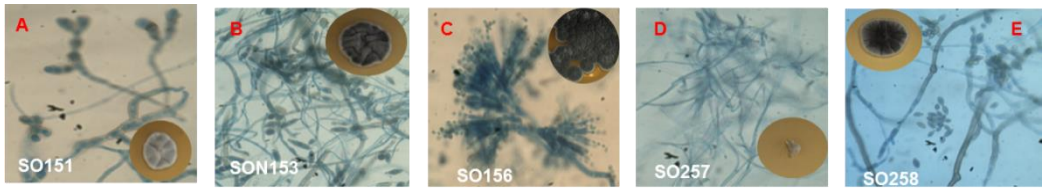



**Figure 4.3b**: Moulds identified in sorghum *ogi.* Isolates were identified by morphological characteristics by wet mount in lactophenol cotton blue under a microscope (bright-field at x1000 magnification). A, B and E: *Cladosporum* sp., C: *Penicillium* sp., D: not identified.

#### 4.2.1.2.2.1 Screening of residual moulds in *ogi* for mycotoxigenic potentials

The presence of biosynthetic pathway genes for mycotoxin (aflatoxin, zearalenone and ochratoxin) production in the fungal isolates was tested (§3.2.1.4). *Aspergillus oryza, Aspergillus tubingensis, Penicillium biourgeianum, Fusarium oxysporum, Cladosporum cladosporioides* and *Cladosporum macrocarpum* detected in *ogi* were investigated for mycotoxin production potential by PCR targeting the ochratoxin, zearalenone, and aflatoxin producing species using the gene primers shown in Table 3.4 to amplify the target regions of the structural genes following the conditions outlined in Table 3.5.

This preliminary screening showed that none of the mycotoxin biosynthetic genes tested were detected by PCR for all the mould species investigated; an example of the results is shown in Fig.4.4.

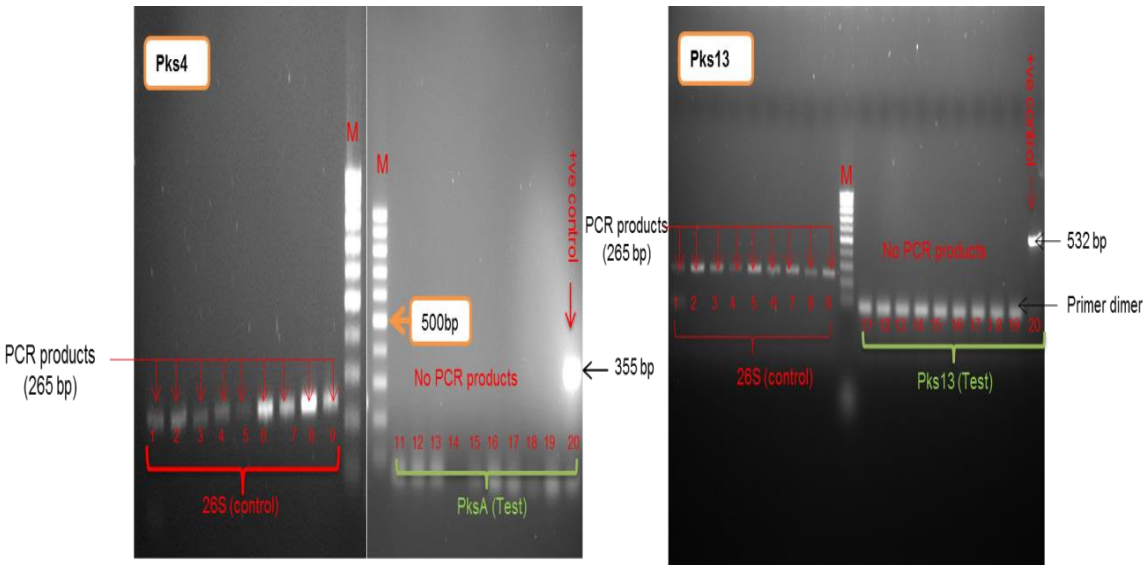



**Figure 4.4:** Detection of zearalenone genes in mould isolates from maize *ogi* by PCR. 1, 11 = *Aspergillus oryzae;* 2, 12 = *Penicillium biourgeianum;* 3, 13 = *Fusarium oxysporum; 4, 14 = Penicillium sp.;* 5, 15 = *Aspergillus sp*.; 6, 16 = *Cladosporum sp.;* 7, 17 = *Cladosporium* sp.; 8, 18 = *Aspergillus tubingensis*, 9, 19 = *Cladosporum cladosporioides,* 20 = *Fusarium graminearum* (positive control), M= 100bp DNA ladder *PKS13 and PKSA = polyketide synthase gene primers for zearalenone. Expected band size: Pks4:355 bp; Pks13: 532 bp. Bands were separated on 1 % agarose gel in 1x TAE buffer at 70 V for 1h 30 min. Blanco

#### 4.2.1.2.2.2 Growth and characterization of substrate utilization and acid tolerance of LAB isolates

Lactic acid bacteria (LAB) are one of the important groups of microbes in cereal fermentation. To better understand the physiological behaviour and survival potential of the LAB isolates during natural fermentation of *ogi* and to enhance the selection of important species for use as starter cultures, assessment of carbohydrate utilization, optimal growth temperature (at 4, 15, 25, 30, 37, 42, and 55$^{o}$C for 12 and 24 h) and acid tolerance were carried out on selected group 3 and 4 isolates. The results of growth at different temperatures and carbohydrate utilization and the identities of LAB are presented in Tables 4.10 and 4.11. Glucose, fructose, sucrose, mannose, cellobiose, and maltose were mostly fermented while rhamnose was the least utilized. Only 17 isolates were able to ferment glucose and produce $CO_2$ indicating the presence of 17 heterofermentative LAB species in *ogi* fermentation. Spectrophotometric measurement of the degree of microbial growth at pH 6.2 after 24 h of incubation at $A_{600}$ showed significant increase in cell density of 8 isolates (MO131, MO132, MO134, MO233, SO131, SO132 SO134 and SO137) which exhibited optimal growth at 30-37$^{o}$C with absorbance of 1.6-1.8 at 600nm. When tested for acid tolerance, a similar growth pattern was observed for both maize and sorghum isolates. At pH 3, growth was observed in 3 h but plateaued when tested at 24 h (Fig.4.5) However, no significant growth was observed in all the isolates at pH 2 from 2-24 h (Fig.4.5)

**Table 4.10:** Growth and carbohydrate fermentation of LAB isolates from *ogi.*

| **Test** | **†n=52** | | |
|---|---|---|---|
| | +ve | w+ | -ve |
| Glucose | 40 | 7 | 5 |
| Fructose | 40 | 7 | 5 |
| Lactose | 18 | 22 | 12 |
| Mannitol | 15 | 10 | 27 |
| Sucrose | 36 | 4 | 12 |
| Galactose | 28 | 12 | 12 |
| Mannose | 39 | 5 | 8 |
| Sorbose | 14 | 4 | 34 |
| Sorbitol | 16 | 15 | 21 |
| Inositol | 11 | 8 | 33 |
| Cellobiose | 35 | 4 | 13 |
| Melibiose | 22 | 9 | 21 |
| Maltose | 38 | 5 | 9 |
| Raffinose | 17 | 20 | 15 |
| Rhamnose | 7 | 4 | 41 |
| Gas production (Glucose) | 17 | 2 (ND) | 37 |
| | | | |
| Growth (†n=17) | +ve | -ve | |
| 4$^{o}$C | 3 | 14 | |
| 15$^{o}$C | 16 | 1 | |
| 25$^{o}$C | 17 | 0 | |
| 30$^{o}$C | 17 | 0 | |
| 37$^{o}$C | 17 | 0 | |
| 42$^{o}$C | 9 | 8 | |
| 55$^{o}$C | 16 | 1 | |

†Number of isolates tested. Growth was tested in MRS broth for presumptive heterofermentative isolates. W+: weak positive, ND: not detected. Increase in media turbidity reflects bacterial growth and was recorded as positive.

**Table 4.11:** Identities of LAB isolates from maize and sorghum *ogi.*

| **isolates** | **Species identity** |
|---|---|
| MO131 | *Enterococcus* sp |
| MO132 | *Enterococcus durans* Hmc 2 |
| MO134 | *Enterococcus* sp |
| MO233 | *Enterococcus faecium* |
| SO131 | Not identified |
| SO132 | *Pediococcus pentosaceus* strain RU28-2 |
| SO134 | *Enterococcus* sp |
| SO137 | *Pediococcus pentosaceus* strain GC16 |

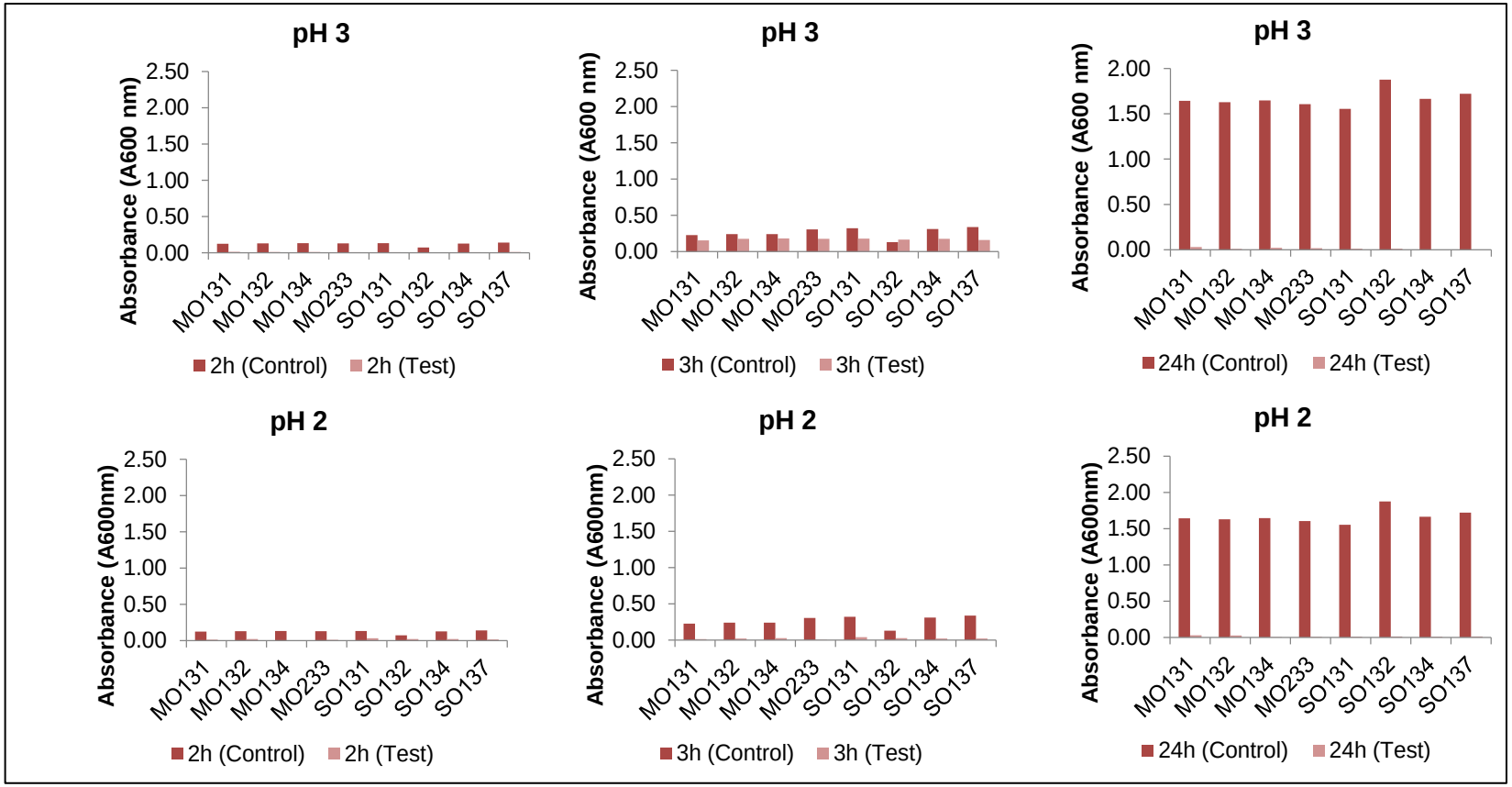



**Figure 4.5**: Acid tolerance of LAB isolates in MRS broth. Growth was monitored for 2, 3 and 24 h at: pH 2 and 3 (tests) and pH 6.2 (controls). Incubation temperature: 37$^{o}$C, MO: maize *ogi*, SO: sorghum *ogi.*

#### 4.2.1.2.3 Fingerprinting of bacterial isolates by Pulsed Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis is thought to be a highly discriminating molecular tool suitable for typing of a wide range of microbes. Dereplication of Isolates presumptively identified as lactic acid bacteria was carried out by this technique in order to assess their relatedness and select representative isolates for identification by sequencing. Isolates from different *ogi* samples classified as LAB (based on phenotypic properties) were investigated by comparison of their DNA fingerprints obtained from restriction enzyme digest with *Sma*I and analysis of fragments by PFGE as outlined in §3.2.3.6.3.

All bacterial isolates on MRS agar were identified as cocci by microscopic examination. Seven band patterns were visually evident out of 12 different PFGE pulsotypes (Appendix. 4.3a) identified in both maize and sorghum *ogi*. The number of bands ranged from 14 -20 with molecular weight ranging between 15,000–291,000bp as estimated from the mid-range PFGE marker.

DNA fingerprints of the isolates were compared by FPQuest software using the Dice coefficient and the Unweighted Pair Group Method of Arithmetic mean (UPGMA) at 1.0% optimization and 1.0% band tolerance position. Lanes without bands were not analysed. Overall analysis of the dendogram (Fig. 4.6a) revealed seven clusters with 2 outliers to groups I (SO136) and IV (MO237). Comparison at similarity level of 79% ($p<0.01$) showed 4 clusters with similarities between sub groups ranging from 82.1%-91.4%.

Microscopic examination of isolates on M17 agar revealed both cocci and rod-shaped bacteria. Different band patterns were observed although most of the isolates (63%) separated at a much lower level than the others. However up to 19 different pulsotypes were visually identified (Appendix.4.3b). These showed more than 15 bands with molecular weights ranging between 150–194,000bp as estimated from the low-range PFGE marker.

Overall comparison of the DNA fingerprints of M17 isolates by Dice coefficient and the Unweighted Pair Group Method of Arithmetic mean (UPGMA) at 1.0% optimization and 1.0% band tolerance position showed 21 clusters with 6 outliers (Fig. 4.6b). Normally this approach is used to compare isolates of same species but here different bacteria were compared in an attempt to group and select representative isolates for sequencing and as such related patterns were less expected. Similarity comparison at 54% ($p<0.01$) indicated 5 clusters with similarities between sub groups ranging from 62%-100%. Three outliers to group II (SO213 and SO11) and group VI (SO212) were observed. The PFGE pattern showed some heterogeneity and isolates from different sources were observed in the same cluster except clusters IV and VI. It is also notable that some isolates (SO113:SO118, MO116:MO119 and MO212:MO215) were indistinguishable by the *Sma*I restriction profile used in this study and showed 100% similarity.

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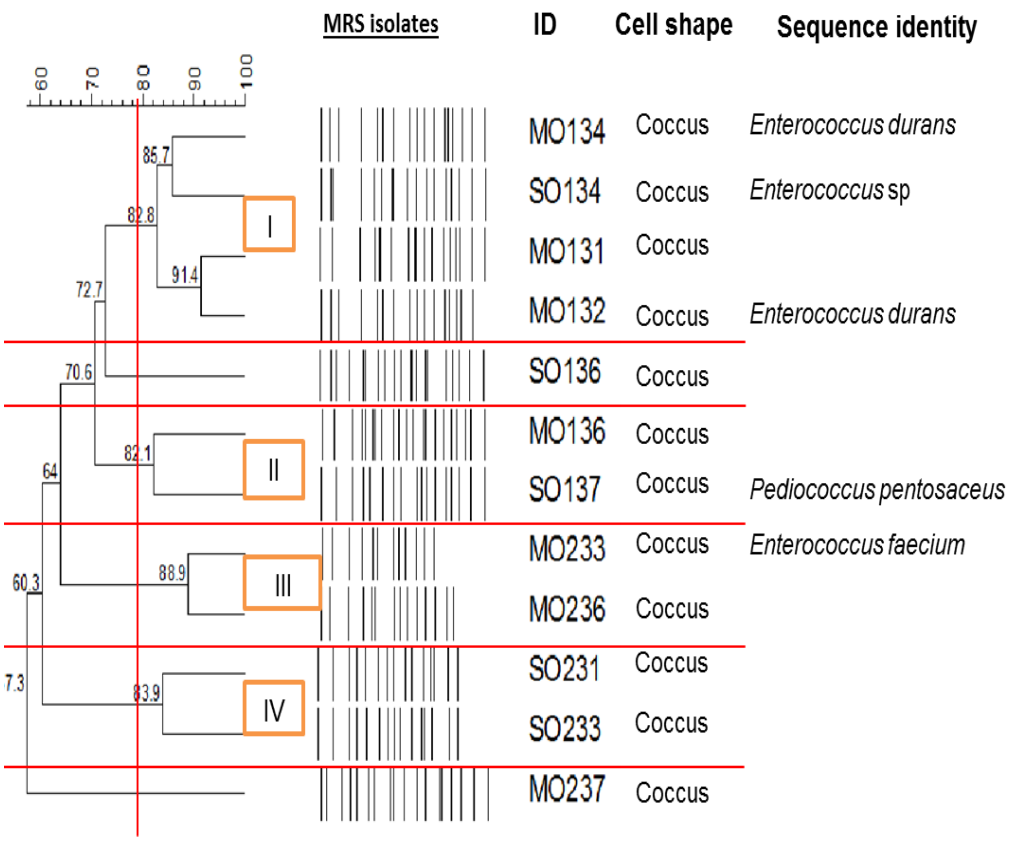



**Figure 4.6a:** Similarities of pulsotypes of MRS isolates from *ogi* analysed using Dice coefficient and UPGMA cluster analysis by FPQuest software at 1.0% optimization and 1.0% band tolerance position. MO= isolates from maize *ogi.* SO= isolates from sorghum *ogi.*

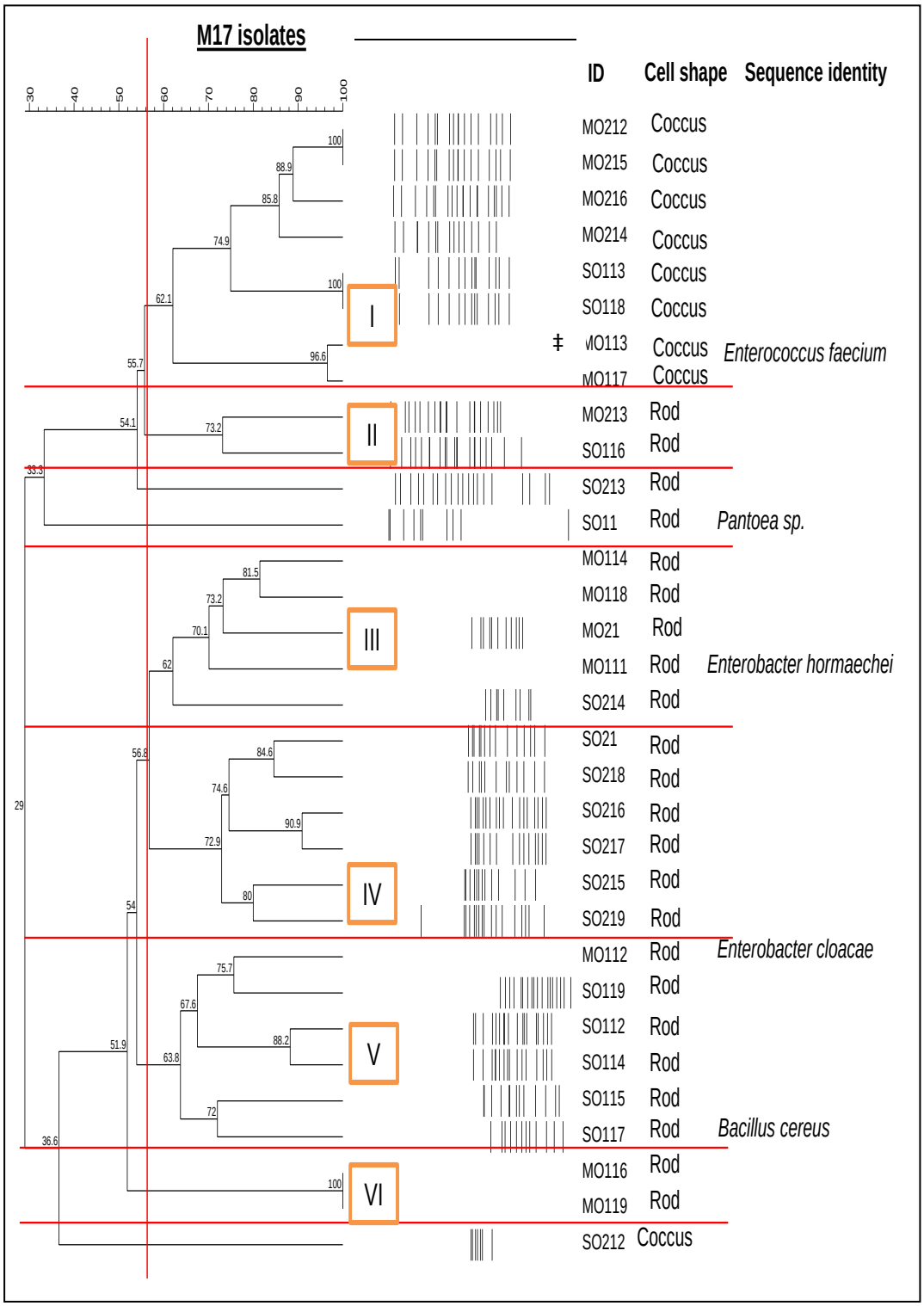



**Figure 4.6b:** Similarities of pulsotypes of M17 isolates from *ogi* analysed using Dice coefficient and UPGMA cluster analysis by FPQuest software at 1.0% optimization and 1.0% band tolerance position. MO= isolates from maize *ogi.* SO= isolates from sorghum *ogi,* MO= isolates from maize *ogi,* SO= isolates from sorghum *ogi.* ‡Bands formed at lower levels (Appendix 4.3b) are beyond the width of the PFGE pattern shown and only the upper levels without bands are seen in the clusters.

#### 4.2.1.2.4 Identification by sequencing of 16SrRNA bacterial and 26S rRNA fungal genes

Representative isolates were selected for sequence analysis, from each group of microbes based on cultural identification and cluster analysis of PFGE DNA fingerprints. PCR amplification of 16S rRNA genes and 26S rRNA fungal genes were carried out as in Section 3.2.3.5.1. Results of nucleotide BLAST analysis of the DNA sequences of microbial isolates are shown in Table 4.12. Microbial identifications obtained by sequencing were very similar to those presumptively obtained by phenotypic methods. *Enterobacter*, *Pantoea* and *Enterococcus* species were the dominant organisms identified in both maize and sorghum *ogi* as indicated by the sequence result, however a combination of the microbiological count and molecular identification over the fermentation period would give a better identification of the dominant species. *Pediococcus pentosaceus* and *Bacillus* sp were also detected in sorghum *ogi* although *P. pentosaceus* was not fully identified by the API technique. Both phenotypic and molecular methods identified similar species of moulds in both maize and sorghum *ogi*. *Aspergillus* and *Fusarium* sp were present in maize while *Cladosporum* was identified in both maize and sorghum *ogi* (Table 4.12). *Fusarium* sp detected in sorghum grain was not found in the *ogi.*

#### 4.2.1.2.5 Analysis of microbial community in *ogi* by denaturing gradient gel electrophoresis (DGGE)

Results of DGGE analysis of the microbial diversity of *ogi* from PCR amplification of 16S (V6-V8 region) and 26S rRNA of bacterial and fungal genes respectively, extracted directly from the *ogi* samples and bulk cells are presented in Table 4.13. Diversity of microbes observed with the direct microbial DNA extraction from the samples and the DNA extraction from cultured cells varied in each *ogi* although not all representative band amplimers after purification produced the required DNA concentration for sequencing. The DGGE profile image of direct DNA extraction showed bands ranging from 1-5 in maize and 1-6 in sorghum. *Serratia* sp and *Enterobacter* sp (Enterobacteriaceae) were identified in maize *ogi* with 98-99% ID and

a lactic acid bacterium (*Lactobacillus rossiae,* 96% ID) was only identified in sorghum *ogi*.

**Table 4.12:** Identities of 16S and 26S rRNA gene sequence of PCR amplimers (with V3 and NL1: LS2 primers respectively) from pure isolates from maize and sorghum *ogi.*

| *Source | Primer | Accession number | E-value | % identity | Closest relative |
|---|---|---|---|---|---|
| **M17 isolates 30°C** | | | | | |
| MO111 | V3 | KC315760 | 0.0 | 100 | *Enterobacter hormaechei* |
| MO112 | V3 | GU459207 | 4e-76 | 100 | *Enterobacter cloacae* |
| SO111 | V3 | KC833511 | 5e-74 | 99 | *Pantoea sp.* |
| SO113 | V3 | JX275809 | 1e-71 | 99 | *Enterococcus faecium* |
| SO117 | V3 | HE804778 | 2e-74 | 100 | *Bacillus cereus* |
| **MacConkey isolates 30°C** | | | | | |
| MO121 | V3 | JX112354 | 2e-75 | 100 | *Enterobacter* sp. |
| SO121 | V3 | AY659872 | 2e-75 | 100 | *Pantoea* sp. |
| **MRS isolates, 30°C** | | | | | |
| MO131 | V3 | DQ305313 | 2e-69 | 99 | *Enterococcus* sp |
| MO132 | V3 | KC845211 | 6e-37 | 100 | *Enterococcus durans* Hmc 2 |
| MO134 | V3 | KC845211 | 4e-71 | 99 | *Enterococcus durans* Hmc 2 |
| MO135 | V3 | KC884694 | 3e-73 | 99 | *Pantoea sp* MHK15 |
| SO132 | V3 | KC836678 | 3e-72 | 99 | *Pediococcus pentosaceus* strain RU28-2 |
| SO133 | V3 | KC836678 | 3e-72 | 100 | *Pediococcus pentosaceus* strain GC16 |
| SO134 | V3 | DQ305313 | 1e-71 | 100 | *Enterococcus* sp |
| SO135 | V3 | DQ305313 | 4e-71 | 100 | *Enterococcus* sp |
| SO137 | V3 | JN851781 | 3e-72 | 100 | *Pediococcus pentosaceus* strain GC16 |
| **RBCA isolates, 25°C** | | | | | |
| MO151 | NL1:LS2 | KJ650341 | 0.0 | 100 | *Aspergillus oryzae* |
| MO152 | NL1:LS2 | HM469395 | 0.0 | 100 | *Penicillium biourgeianum* |
| MO153 | NL1:LS2 | HQ377257 | 0.0 | 100 | *Fusarium oxysporum* |
| MO259 | NL1:LS2 | KJ650338 | 0.0 | 100 | *Aspergillus tubingensis* |
| MO2510 | NL1:LS2 | KF611802 | 0.0 | 100 | *Cladosporum cladosporioides* |
| SO151 | NL1:LS2 | KC311478 | 0.0 | 100 | *Cladosporum macrocarpum* |

*Red numbers represent two different sources of grains: 1 northern and 2 eastern; maize (M) and sorghum (S).

The result of DGGE profile analysis of cultivable community of 16S rRNA sequence of bacterial and 26S rRNA sequence of fungal genes amplified revealed a wider microbial community in both maize and sorghum *ogi* with profile images showing 1-8 bands in maize and 1-3 bands in sorghum (Appendix 4.5). Microbial species identified by bulk cell method differed from that of direct DNA extraction except for *Enterobacter* sp detected by both methods in maize *ogi*. *Aspergillus* sp and *Penicillum* sp were detected in maize and sorghum *ogi* respectively. DGGE PCR amplimers of cultivated cells of sorghum *ogi* were not all identified due to low DNA recovery. However of all the identified genera, *Enterobacter* was dominant in maize *ogi*.

**Table 4.13** Identities of amplimers obtained from PCR-DGGE analysis of bulk cells and directly extracted microbial DNA of maize and sorghum *ogi*

| ‡Isolates | Primers | Accession number | E-value | % identity | Closest relative |
|---|---|---|---|---|---|
| | | **Direct DNA extraction** | | | |
| MO1 | V6-V8 | EF585319 | 0.0 | 99 | *Serratia marcescens* |
| | V6-V8 | JN542917 | 0.0 | 99 | *Enterobacter* sp. |
| SO1 | V6-V8 | JN863606 | 0.0 | 96 | *Lactobacillus rossiae* |
| | V6-V8 | HM804849 | 0.0 | 100 | *Streptomyces sp.* |
| | | **Bulk cells** | | | |
| **M17 isolates** | | | | | |
| MO1:MI7 37$^{O}$C | V6-V8 | GQ890353 | 0.0 | 99 | *Enterobacter cloacae* |
| **MacConkey isolates** | | | | | |
| MO1: Mac. 37$^{O}$C | V6-V8 | GQ890353 | 0.0 | 100 | *Enterobacter cloacae* |
| **MRS isolates** | | | | | |
| MO1: MRS 25$^{O}$C | V6-V8 | KC990791 | 0.0 | 100 | *Enterobacter cloacae* |
| MO1: MRS 25$^{O}$C | V6-V8 | HE653017 | 0.0 | 99 | *Pantoea agglomerans* |
| **Nutrient agar** | | | | | |
| MO1: NA 37$^{O}$C | V6-V8 | CP000303 | 0.0 | 100 | *Bifidobacterium breve* |
| MO1: NA 37$^{O}$C | V6-V8 | JX946303 | 0.0 | 100 | *Enterobacter asburiae* |
| **RBCA** | | | | | |
| MO1:RBCA 25$^{o}$C | NL1:LS2 | U15508 | 0.0 | 100 | *Aspergillus avenaceus* |
| MO1:RBCA 25$^{o}$C | NL1:LS2 | FJ195795 | 0.0 | 100 | *Aspergillus oryzae* |
| MO1:RBCA 25$^{o}$C | NL1:LS2 | KC146369 | 0.0 | 100 | *Emericella nidulans* |
| MO1:RBCA 25$^{o}$C | NL1:LS2 | KC589439 | 0.0 | 100 | *Aspergillus flavus* |
| SO1:RBCA 25$^{o}$C | NL1:LS2 | JN938947 | 0.0 | 100 | *Penicillium brevicopactum* |
| SO1:RBCA 25$^{o}$C | NL1:LS2 | KC146370 | 0.0 | 100 | *Penicillium aurantiogriseum* |

‡MO: maize *ogi*, SO: sorghum *ogi,* RBCA: Rose Bengal chloramphenicol agar

### 4.2.2 Assessment of other important microbes in *ogi* and grains

#### 4.2.2.1 Acetic acid bacteria (AAB)

Acetic acid bacteria (AAB) have been reported as part of the microbial community in indigenous fermented foods (Schwan and Romos, 2015) which may undergo an extended period of fermentation for up to 72 h. Fifty isolates were obtained from maize (MO1:15 and MO2:15) and sorghum (SO1:8 and SO2:12) *ogi* as described in §3.2.1.1 and presumptively identified as Gram negative, oxidase negative, catalase positive, motile or immotile, non-spore forming, ellipsoidal to rod-shaped which is characteristic of the genus *Acetobacter*. Acetic acid bacteria are not able to produce indole from deamination of tryptophan or gas from glucose fermentation. Therefore for further classification these isolates were assessed for glucose utilization, gas and indole production. All isolates were indole negative. Eight isolates designated as X isolates (MO1=7 and MO2=1) fermented glucose and produced $CO_2$ while 23 isolates (Y isolates) did not ferment glucose and therefore were not AAB. However 19 isolates, Z isolates (MO1=5, SO1=12 and SO2=2) only fermented glucose without gas production and were presumptively identified as AAB.

Characterization by ERIC PCR (§3.2.3.5.3) to further assess, group and select isolates for identification by sequencing, revealed DNA fingerprints with 1-11 DNA fragments of 100-5,000 bp after gel electrophoresis of the PCR products. Presumptive AAB patterns were grouped into 6 clusters at 62.5% ($p<0.01$) Dice coefficient of similarity (Fig.4.7). Clusters I and III were of maize isolates with coefficient of similarities within groups of ≥ 80% and 74.3% respectively, while, IV, V, and VI were of sorghum isolates showing similarities of ≥ 56.7%, 71.1% and 63.5% within groups in each case. Clusters II showed mixed isolates from maize and sorghum with similarities ranging from 74.3% to 100%. Isolates grouped as X and Y were found in clusters III and V respectively, while group Z isolates were found in clusters IV and VI. Surprisingly 16S rRNA sequence identification of representative isolates from group Z presumptively identified as AAB did not indicate the presence of any AAB genera in the group instead different strains of *Enterobacter* species were dominant in both maize and sorghum *ogi* as demonstrated by other analyses.

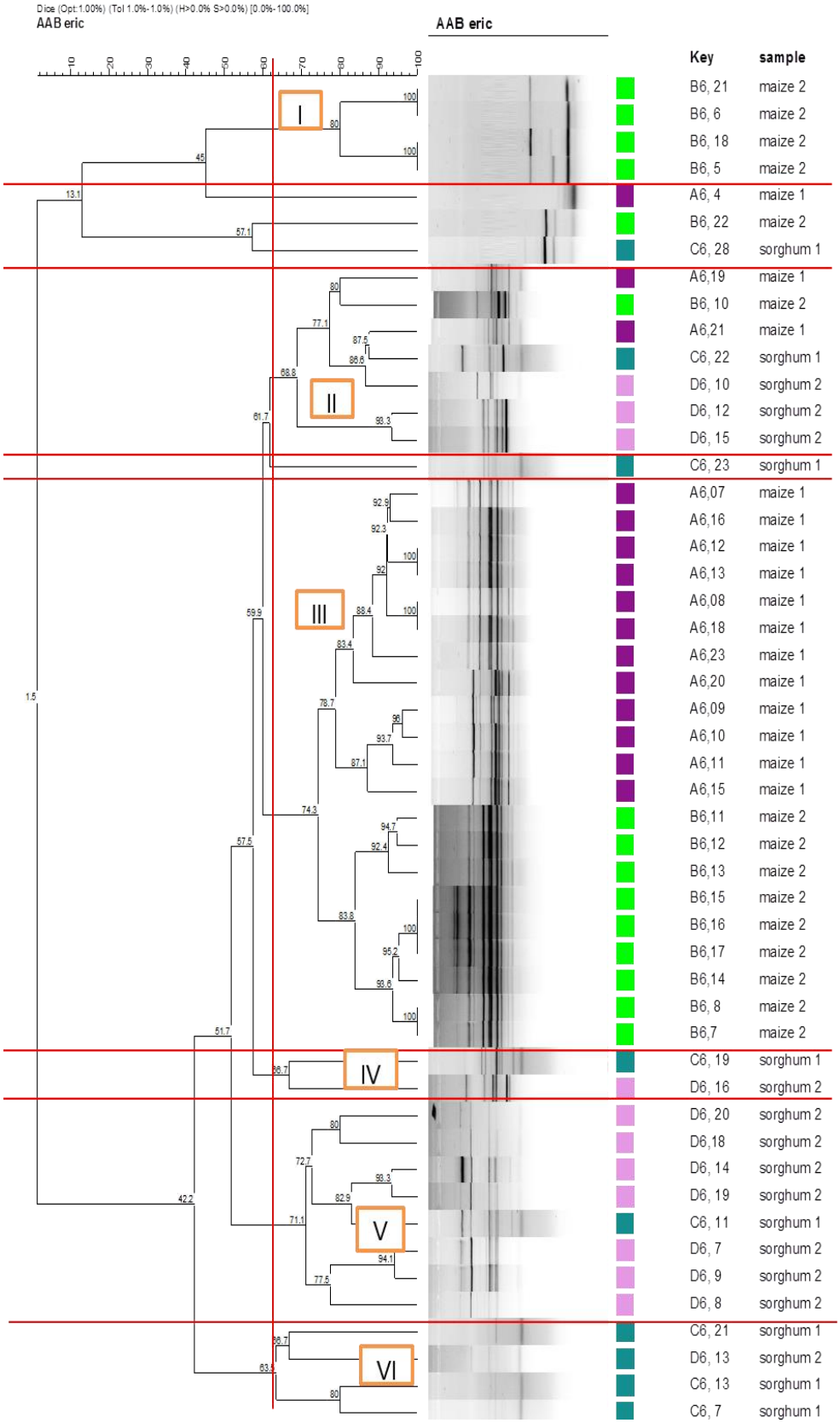



**Figure 4.7:** Dendogram obtained from ERIC PCR fingerprints of presumptive acetic acid bacterial (AAB) isolates from *ogi* analysed by FPQuest software at 1.0% optimization and 1.0% band tolerance position. Two sources of *ogi* from 1: northern and 2: eastern. Source of the isolates are in colour bars MO1: A (**purple**), MO2: B(**green**), SO1: C (**dark cyan**) and SO2: D (**pink**).)

#### 4.2.2.2 Phytate hydrolysing fungi (PHF)

The presence of phytate hydrolysing fungi (PHF) in maize and sorghum grains was also investigated (§3.2.1.2). These were identified morphologically by appearance, hyphae and spore characteristics. Four fungal isolates from maize and sorghum grains were morphologically characterised (Table 4.14a-b). Four isolates were recovered; one from each maize (MG1 from M1; MG2 from M2) and sorghum (SG1 from S1; SG2 from S2) grain sample from both regions. All isolates showed cleared zone around colonies on CPSM which indicates phytase production (Fig. 4.8). Only MG1 and SG2 isolates showed good growth on CPSM with colony diameters ranging from 20–27 mm and 37-85 mm, respectively. MG2 had suppressed growth on CPSM and did not grow at all on CDA, CYA and CREA media. SG1 and SG2 showed poor to moderate growth on CREA medium and changed the colour around the cleared zone from blue to yellow indicating acid production.

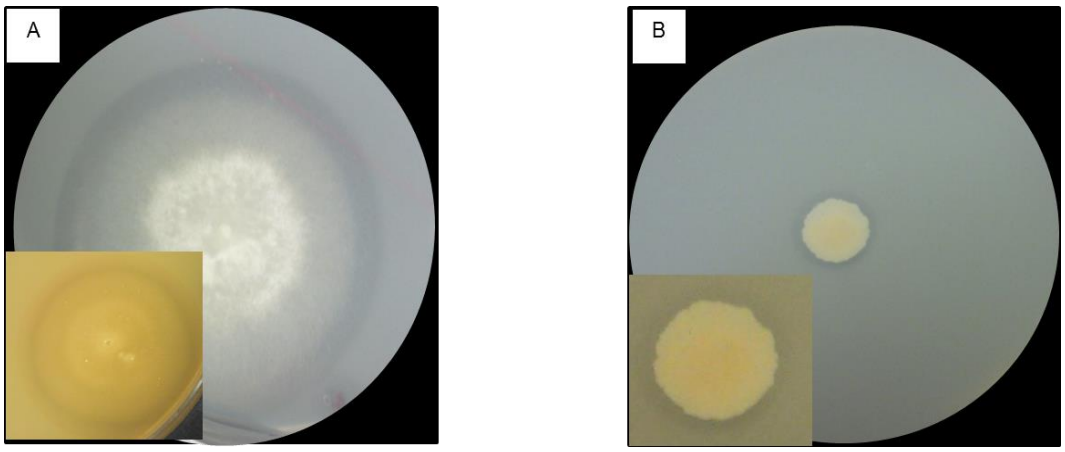



**Figure 4.8:** Fungal isolates showing cleared zone on phytate specific medium plate containing 0.5 % calcium phytate pH 6.5 after 5 days incubation at 30 $^{o}$C. **A**: SG1 *Trametes hirsuta*, **B**: MG2- *Acremonium zeae.*

**Table 4.14a**: Morphological characterization of phytate hydrolysing fungi on different growth media and identification (maize isolates)

| Fungal isolates* | †Medium | Colony characteristics | | | | | | % Identity | Accession number | 26 S rRNA sequence identification |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Average colony size (mm) | texture | Colony colour | | sporulation | Reaction on agar plate | | | |
| | | | | surface | reverse | | | | | |
| **MG1** | CPSM | 27 | Velvety | cream periphery, pink sporulation centre | Peach / cream | moderate | clearing zone around colony | 99 | GQ167229 | *Acremonium zeae* |
| | CDA | 27 | Velvety | cream periphery, peach sporulation centre | | moderate | - | - | - | - |
| | CREA | 20 | Velvety | green periphery, cream sporulation centre | | heavy | - | - | - | - |
| | CYA | 27 | Velvety | peach, raised sporulation centre | | heavy | - | - | - | - |
| **MG2** | CPSM | 13 | Smooth | cream and moist | cream | - | clearing zone around colony | 98 | GQ167229 | *Acremonium zeae* |
| | CDA | ng | | - | - | - | - | - | - | - |
| | CREA | ng | | - | - | - | - | - | - | - |
| | CYA | ng | | - | - | - | - | - | - | - |

MG1; MG 2 (maize isolates) CPSM: calcium specific medium, CDA: czapek dox agar, CREA: creatine sucrose agar; CYA: czapek yeast agar.

**Table 4.14b**: Morphological characterization of phytate hydrolysing fungi on different growth media and identification (sorghum isolates)

| | | Colony characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Colony colour | | | | | | |
| Fungal isolates* | Medium | Average colony size(mm) | texture | surface | reverse | sporulation | Reaction on agar plate | % Identity | Accession number | 26 S rRNA sequence identification |
| **SG1** | CPSM | 9 | powdery | cream periphery, white sporulation centre | cream | moderate | - | 99 | AY855910 | *Trametes hirsuta* |
| | CDA | 10 | velvety | white raised centre | cream | moderate | - | | | |
| | CREA | 5 | | cream, raised centre | light yellow, | | poor growth, zone clearing around the colony | | | |
| | CYA | 38 | velvety | cream periphery, white sporulation centre | white periphery, cream sporulation centre | Heavy | - | | | |
| **SG2** | CPSM | 37 | velvety | brown periphery, cream sporulation centre | Grey/ slightly cream | Heavy | clearing zone around the colony | 100 | KF590135 | *Bipolaris oryzae* |
| | CDA | 90 | velvety | green periphery, grey sporulation centre | brown with veins | Heavy | - | | | |
| | CREA | 22 | velvety | green periphery, grey sporulation centre | cream | Heavy | moderate growth, zone clearing around colony | | | |
| | CYA | 85 | powdery | brown periphery, grey sporulation centre | black | Heavy | | | | |

SG1; SG 2 (sorghum isolates) CPSM: calcium specific medium, CDA: czapek dox agar, CREA: creatine sucrose agar; CYA: czapek yeast agar

An overall assessment of growth of these fungal isolates on the different media suggests acid tolerance potentials making them good candidates for acid fermentations as associated with production of *ogi*. Microscopy results of the morphological characteristics showed similar results for MG1 and MG2.

The nuclear large subunit ribosomal RNA (26S) has been reported to be more diverse in characterization and identification of fungal species (Kurtzman and Robnett, 1998; Sugita and Nishikawa, 2003). Identification of the four phytate hydrolysing fungal isolates by PCR amplification of 26S rRNA gene and sequencing revealed both maize isolates (MG1 and MG2) as closest relatives to *Acremonium zeae* (GQ167229, 98-99% ID and E-value of 6e-92: 3e-95) and sorghum isolates SG1:*Trametes hirsuta* (AY855910, 99% ID and E-value of 6e-102) of the edible species of white-rot fungi and SG2: *Bipolaris oryzae* (KF590135, 100% ID and E-value of 2e-101).

#### 4.2.2.2.1 Phytase activity

The level of inorganic phosphate released by the isolates was used to determine phytase activity as described in §3.5.2. Not all isolated fungal species showed phytase activity when tested enzymatically. *Acremonium zeae* (MG1) released 46.01 µmol and *Trametes hirsuta* (SG1) 34.1 µmol of phytase. *Bipolaris oryzae* (SG2) and *Acremonium zeae* (MG2) did not show significant phytase activity.

### 4.2.3 Starch hydrolysis

Cereal grains are high in starch content. Monosaccharides and disaccharides which are readily available for fermentation by microbes are naturally present in cereal grains (Díaz-Ruiz *et al*., 2003). However availability of these simple carbohydrates may depend on food processing methods and utilization by microbes during fermentation which could cause depletion, making starch the major available substrate for fermentation. Therefore the amylolytic characterization of bacterial and fungal isolates in *ogi* is important to understand their significance during natural fermentation. All isolates classified as LAB and moulds in *ogi* as well as phytate hydrolysing moulds

were tested for amylolytic properties (§3.2.2.11**)**. Bacterial isolates tested showed negative starch hydrolysis. Eight out of 15 tested mould isolates from *ogi* showed positive hydrolysis. These include MO151 (*Aspergillus oryzae*), MO152 (*Penicillium biourgeianum*), MO251 (*Penicillium* sp), MO252 and MO257 (*Aspergillus* sp), SO151 (*Cladosporum macrocarpum*), SO156 (*Penicillium* sp) and SO256 (*Cladosporum* sp). *Fusarium oxysporum* (MO153) showed a weak positive reaction with very small hydrolysis diameter. Only two of the phytate hydrolysing fungi (MG1: *Acremonium zeae* and SG1: *Trametes hirsuta*) showed positive starch hydrolysis.

### 4.3 Discussion

A combined approach of cultural and molecular methods of analysis was applied to investigate the microbial community of *ogi* and grains. The goal of this study was to identify the predominant bacterial and fungal species and their levels during fermentation of *ogi.* Traditional preparation of fermented foods involves mixed culture fermentation with complex microbial flora due to uncontrolled inoculation. Understanding the taxonomy of the microbial community in these foods has been a major challenge. The microbiology of *ogi* is complex and is yet to be elucidated. Microbial ecology of traditionally fermented amylaceous cereal foods such as *ogi* have mostly been studied by culture dependent techniques (Nout, 1991; Halm *et al*., 1993), however recent studies (Ampe and Miambi, 2000; Oguntoyinbo *et al*., 2011) have reported on the use of culture independent approaches such as rDNA PCR with amplimers separated by denaturing gradient gel electrophoresis (DGGE) to study the bacterial community of fermented maize products such as *pozol*, *poto-poto* and *ogi*. While each study has focused mostly on diversity of the bacterial community of traditionally pre-made *ogi*, the coexistence and impact of the fungal community which may influence the metabolic activities (Navarrete-Bolaños *et al*., 2007) and overall microbial population during the fermentation is less considered. This may have contributed to the inconsistency in microbial populations and species identified in *ogi* as seen in the literature (Ampe *et al*., 1999; Ampe and Miambi, 2000; Oguntoyinbo *et*

*al*., 2011) although similar genera may be present as observed in this study. Two sources of maize (M) and sorghum (S) (1: eastern and 2: northern grains) were studied. The endogenous microbial species are known to be affected by certain ecological factors which act on the consortium resulting in specific species association and population variations in cereal grains (Hammes *et al*., 2005). This effect explains the variations in microbial counts observed between the same types of grains obtained from two different sources as demonstrated in this study. Higher counts in M2 and S2 (maize and sorghum from East) suggests a regional effect and could be attributed to handling and storage conditions (temperature and humidity) of the grains. Compositional differences in the grains could also contribute to the level and type of organisms growing on each substrate. High water activity ($a_w$)>0.70 (Olstorpe and Passoth, 2012) in the grain during storage may suggest high endogenous hydrolytic activities which enhance continuous supply of free sugars to the microbes (Hammes *et al*., 2005). This may explain the higher count in yeast and moulds in M1 and S2 compared to M2 and S1. Lower counts observed with MRS isolates compared with M17 are indication of growth of other aerobic bacterial species apart from *Lactococcus* on M17 which are not able to grow anaerobically and under the differing selective system on MRS. As expected microbial load increased during fermentation with MRS and M17 isolates increasing preferentially in *ogi*. Total aerobic count was lower than expected due to possible competition and antagonistic activities among the groups resulting in stress and death or incompatibility with aerobic growth. Thus this was not a reflection of the total population of individual groups in both maize and sorghum grains and *ogi*.

Lactic acid bacteria would be expected to reduce the pH of the fermentation medium due to lactic acid production and this may prevent the growth of other organisms. However despite the differences in microbial population, pH of the fermentation changed to a similar level (4.80-5.04) in all the samples. Traditionally fermented foods are known to have low pH. Such foods have their end pH ranging from 3.0-4.8 such as soy sauce (pH 4.6-4.8), *trahana* (made from LAB fermentation of wheat, milk *&* vegetable): pH 3.8–4.2 (Blandino *et al*., 2003), *pozol* (fermented maize dough): pH 4.6

(Wacher *et al*., 1993), togwa: pH 3.1–3.3 (Mugula *et al*., 2003) and *ogi* with pH 3.0-4.8 (Banigo and Muller; 1972, Omemu, 2011; Akinleye *et al*., 2014). The end pH of *ogi* fermentation in this study (4.8-5.04) was comparable to the range of values reported in the literature. Unfavourable pH is thought to extend the lag phase, decrease cell concentration and increase mortality in the stationary phase (Brock, 1969; Russell and Dombrowski, 1980). The lower and upper growth pH limits of bacteria of the Enterobacteriaceae group are 3.0 and 9.0 respectively however they may just survive and grow minimally at pH 4.20-4.90 (Baylis *et al*., 2011b) which is similar to the pH observed in these fermentations and this could explain the counts on MacConkey agar which did not decrease but either increased or remained the same. Nout (1991) reported the disappearance of Enterobacteriaceae below pH 4.5 in fermented infant maize formula. The increase in yeast and mould count in MO1 and SO2 suggests the presence of acid tolerant species.

Microbes originally present in raw materials initiate the fermentation and facilitate the emergence of other groups which impact on the overall quality of the food. The interaction of several microbes ranging from bacterial to fungal species present in mixed fermentations results in the unique sensory and nutritional properties of the foods (Chisti, 1999). Variations in microflora of the same product fermentations occur and may be attributed to the initial substrate population and their participations over the fermentation period resulting in changes in the dominant flora (Blandino *et al*., 2003). Similar species of the different groups of organisms investigated were detected in the same types of grains suggesting possible similarities in metabolic activities and quality of *ogi.* On the other hand it is also worth knowing that some groups of the Enterobacteriaceae identified in the maize and sorghum grains by phenotyping such as *Ewingella americana, Cronobacter sakazakii* and *Rahnella aquatilis* were not detected by PCR-DGGE although some minor populations could be represented in the bulk cell plate. However some microbial species appear to be resistant to lysis by enzymes during DNA extraction (Kafili *et al*., 2009) and this may have contributed to the slight differences observed in the diversity of species identified by phenotypic and the bulk cell/direct microbial DNA extraction methods. Nevertheless, the combination

of the culture-independent and culture-dependent methods of analysis by PCR-DDGE is a very useful tool in assessing the microbial communities in *ogi*.

Limitations in the identification of microbes to the species level has been attributed to the use of short amplicons and the reliability of microbial species identification increases with the length of the variable regions amplified (Schmalenberger *et al*., 2001; Yu and Morrison, 2004). Due to the differences in the degree of sequence diversity of the 16S rRNA hypervariable regions, the ability to distinguish among several bacterial species by only one region is also limited (Chakravorty *et al*., 2007). Thus combined amplification of two regions with different degrees of sequence heterogeneity may be more discriminatory in distinguishing the available species in *ogi* fermentation. Therefore a primer set targeting the V6-V8 hypervariable regions (having the attributes of high degree of sequence heterogeneity (V6) and conservation (V8) of the 16S rRNA was further used in this study to support the previous identification with V3 primers which may be limited by the short partial sequence of the hypervariable region (Yu and Morrison, 2004). Again this primer set has successfully been used in the analysis of microbial communities in fermented food such as cheese (Randazzo *et al*., 2002).

Analysis of the direct DNA extraction DGGE profile of amplified 16S rRNA bacterial genes using V6-V8 primers revealed slightly higher microbial diversity in *ogi* compared to the grain samples. The cultivated bulk cell analysis showed that community diversity in grains from the northern region was higher than that of the eastern grains which mostly showed closest relatives to *Aeribacillus* and *Bacillus* as dominant species. It was noticeable that some species in the original grain seem to have disappeared during fermentation and as such were not dectected in the *ogi.* Closest relatives to *Pantoea* and *Enterococcus* genera were identified in both grains and *ogi*. *Bacillus sp., Cronobacter sakazakii, Ewingella americana* and *Micrococcus sp* identified in maize grain were not found in the *ogi* and *Serratia sp.* identified in maize *ogi* and *Streptomyces* sp., *Pediococcus pentosaceus* and *Lactobacillus rossiae* in sorghum *ogi* were not detected in the grain. Although *Serratia* spore is not common

and only few report is available on spore-forming *Serratia* sp. (Ajithkumar *et al*., 2003), the proliferation of microbes such as *Serratia* and *Streptomyces* in the sorghum *ogi* suggests that they may have been present as spores in the grains which then germinated and grew in the fermentation. *Streptomyces* have been reported as antibiotic producers (Emerson de Lima Procópio *et al*., 2012) and may be significant in the microbial populations during *ogi* fermentation. Although not found in the *ogi*, *Cronobacter sakazakii* (*Enterobacter sakazakii*) is an important pathogen associated with infant powdered milk formula and has been thought to originate from milk, water but could be from plant sources as evident in this present study (Fakruddin *et al*., 2013). The disappearance of bacterial species during traditional fermentations has been attributed to the inability to resist the acid medium and antimicrobial metabolites (such as diacetyl and bacteriocins) produced mostly by LAB species (Escalante *et al*., 2008) indicating the production of acid by individual identified species of organisms which may be involved directly or indirectly in *ogi* fermentation.

Lactic acid bacteria (LAB) are fastidious, homofermentative or heterofermentative organisms although some can grow in unfavourable environments making them available in both plant and animal substrates (Hammes *et al*., 2005; König and Fröhlich, 2009). Apart from obligate homofermentative *Pediococcus pentosaceus* and heterofermentative *Lactobacillus rossiae* detected in sorghum *ogi* and *Bifidobacterium breve (*an endosymbiotic bacteria of the Bifidobacteriaceae) in maize *ogi, Enterococcus* was mostly dominant in both maize and sorghum fermentations. Although *Enterococcus* has been reported to play an important role in fermentation through proteolytic and lipolytic activities, and is frequently isolated from fermented foods such as cheeses, it is considered undesirable and indicates faecal contamination. The presence of these genera of LAB in *ogi* except *Bifidobacterium breve* is not surprising as they are naturally found in plant materials (Hammes *et al*., 2005) while the source of *B. breve* in maize *ogi* remains unclear. *Bifidobacterium breve* although not known for use in fermented foods, undergoes fermentation metabolism and has probiotic properties (Shimakawa *et al*., 2003; Hammes *et al*., 2005) making its presence in maize *ogi* (MO1) beneficial. The interaction of

homofermentative and heterofermentative LAB in food fermentation is important in determining the ratio of lactic to acetic acid levels (acidification quotient, QF) (Coppola *et al*., 1998; Di Cagno *et al*., 2007).

*Micrococcus* and *Staphylococcus* species are Gram positive, catalase positive non-sporeforming aerobic cocci widely present in animal products and may be present in foods of plant origin through contamination by handling. These have mostly been involved in natural fermentation of sausages and are known to enhance flavour and colour rather than acid production as they reduce nitrite and hydrolyze lipids and proteins (Di Maria *et al*., 2002; Hammes *et al*., 2005). *Micrococcus* sp (*luteus*) is also known to inhibit pathogens such *Staphylococcus* sp., *Klebsiella* sp. and *Pseudomonas* sp (Di Maria *et al*., 2002; Umadevi and Krishnaveni, 2013). The presence of *Staphylococcus xylosus* and *Micrococcus luteus* in maize may result in a unique aroma profile in maize compared with sorghum *ogi.* The involvement in food fermentation of *Kytococcus sedentarius* (family: *Dermatophilaceae*) identified in maize is not known. However it is mostly associated with the human skin flora and is known to produce protease enzymes (Becker *et al*., 2002; Longshaw *et al*., 2002) and may have been present through human contamination during harvesting.

*Bacillus* species are widely present in foods as contaminants and are implicated in food spoilage (Hammes *et al*., 2005). These are natural soil organisms and may have been present on the grains by contamination. However the non-pathogenic strains of *B. subtilis* known for utilization and breakdown of indigestible oligosaccharides and polysaccharides and exhibition of proteolytic activities have been found useful in fermentation of soybeans (Shresthai *et al*., 2010) and in African fermented proteinous oil seeds like locust beans (Odunfa, 1985). The ability of *B. subtilis* to degrade aflatoxin has been reported (Kazuhiro, 1996) and as such its presence in maize may be quite significant in improving the safety of *ogi* since *Aspergillus* known for aflatoxin production is a major mould found in maze grain. On the other hand *B. subtilis* could cause an opportunistic infection (Oggioni *et al*., 1998) but this has been reported to be generally low or absent (de Boer and Diderichsen, 1991). Similarly *B. cereus* is mostly

associated with diarrhoeic and enteric toxins (enterotoxin) and its presence in *ogi* in highly undesirable.

Research has also shown the ability of *Bacillus* to release phenolic compounds in soybeans (Moktan *et al*., 2008) suggesting the potential presence of higher levels of phenolic compounds in naturally fermented *ogi* than the unfermented grain.

*Enterobacter*, *Pantoea* and *Serratia* belonging to the *Enterobacteriaceae* are widely distributed and associated with soil and plant materials. *Enterobacter cloacae* could be found in the normal gut flora and is not a primary pathogen but has been found beneficial in bio-control of plant diseases. However *Enterobacter aerogenes* identified in sorghum and *Enterobacter ludwigii* and *Serratia marcescens* found in maize grain and *ogi* are nosocomial pathogens found in water, soil and plants (Baylis *et al*., 2011a). A novel species, *Enterobacter asburiae* (known to ferment melibiose and rhamnose differentiating it from other species of *Enterobacter*), not previously identified in maize grain (Brenner *et al*., 1998) was identified in only one maize *ogi* sample (MO1)

Other bacterial groups not well known in food fermentation were also identified in this study. *Acinetobacter* species detected in sorghum are mostly isolated from nosocomial infections. However *Acinetobacter calcoaceticus* produces bio-emulsifiers known as emulsan (Brown and Cooper, 1992) and biodispersan (Shabtai, 1990). Hwanga and Draughton (1994) also reported the ability of *A*. *calcoaceticus* to degrade ochratoxin A. However the presence of nosocomial pathogens in food is undesirable and suggests poor hygiene and possible human and animal contaminations. Minimizing cereal contamination in the field and controlling the levels of these organisms during fermentation are highly important to improve the safety of *ogi*. Interestingly, *Streptomyces* and *Amycolatopsis mediterranei* are good antibiotic producers (Bala *et al*., 2004). However antibiotic producing species are not favourable in fermentation as the activities of desirable species could be inhibited. *Aeribacillus pallidus,* a species widely found in the environment, are not pathogens but are

capable of producing enzymes such as amylase which could favour food fermentation and bio-emulsifiers for industrial applications (Zheng *et al*., 2012).

Acetic acid bacteria (AAB) were not detected in any grains and respective *ogi* samples. AAB are mesophilic, appear very late in fermentations (up to 144 h) (Laureys and De Vuyst, 2014), are acid tolerant but grow optimally between pH 5.3-6.3. Apart from the slight variation in requirements for growth when compared to fermentation conditions in *ogi* (pH< 5; 72h), characterization of AAB is thought to be difficult due to problems in strain identification and cultivation (Kittelmann *et al*., 1989). Culture media tend to pick up other microbial groups such as *Enterobacter* with similar cell morphology.

Yeast and moulds form part of the microbiota in *ogi* fermentation (Blandino *et al*., 2003; Greppi *et al*., 2013). Different genera of yeast and mould in *ogi* have been identified and these varied greatly from one study to another which may be attributed to different sources of contamination in the initial grains. Only one report was found on molecular identification (PCR-DGGE) of yeasts and moulds in *ogi* with NL1 and NL4 primers spanning the D1 domain of 26S fungal genes (Greppi *et al*., 2013) which identified only yeast species (*Clavispora lusitania, Candida krusei*, *Pichia kudriavzevii* and *Delkkera bruxellensis*) by direct DNA extraction from samples. The result of the current study revealed a wider fungal community consisting of yeasts (*Pichia* and *Hyphopichia burtonii*, *Meyerozyma caribbica* and *Saccharomyces pombe)* in maize and moulds (*Aspergillus*, *Cladosporum*, *Eupenicillium*, *Penicillum* and *Fusarium* sp) in maize and sorghum by PCR-DGGE of bulk cells on both MRS and RBCA. Yeasts common in traditional cereal fermentation such as *ogi*, *kenkey*, and *enjera* are *Saccharomyces* (Caplice and Fitzgerald, 1999; Blandino *et al*., 2003; Kohajdová and Karovičová, 2007), *Candida* (Caplice and Fitzgerald, 1999; Greppi *et al*., 2013) and *Pichia* sp. (Greppi *et al*., 2013). Yeasts are known to release volatile compounds such as thiols which improve the flavour of fermented foods (Banigo *et al*., 1974). However studies have shown that co-existence of yeast species with other fermentation microbes such as LAB result in a more complex interaction and development of

flavour and texture as well as reduce the chances of the yeast causing spoilage (Blandino *et al*., 2003; Fleet, 2006). The presence of yeast in maize may encourage the growth of LAB through production of carbondioxide, pyruvate, propionate and succinate during fermentation (Leroy and Pidoux, 1993) and this may make maize a better grain for natural fermentation of *ogi*. Again the presence of yeasts in both maize samples (M1 and M2), may result in higher aroma profiles in maize *ogi* than sorghum *ogi* following good interaction between yeasts and other microbial groups such as LAB present in the fermentation.

Moulds are known for food spoilage, however their ability to produce hydrolytic enzymes such as amylase, esterase and phytase, thus improving flavour characteristics and breaking down antinutrients, make them potentially useful in cereal fermentations. Interestingly preliminary screening of moulds (*Aspergillus oryza, Penicillium biourgeianum, Fusarium oxysporum, Cladosporum cladosporioides*, *Cladosporum macrocarpum* and *Aspergillus tubingensis*) from *ogi* in this study by PCR showed that mycotoxin biosynthetic genes were not identified indicating that these species are not able to produce mycotoxins. Although the production of mycotoxin (ochratoxin A) by *Aspergillus tubingensis* in liquid culture has been reported (Giancarlo Perrone *et al.*, 2006), another study reported the inability of *A. tubingensis* strain 'T' type to produce ochratoxin A (Accensi *et al*., 2001). This discrepancy has been attributed to the methods of analysis and ability to distinguish between the strains thus the need for a combination of biochemical and molecular methods in toxicological analysis of fungi (Giancarlo Perrone *et al*., 2006). Control and prevention of moulds or microbial degradation of mycotoxins (Juodeikiene *et al*., 2012; Gerez *et al*., 2013) during fermentation should be considered to improve the safety of *ogi*. LAB are expected to show antagonistic effects on moulds during fermentation (Daly, 1991; Oyewole, 1997; Juodeikiene *et al*., 2012) therefore the recovery of some species in *ogi* suggests acid tolerance ability. Apart from *Aspergillus* species dominant in maize which is known for commercial production of phytase enzyme (Gontia-Mishra *et al*., 2013), the presence of *Acremonium* and *Trametes* genera (discovered in this study by isolation on specific phytate enriched medium) which showed the ability of hydrolysing

phytate in both maize and sorghum suggests there may be improved mineral availability in both naturally fermented maize and sorghum *ogi.*

### 4.4 Conclusions

This study has demonstrated that a wide range of microbes are responsible for natural fermentation of *ogi*. Although *ogi* has been reported to be fermented by LAB, they were not the major part of the unfermented or the fermented product in this study. In addition to LAB, *Micrococcus*, *Staphylococcus*, *Pantoea*, *Bacillus* and yeast were initially present and the predominant microbes included species of *Enterobacteriacae* and moulds indicating grain contamination. The presence of non-acid producing species such as *Micrococcus* and *Bacillus* which exhibit proteolytic activities resulted in low acid pH of 4.8 - 5.0 and created an optimal growth environment for pathogenic organisms detected in *ogi*. Acetic acid bacteria were not identified as part of the diverse community. The microbiota and counts of each microbial group differed between maize and sorghum from different sources. While some organisms disappeared, others proliferated in the course of the fermentation. Three LAB genera (*Lactobacillus*, *Pediococcus* and *Enterococcus*) were identified in this study but only *Pediococcus pentosaceus* from sorghum *ogi* and *Enterococcus* species from maize and sorghum *ogi* were recovered by cultivation. *Pediococcus pentosaceus* fermented glucose, fructose, sucrose, mannose, cellobiose and maltose and did not ferment rhamnose which is classical of this genus. Utilization of sucrose may enhance rapid cell growth and generation of metabolites (Cho *et al*., 2006). Interestingly two fungal isolates (*Acremonium zeae* and *Trametes hirsuta*) showed significant phytase activity. Therefore since no report was found in the literature on the use of *Pediococcus pentosaceus* in *ogi* fermentation and because *Enterococcus* is unacceptable as a starter culture, the application of *P. pentosaceus* in *ogi* fermentation as a single and mixed starter cultures with *Acremonium zeae* and *Trametes hirsuta* was the next area investigated.

# CHAPTER 5

# NATURAL FERMENTATION OF *OGI*

## 5.1 Introduction

The basic components of whole maize and sorghum grains are the starchy endosperm, germ and bran (Okarter and Liu, 2010). Bioprocesses occurring during natural fermentation are thought to affect the organoleptic and nutritional quality of *ogi* (Chavan and Kadam, 1989). The levels of total carbohydrate, non-digestible polysaccharides and oligosaccharides all decrease during natural fermentation (Nout and Motarjemi, 1997).

Phenolics found in pericarp, testa, and aleurone layers (free phenolics) or in cell walls (bound phenolics) of grains are secondary plant metabolites (Troszyńska and Ciska, 2002; Dykes and Rooney, 2006; Butsat *et al*., 2009). They are capable of inhibiting non-haem iron absorption (Brune *et al*., 1989; Tuntawiroon *et al*., 1991) but have biological potential as antioxidants, anti-inflammatory, anti-atherosclerosis, and anti-carcinogenic agents (Hodzic *et al*., 2009).

Phytate (myo-inositol hexaphosphate) is abundant in cereal grains and enzymatic dephosphorylation of phytate to myo-inositol bis-, tris-, tetra-, penta- or hexaphosphate (IP2-IP6) is well known (Hartman, 1979; Sandberg and Ahderinne, 1986; Eklund-Jonsson *et al*., 2006). Apart from microbial phytases, endogenous phytases in grains are activated during fermentation (Svanberg and Sandberg, 1988). Release of phosphorus and increases in niacin, riboflavin and available lysine were reported during fermentation of whole grain corn meal (Lopez *et al*., 1983), achieved by enzymatic activities of a succession of microbes which derive energy from fermentable sugars obtained by hydrolysis of starch in the grain (Nout and Motarjemi, 1997).

*Ogi* is a fermented starch associated with poor nutrient bioavailability due to antinutrients (phytates) inherent in the grains which form complexes with divalent

cations (zinc, calcium magnesium) making them unavailable. It has been reported to be inadequate as an infant complementary food due to the percentage decrease in nutrients (protein, 13.86%; lipid, 9.33%; fibre, 53.95%; and ash, 38.46%) after traditional processing (Ebuehi and Akinwande, 1991). Efforts have been made to improve the nutritional value of *ogi* due to its great importance as one of the basic staples and infant complementary food in Africa. However this has focused mostly on fortification to improve the carbohydrate and protein compositions with no record found on the other nutrient components, thus creating the need to investigate the biodegradation and levels of the bioactive substances in cereal grains in relation to nutrients bioavailability in *ogi*.

Apart from nutritional changes, development of aroma compounds and changes in flavour also occur during natural fermentation (Nout *et al*., 1989). Annan *et al*. (2003) reported the presence of carbonyl compounds, alcohols, organic acids and phenolic compounds during traditional fermentation of maize dough with high levels of carbonyl compounds at the early stage of fermentation. Volatile phenols contribute to the flavour of fermented plant foods and are derived from precursors such as hydroxycinamic acids (*p*-coumaric acid and ferulic acid) which are present in maize and sorghum.

It is well known that different types and varieties of grains differ in nutritional and physicochemical compositions and that various compounds such as phytate exist in different complex forms in these grains (Kowieska *et al*., 2011; Koehler and Wieser, 2013). Both indigenous and microbial enzymes are involved in natural fermentation of cereal grains. Enzymes such as phytases, esterases, amylases and proteases at optimal pH, hydrolyse phytate, phenolic acids, starch and amino acids and contribute to aroma development. In Chapter 4, a wide range of bacterial and fungal species associated with the natural fermentation of *ogi* were identified and it was anticipated that the uniqueness and complexity of each fermentation microbiota would influence enzymatic activities. It was therefore hypothesized that the level of enzyme activity

would depend on the food matrix and metabolism of these microbes during fermentation.

Therefore this study aimed to examine the effect of natural fermentation on nutritional and sensory characteristics of *ogi.* To be able to assess the changes resulting from fermentation, hydrolysis of starch, non-starch polysaccharides, phytate and phenolic compounds were investigated in both the original grains and *ogi*. Viable count was also performed over the fermentation period.

## 5.2 Methods

### 5.2.1 Microbial count, pH and acid production

Microbial load in steep water was determined (§3.2.1) with LAB counts based on colonies showing zone of clearing (differentiating LAB from yeast by acid production) on MRS supplemented with 1% $CaCO_3$ (Chang *et al*., 2013). Acid production was monitored by measurement of pH and individual acids analysed by HPLC as described in §3.6.1. Lactic, acetic and malic acids are the major organic acids associated with cereal fermentation (Kohajdová and Karovičová, 2007) and as such these were investigated in this study.

### 5.2.2 Starch analysis

Starch content was determined using the starch assay kit (Sigma-Aldrich), by applying the dimethylsulphoxide (DMSO) method (§ 3.3.1) to solubilize the starch before glucose assays following the manufacturer's procedure (Sigma-Aldrich).

## 5.3 Results

The component yields after processing of *ogi* was evaluated. The weight (dry basis) of *ogi* recovered from 200g of each grain was 141 g and 147 g in maize (M1 and M2) and 134 g and 122 g in sorghum (S1 and S2). Similarly the recovery of pomace was 85.30 g and 76.2g in M1 and M2, and 77.60 g and 86.0 g in S1 and S2 respectively.

### 5.3.1 Microbial count and pH

The results of microbial counts on four different culture media (M17, *Lactococcus*; MRS, total lactic acid bacteria; MacConkey, Enterobacteriaceae; and RBCA, Yeast and mould) and pH changes in the steep water collected from natural fermentation of *ogi* are presented in Fig. 5.1 and Table 5.1, respectively. Viable cell count and pH were monitored at 6-12 h intervals in order to assess the dynamics in growth of microbes over the fermentation period. The viable cell populations and pH changed during fermentation. Overall the highest total viable population was mostly observed at 24 h in both maize and sorghum fermentations which presumably represent the initial log phase, before decreasing at 30 h.

In maize fermentations, changes in microbial populations followed a relatively similar trend in both M1 and M2 (Fig. 5.1). The increase in viable population in maize within 12-24 h may have resulted in competitive growth at 30 h except MRS isolates which increased exponentially and M17 which remained static. However M17 and MacConkey isolates showed a more stable population than the RBCA group in both M1 and M2 fermentations. The yeast and mould populations showed negative correlation with the LAB population suggesting antagonistic interaction between the two groups. Comparison of the overall count revealed a shift in population at 6-12 h and 30–36 h of fermentation in M1 and M2. Again probably due to the slightly higher pH in M2 fermentation, enterobacteria count was 54% higher than in M1.

Sorghum fermentations showed similar trends in viable counts in both S1 and S2 within the first 24 h except for the yeast and mould populations which decreased by ~ 5 log cfu/ml after 12 h and 24 h in each case. In S1 fermentation, cell counts on M17 decreased while MRS and MacConkey increased after 12 h. The viable count on MacConkey decreased consistently from 24 h to 72 h by ~log 3 cfu/ml and may be attributed to the antagonistic effect of the LAB population in (Fig 5.1) as MRS population increased at 24 h and remained stable up to 72 h. Again yeast and moulds populations decreased to log 2 cfu/ml at 30–36 h, proliferated at 48 h and then became stable for 6 h before decreasing finally by log 2.0 at 72 h.

In S2 fermentation, the highest microbial concentration was recorded on M17 (8.08 log cfu/ml) at 12 h which remained static after 30 h up to 7.21 log cfu/ml at 72 h. The viable population of enterobacteriaceae in S2 fermentation increased to 7.91 log cfu/ml at 24 h and only decreased by 13% after 72h despite the decrease in pH to 4.82. Decrease in viable populations of yeast and mould was also observed at 30 h only. Overall S1 and S2 showed similar trend in population dynamics mostly in the first 30 h of fermentation.

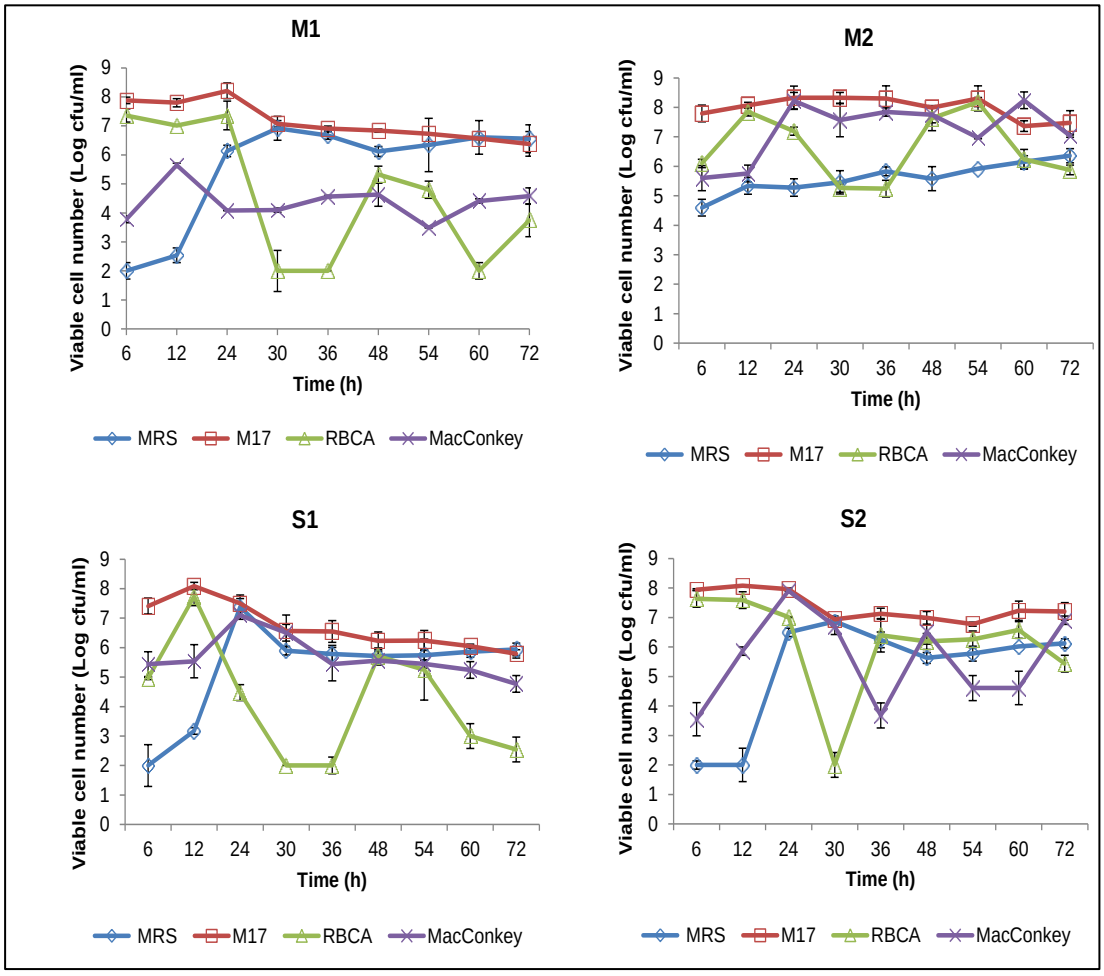



**Figure 5.1**: Microbial population in steep water during natural fermentation of *ogi*. Media: M17 for *Lactococcus*, *MRS; total lactic acid bacteria, MacConkey; Enterobacteriaceae and RBCA; Yeast and mould. *Medium was supplemented with 1% $CaCO_3$ and only colonies with zone of clearing were counted as LAB.

**Table 5.1**: pH values during natural fermentation of *ogi*

| | †pH | | | |
|---|---|---|---|---|
| **Time (h)** | M1* | M2 | S1 | S2 |
| **0** | 7.06 (0.04)[3] | 7.08 (0.57)[4] | 7.30 (0.12)[2,3] | 7.40 (0.13)[5] |
| **6** | 6.02 (0.03)[2] | 6.63 (0.17)[3] | 6.51 (0.08)[3] | 6.11 (0.06)[4] |
| **12** | 6.16 (0.35)[2] | 6.42 (0.06)[3] | 6.37 (0.20)[3] | 6.08 (0.11)[4] |
| **24** | 5.77 (0.49)[2] | 5.86 (0.99)[2] | 5.57 (0.62)[1,2] | 5.77 (0.21)[3,4] |
| **30** | 5.10 (0.11)[1] | 5.71 (0.13)[2] | 5.03 (0.99)[1,2] | 5.21 (0.14)[1,2] |
| **36** | 4.98 (0.14)[1] | 5.70 (0.20)[2] | 5.22 (0.57)[1,2] | 5.12 (0.17)[1,2] |
| **48** | 5.16 (0.05)[1] | 5.98 (0.16)[2] | 5.30 (0.13)[1,2] | 5.37 (0.21)[2,3] |
| **54** | 5.17 (0.27)[1] | 5.80 (0.17)[2] | 5.22 (0.14)[1,2] | 5.54 (0.18)[2,3] |
| **60** | 5.05 (0.04)[1] | 5.80 (0.14)[2] | 5.20 (0.03)[1,2] | 5.33 (0.35)[1,2] |
| **72** | 4.80 (0.07)[1] | 5.04 (0.07)[1] | 4.80 (0.15)[1] | 4.82 (0.03)[1] |

*Two different sources of grains: 1 northern and 2 eastern; maize (M) and sorghum (S).

†Data are means ± standard deviation in parentheses of four replicates from duplicate determinations (n=4).Means in the same column with same superscripts (numbers) are not significantly different (p>0.05)

The pH changed to a similar level in all fermentations, reducing from 7.06-7.08 to 4.80–5.04 in maize and 7.30–7.40 to 4.80–4.82 in sorghum (Table 5.1). Although not significantly different in M2 and S1, decrease in pH was observed at 24-30 h which corresponded with acid production and showed positive correlation with viable population on RBCA in all the fermentations. Decrease in bacterial viable count was also observed as pH decreased from 24-30 h, as seen with M1 and S2 on M17 agar, S2 and M2 on MacConkey agar and S1 on all media (Fig. 5.1). The observed reduction in viable populations may be attributed to competition or other unfavourable conditions (toxicity and nutrient scarcity) present at the stationary phase.

### 5.3.2. Recovery of starch and non-starch polysaccharides in *ogi*

The results for starch and non-starch polysaccharide (NSP) content of maize and derived *ogi* fractions are presented in Fig. 5.2. The starch content of maize grain was 79.45 g/100g and 81.25 g/100g in M1 and M2, respectively. Recovery of starch in the *ogi* in MO1 and MO2, accounted for 61% and 64% of the amount in the original grains, respectively. Losses through the pomace in MO1p and MO2p accounted for 8.7% and 5.0% of the original grain content, respectively. Starch content was not significantly different ($p>0.05$) in the two maize grain samples (M1 and M2) and this was also true for the respective maize *ogi* (MO1 and MO2) and pomace (MO1p and MO2p).

The average starch content of sorghum grain was 91.66 and 85.88g/100g in S1 and S2, respectively. Recovery of starch in the *ogi* (SO1 and SO2) varied significantly ($p<0.05$) and accounted for 63% and 51% of the original grain content in S1 and S2, respectively (Fig 5.2). The amount of starch lost through the pomace (SO1p and SO2p) was 9.6% and 8.3% of the starch originally in the grain.

The total non-starch polysaccharide (NSP) content in the maize was 16.24 and 21.34g in M1 and M2. Recovery in *ogi* (MO1 and MO2) accounted for 41% and 43% of the total NSP in the grains, respectively. The fraction recovered in the pomace (MO1p and MO2p) showed 31% and 23% of the amount originally present in the grain. Overall recovery in maize *ogi* and pomace showed values 72% and 66% of the original grain content.

In sorghum total NSP content was similar (p>0.05) 14.98 and 17.56 g in S1 and S2, respectively. Recovery in the *ogi* showed 31% and 24% of the total grain content in S1 and S2, respectively. The pomace (SO1p and SO2p) in each case showed values of 30% and 24% of the total originally in the grain. Overall, NSP recovery in *ogi* and losses through the pomace accounted for 61% and 47% of the original total in the grain in S1 and S2, respectively. Overall results in maize and sorghum indicated the retention of 61-64% and 51-63% starch and 41-43% and 24-31% fibre, respectively, thus the major energy value is not lost during fermentation.

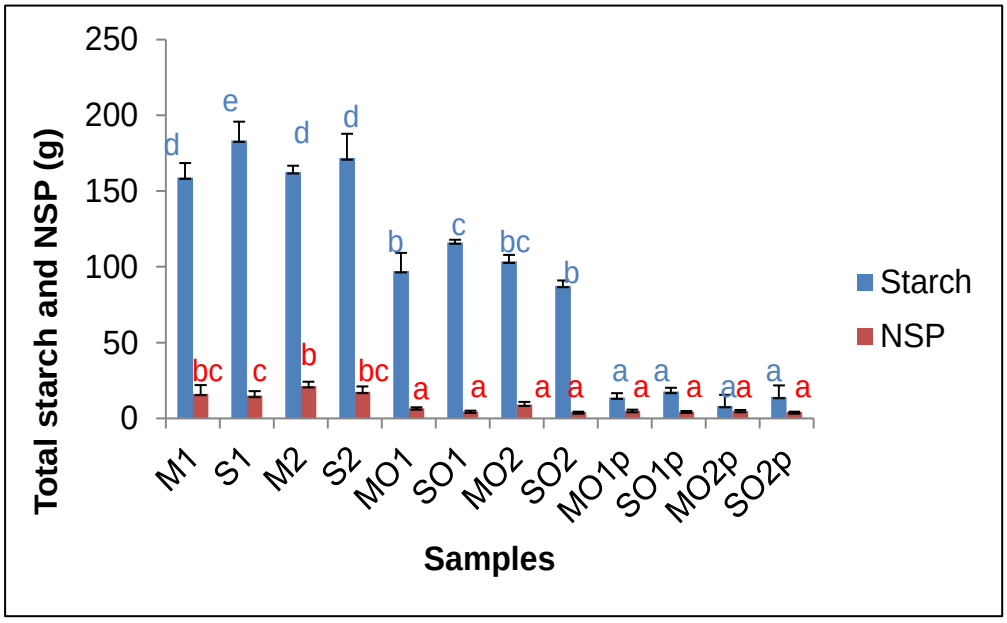



**Figure 5.2:** Total starch and non-starch polysaccharide (NSP) content of maize and sorghum grain and derived *ogi* fractions.

*Two different sources of grains: 1 northern and 2 eastern, M: maize, S: sorghum, *ogi* (MO, SO), p: pomace (residue). NSP: Non-starch polysaccharide. Data are means ± standard deviation of four replicates from duplicate determinations (n=4). Same coloured bars with same superscript are not significantly different (p>0.05).

### 5.3.3 Compositional analysis of NSP

Non-starch polysaccharide (NSP) is a complex mixture of polymers. HPLC analysis of the monomer sugars resulting from the hydrolysis of the NSP was performed in order to understand the type and levels of polymers present and their potential hydrolysis in *ogi* fermentation. Among the 7 monomers and 1 dimer analysed (glucose, xylose, arabinose, galactose, mannose, rhamnose, fructose and cellobiose) only 3 monomers

(glucose, xylose and arabinose) were consistently detected in both maize and sorghum with glucose being the mostly abundant. These sugars can be used to estimate the level of NSP thus indicating the amount of fibre in the food matrix. Glucose, xylose, mannose, galactose and arabinose reflect the hemicelluloses while glucose and cellobiose reflect cellulose (Pérez *et al*., 2002).

The HPLC analysis of sugars in *ogi* and grain is presented in Table 5.2. Maize grains (M1 and M2) showed similar sugar composition. The high levels of glucose may have resulted from mixed linkage beta–D-glucans, and the arabinose and xylose from the arabinoxylans which form the major hemicellulose in cereal grains. NSP from *ogi* consisted entirely of glucose suggesting that this fraction contains only the beta-D-glucans (found in the grain endosperm) while the pomace also contains arabinose and xylose showing the presence of the arabinoxylans.

**Table 5.2:** HPLC analysis of sugars from NSP extract (concentration, g/l)

| Samples | Glucose | Xylose | Arabinose |
|---|---|---|---|
| M1 | 3.20 | 0.37 | 0.16 |
| MO1 | 7.21 | 0.00 | 0.00 |
| MO1P | 4.85 | 2.32 | 0.93 |
| M2 | 5.18 | 0.62 | 0.12 |
| MO2 | 11.1 | 0.00 | 0.00 |
| MO2P | 3.91 | 2.87 | 1.13 |
| S1 | 3.81 | 0.00 | 0.07 |
| SO1 | 5.22 | 0.00 | 0.00 |
| SO1P | 5.89 | 1.07 | 0.65 |
| S2 | 4.03 | 0.00 | 0.08 |
| SO2 | 5.43 | 0.00 | 0.00 |
| SO2P | 5.92 | 1.15 | 0.69 |

*Two different sources of grains: 1 northern and 2 eastern, M: maize, S: sorghum, SO: sorghum *ogi*, MO: maize *ogi*, p: pomace (residue).

In sorghum (S1 and S2) glucose was predominantly present, again indicating the presence of the major hemicellulose (beta-D glucan). Surprisingly xylose was not detected in either sorghum grain and only a small amount of arabinose was present and this might suggest the presence of some arabinans but in general is indicative of the predominance of the beta-D glucans in this grain. However, the pomace in this case has both xylose and arabinose suggesting the accumulation of arabinoxylans in the bran fraction.

### 5.3.4 TPC levels in grain and fermentation fractions

TPC levels in the steep water during the fermentations of maize and sorghum grains are shown in Fig. 5.3. TPC levels increased consistently over the 72 hours of fermentation reaching 26 and 40 mg GAE in maize M1 and M2, respectively. Sorghum exhibited a higher TPC level reaching 51 and 61 mg GAE in S1 and S2, respectively. Interestingly whilst TPC from the maize increased consistently over the 72 hour fermentation, in the case of both S1 and S2 the TPC appears to have reached a maximum after only 48 hours and then levels in the steep water plateaued.

Total phenolics in the original grain and recovery of phenolics in the *ogi*, pomace (residue), steep and wash waters are shown in Fig.5.4. The overall recovery of total phenolics was 99%, 84%, 101% and 114% for M1, M2, S1 and S2, respectively. Thus recovery from fermentations using M1 and S1 grains totalled approximately100%, however, only 84% of “phenolics” were accounted for from the fermentation with M2 and a total of 114% was observed with the S2 grain. In maize, total phenolic acid content ranged from 410–437 mg GAE/100g of grain. The recovery of phenolics in maize *ogi* varied significantly between samples ($P < 0.05$) with values of 49% and 39% of the original grain total in MO1 and MO2, respectively. The total phenolics in the pomace (MO1P and MO2P) were 39% and 31% of the grain content, respectively.

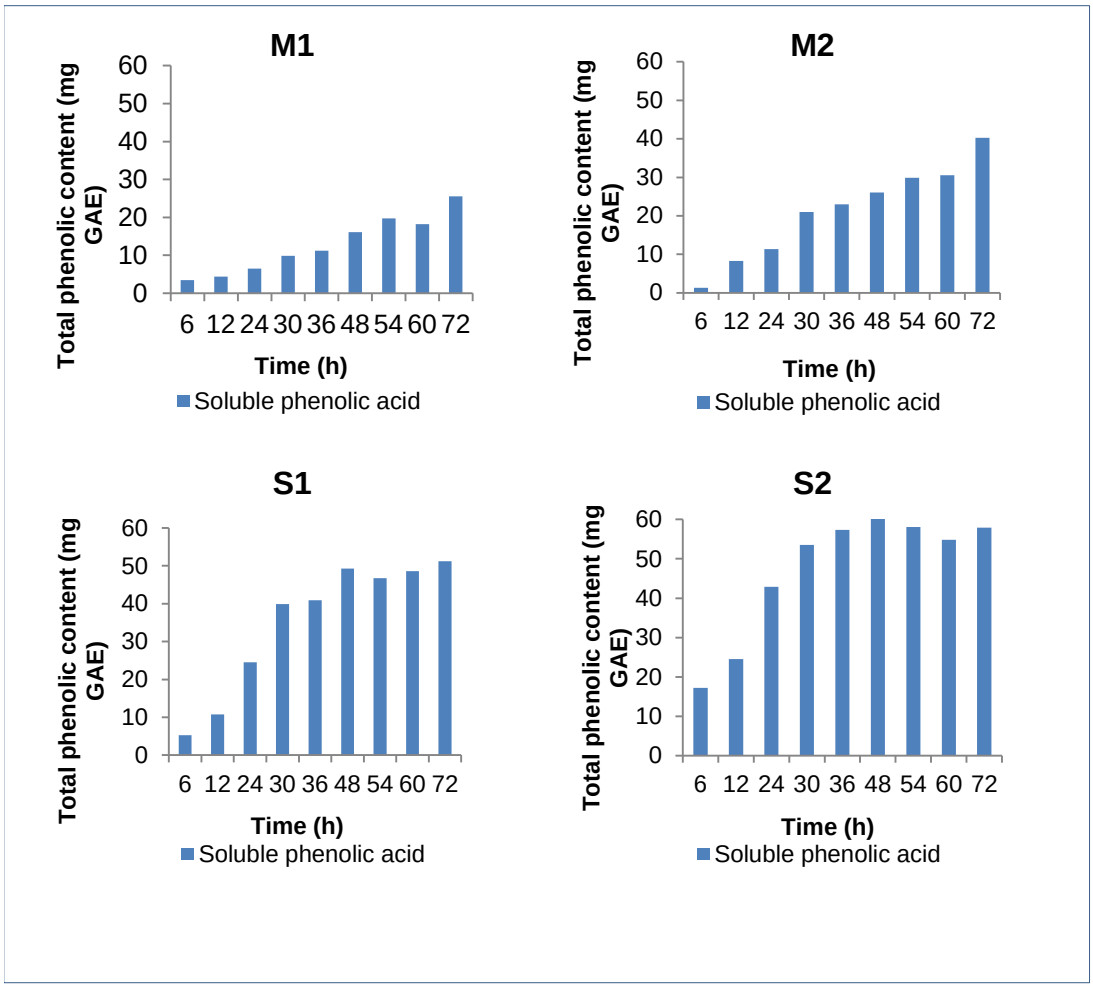



**Figure 5.3:** TPC levels in the steep water during natural fermentation of *ogi.* Grains: maize (M1; M2), sorghum (S1; S2). Values are presented as the total amount in the steep water.

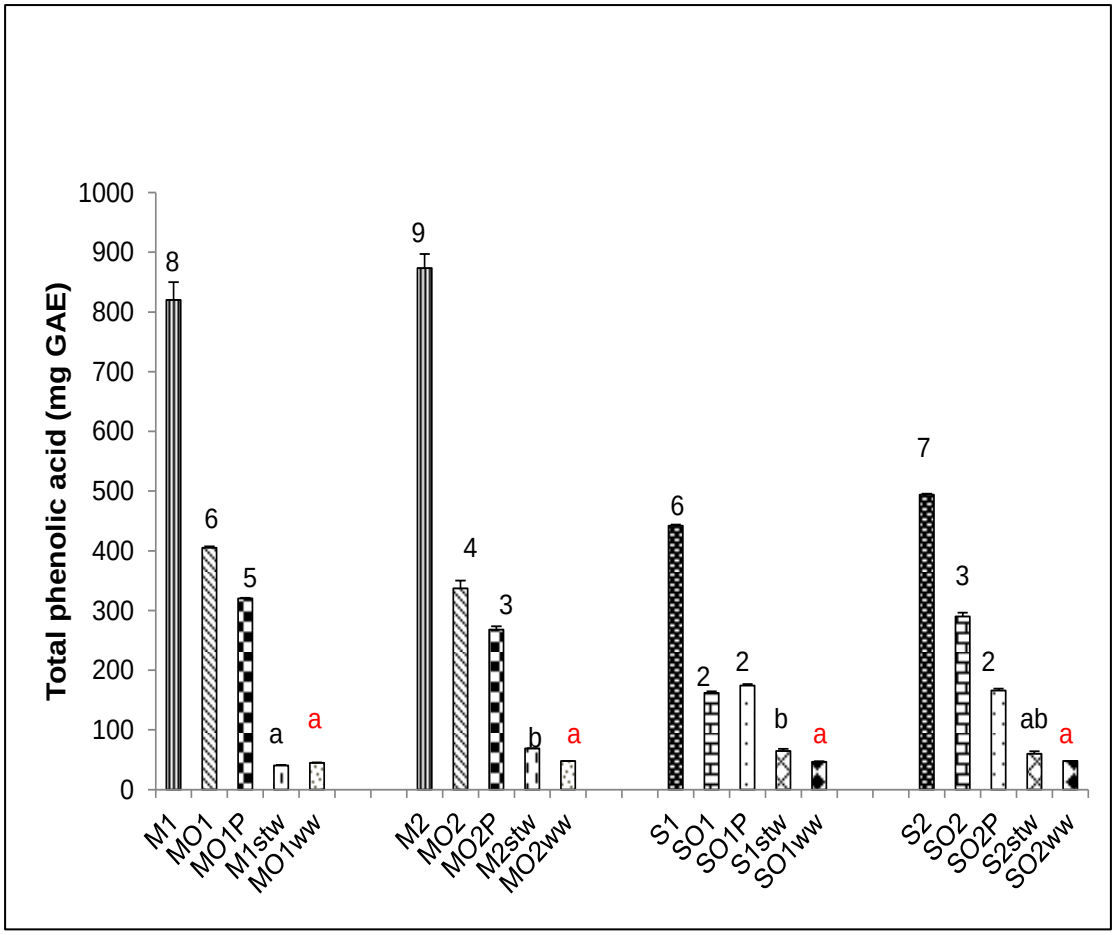



**Figure 5.4:** Phenolic acid content of grains and fractions collected during natural fermentation of *ogi*.

Grains: maize (M1; M2), sorghum (S1; S2), ***Ogi***: maize (MO1; MO2), sorghum (SO1; SO2), **pomace**: maize (MO1P; MO2P), sorghum (SOP1; SOP2), **steep water**: maize (M1stw; M2stw), sorghum (S1stw; S2stw), **wash water**: maize (MO1ww; MO2ww) and sorghum (SO1ww; SO2ww). Data are means ± standard deviation of six replicates from three determinations (n=6), same numbers or letters are not significantly different ($p>0.05$).

In sorghum total phenolics was 221 and 247 mg GAE/100g of grain for S1 and S2, respectively. The recovery of phenolics in the *ogi* varied significantly between samples ($P < 0.05$) with values of 37% and 59% for SO1 and SO2, respectively. Total phenolic content in the pomace was 39% and 34% of the original grain total in SO1P and SO2P, respectively.

Phenolics lost in steep water and wash water combined were 12% and 13% of the original total in M1 and M2, respectively. This leaching effect was more evident with

sorghum grain as compared to maize ($P<0.05$) with the combined steep and wash water content being 25% and 22 % for S1 and S2, respectively.

The values above represent the TPC recoverable from the grain or *ogi* fractions following direct saponification of the sample. However, for nutritional significance it may be important to monitor the amount of phenolics that are either freely soluble or are covalently bound to the food matrix. Thus in a separate experiment these two phenolic fractions were assessed in the grain, pomace and *ogi* (Figure 5.5).

Bound phenolics in maize grain were 254 and 261 mg GAE/100g in M1 and M2, respectively. This was about double the 123 and 132 mg GAE/100g in S2 and S1, respectively found in sorghum grain. The soluble phenolics in each case were 42 and 56 mg GAE/100g for M1 and M2 and 73 and 51 mg GAE/100g for S1 and S2. Thus the bound fraction represents 89% of the total in M1, 82% in M2, 64% in S1 and 71% in S2

In all four fermentations “bound” TPC (in *ogi* and pomace) showed a recovery of between 130–132% and 100%–101% of the total bound phenolics present in maize and sorghum grains, respectively. Overall total soluble phenolics (in *ogi*, pomace, steep water and wash water) showed 153—196% in maize of the soluble fraction in the original grain. In the sorghum recovery of soluble phenolics gave -141-207% GAE in S1 and S2 of total soluble phenolics in the grain. In maize and sorghum grains, similar levels of the soluble fraction were observed in each case except for S1 which has significantly higher levels. *Ogi* showed significantly less free phenolics compared to the bound and this may reflect seepage into steep water.

It was also noted that the sum of the bound and soluble phenolics recovered separately in both maize and sorghum grain and fractions were often different to that obtained by direct extraction by saponification of the samples. This may reflect experimental problems in comparing these two methods. One possible reason being that, it is known that some phenolics (and possibly other compounds that could interfere with the TPC assay) are unstable under alkaline conditions. Thus differences between the two methods could arise if compounds extracted in the soluble

(methanolic) fraction represent chemicals that are unstable in the direct alkaline saponification.

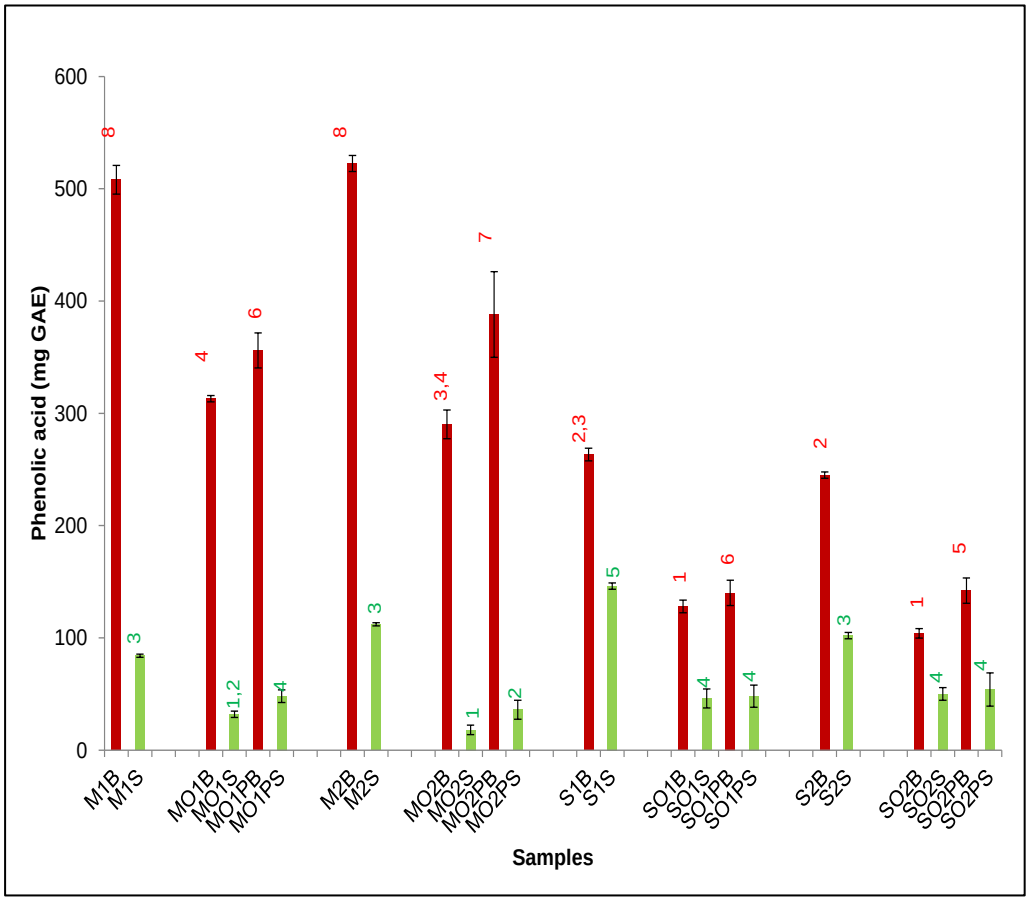



**Figure 5.5:** Bound and soluble phenolic acid recovery in maize and sorghum *ogi*.

Bound phenolics: B (red columns), Soluble phenolics: S (green columns) Grains: maize (M1; M2), sorghum (S1; S2), *Ogi*: maize (MO1; MO2), sorghum (SO1; SO2), maize pomace (MO1P; MO2P), sorghum pomace (SO1P; SO2P) Data are means ± standard deviation of six replicates from three determinations (n=6). Same coloured columns with the same numbers are not significantly different ($p>0.05$).

To determine whether microbial population density affected the release of phenolics, correlation between microbial population changes and release of soluble phenolics into the steep water was assessed by replotting values of TPC released every 6–12 h interval during fermentation against the respective counts from steep water on M17 and MRS agar at each time point (Fig. 5.6). Phenolic leaching with time followed a similar pattern in all grains but was higher in sorghum. During the initial stages

leaching appears to be linear with time in all cases. There was a significant drop in the release of phenolics after 60 h of fermentation in all the grains except S1 which reduced earlier at 54 h. Of the two groups of organisms tested only MRS populations seemed to show slight positive correlation with the increase in TPC at the early stage of the fermentation.

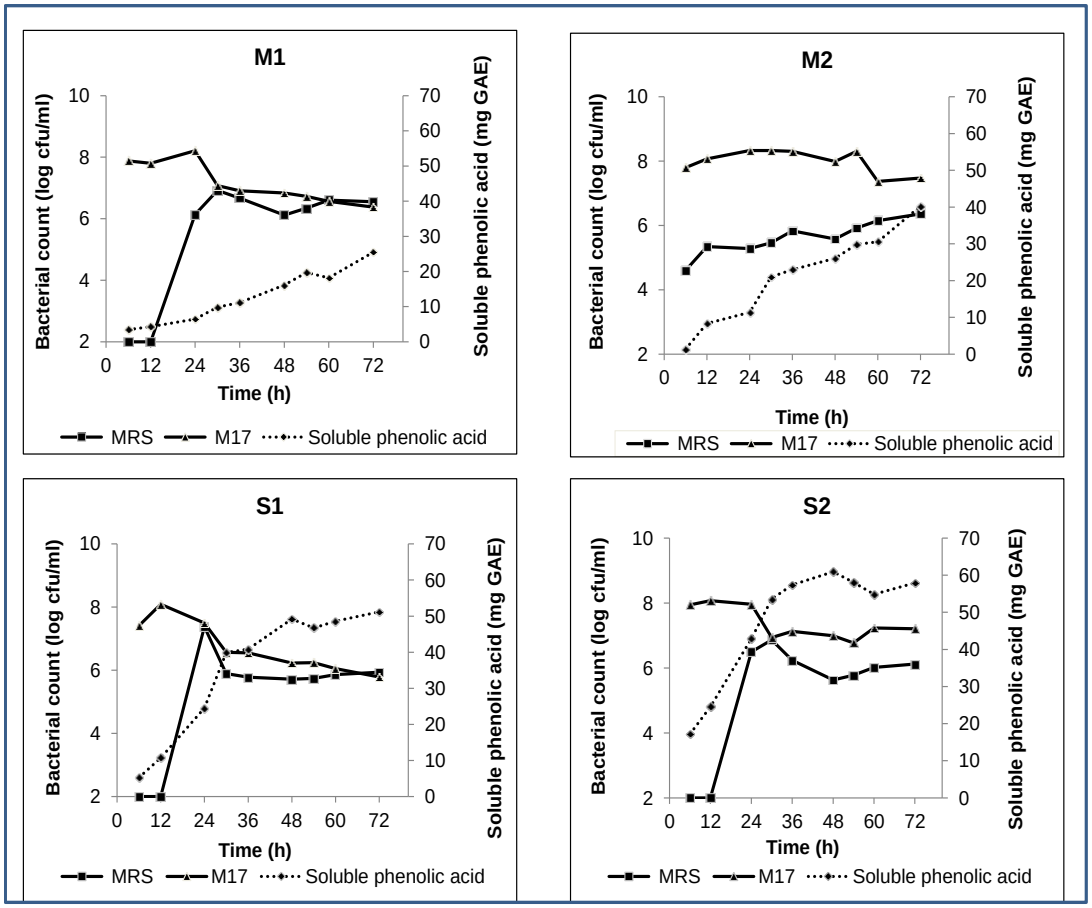



**Figure 5.6:** Correlation of microbial population changes and leaching of phenolic acid during natural fermentation of *ogi*. Maize (M1;M2) and sorghum (S1;S2). Data are means of four replicates from duplicate determinations (n=4).

### 5.3.5 Phytate levels in grain and fermented fractions

Phytate levels in the original grains and resultant *ogi* were determined (Fig.5.7). Phytate levels in both maize (M1 and M2) and in S1 sorghum grains were similar at around 0.3-0.4g/100g. These varied significantly ($p<0.5$) from the amount in S2 which gave 0.53g/100g of phytate. The fermentation utilised 200g of grain and total phytate content in the resultant *ogi* were similar ($p>0.05$) in both maize samples at 0.15 g but differed significantly from the levels in sorghum *ogi* at 0.24 g and 0.51g in SO1 and SO2, respectively. This accounted for 18–25% and 40-48% of the initial grain contents of phytate in maize and sorghum, respectively. Phytate lost through the steep water and pomace is shown in Table 5.3. Reduced phytate level in *ogi* suggests phytase activity during fermentation. Higher percentage of phytate based on the original total in grains was observed in M1stw and S2stw suggesting different levels of activity in the two fermentations.

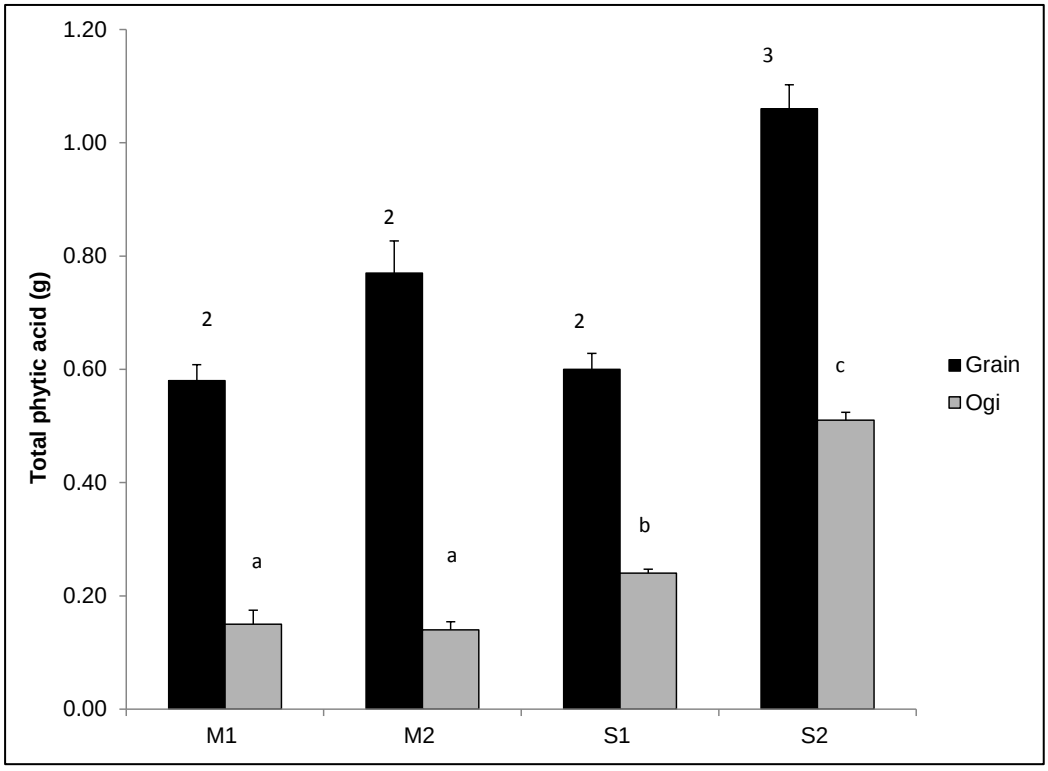



**Figure 5.7:** Total Phytic acid in grains and in *ogi* after natural fermentation.

Values represent the total amount in 200g of grain in the resultant *ogi*. Maize and sorghum grains (M1; M2 and S1; S2), maize and sorghum *ogi* (MO1; MO2 and SO1;SO2). Data are mean ± standard deviation of four replicates from duplicate determinations (n=4). Similar numbers or letters on same coloured bars are not significantly different ($p>0.05$).

**Table 5.3**: Phytate levels in steep water and pomace (residue) after fermentation of *ogi*

| Samples | Phytate (g) | ‡% recovery based of original grain content |
|---|---|---|
| **Maize** | | |
| M1stw | 0.04 | 6.90 |
| M1p | 0.04 | 5.20 |
| M2stw | 0.00 | 0.00 |
| M2p | 0.02 | 2.60 |
| **Sorghum** | | |
| S1stw | 0.06 | 5.70 |
| S1p | 0.02 | 1.80 |
| S2stw | 0.28 | 25.00 |
| S2p | 0.03 | 2.83 |

Maize grain (M1; M2), sorghum grain (S1; S2), stw (steep water), p (pomace); Data are means of four replicates from duplicate determminations (n=4)
‡Recovery was based on original grain contents.

### 5.3.6 Production of acids and aroma compounds

#### 5.3.6.1 HPLC analysis of organic acids

It was shown above that, as expected, all the fermentations demonstrated a drop in their pH. The levels of the three most common organic acids - lactic, acetic and malic acid were thus determined in the steep water throughout the fermentations (Figure 5.8). Lactic acid concentration displayed a similar trend in all fermentations showing the maximum level at 30 h. and then remained static after 48 h except in M2. This trend was apparently similar to that observed with the viable cell growth on MRS and RBCA agar in M1, S1 and S2 (Fig. 5.1) and showed correlation with the drop in pH at

24-36 h (Table 5.1). Higher concentration of lactic acid was recovered in the northern grains (M1, S1) than in the respective eastern grain (M2, S2). In M2 it is notable that lactic acid concentration dropped at 36-48 h and was again detected within 54 h. The sudden appearance of lactic acid showed correspondence to a sharp decrease of up to 2 log cfu/ml observed with viable population on RBCA and this may have reduced the yeast population and utilization of lactic acid. Further accumulation of lactic acid in M2 also showed correspondence to increase in the LAB population on MRS. The disappearance of lactic acid also correlated with further proliferation of yeasts at 36-48 h in M2. Although *Hyphopichia burtonii* identified in M2 (Chapter 4, Table 4.7) has been reported as an acid tolerant species (Papalexandratou and De Vuyst, 2011), it may have shown some sensitivity to acid in this fermentation resulting in suppression of growth and decline in the viable cell population during the fermentation.

In sorghum minimal level of lactic acid was detected in S1 and S2 fermentations (Fig.5.8A). Accumulation of lactic acid in S1 fermentation was higher and more consistent than in S2. Similar concentrations were mostly detected in both fermentations at 30–48 h while significant variations were observed at 12–24 and 54–72 h ($p<0.05$). Although increase in lactic acid concentration paralleled the decrease in viable population of yeast and moulds on RBCA at 30–36 h, this was not clearly evident over the fermentation period except at 72 h which may be due to instability in the total microbial populations and activities.

Acetic acid was detected in both maize fermentations (Fig. 5.8B) in amounts ranging from 0.10–1.90 and 0.1–2.18 g/l in M1 and M2, respectively. Production of acetic acid was highest at 72 h and similar ($p>0.05$) all through the fermentation period in both M1 and M2 except at 36 h. Higher level of acetic acid was recovered in M1 at 36 h which agreed with the corresponding low pH observed at this time. Again correlation observed with concentrations of acetic acid and lactic acid in both maize fermentations suggests possible microbial conversion (mostly by LAB) of lactic acid to acetic acid (Oude-Elferink *et al*., 2001); the absence of acetic acid bacteria was previously established (Chapter 4). Again acetic acid could also be generated through the glycerol pathway.

Acetic acid concentration in S1 and S2 fermentations were similar except at 60 h and ranged from 0.31–1.87 g/l and 0.34–1.29 g/l in each case. The levels of acetic acid in both fermentations were found to increase consistently and showed correspondence with the decrease in lactic acid concentration.

Malic acid was also detected at lowest levels than lactic and acetic acids within 6 h in both maize fermentations (Fig. 5.8C). The concentration ranged from 0.06–0.12 g/l during the exponential growth periods detected at 6-12 h in M1 and 12–24 h in M2. The highest amount of malate was produced at 30 h in M1 (0.73 g/l) and 54 h in M2 (0.64 g/l) at pH 5.10–5.80 respectively. At 72 h malic acid was not detected in M2 while 0.12 g/l was present in M1. Accumulation of this acid was found at 30-48 h and similar levels in both S1 and S2 fermentations ($p>0.05$). This was detected within 6 h of fermentation but disappeared at 12-24 h. The highest levels of malate was detected at 48 h in S1 (0.18 g/l) and 0 h in S2 (0.64 g/l) respectively.

#### 5.3.6.2 Analysis of volatile aroma compounds by gas chromatography mass spectrophotometry (GC-MS)

##### 5.3.6.2.1 Volatile compounds identified in naturally fermented *ogi*

Headspace solid phase microextraction (SPME) and gas chromatography-mass spectrometry was used to evaluate the volatile components in *ogi* fermentation as described in §3.6.2. This analysis identified 29 major compounds classified as alcohols (7), ketones (2), esters (11), aldehydes (1), carboxylic acids (4), sulphur containing compounds (2) and benzene-based (2) compounds (Table 5.4).

Since signal intensity indicated by the peak area is created in relation to compound concentrations, and gets bigger as concentration increases, high peak intensity may suggest high concentration. Thus the abundance of each compound in *ogi* could be compared although no accurate quantification was done in this study.

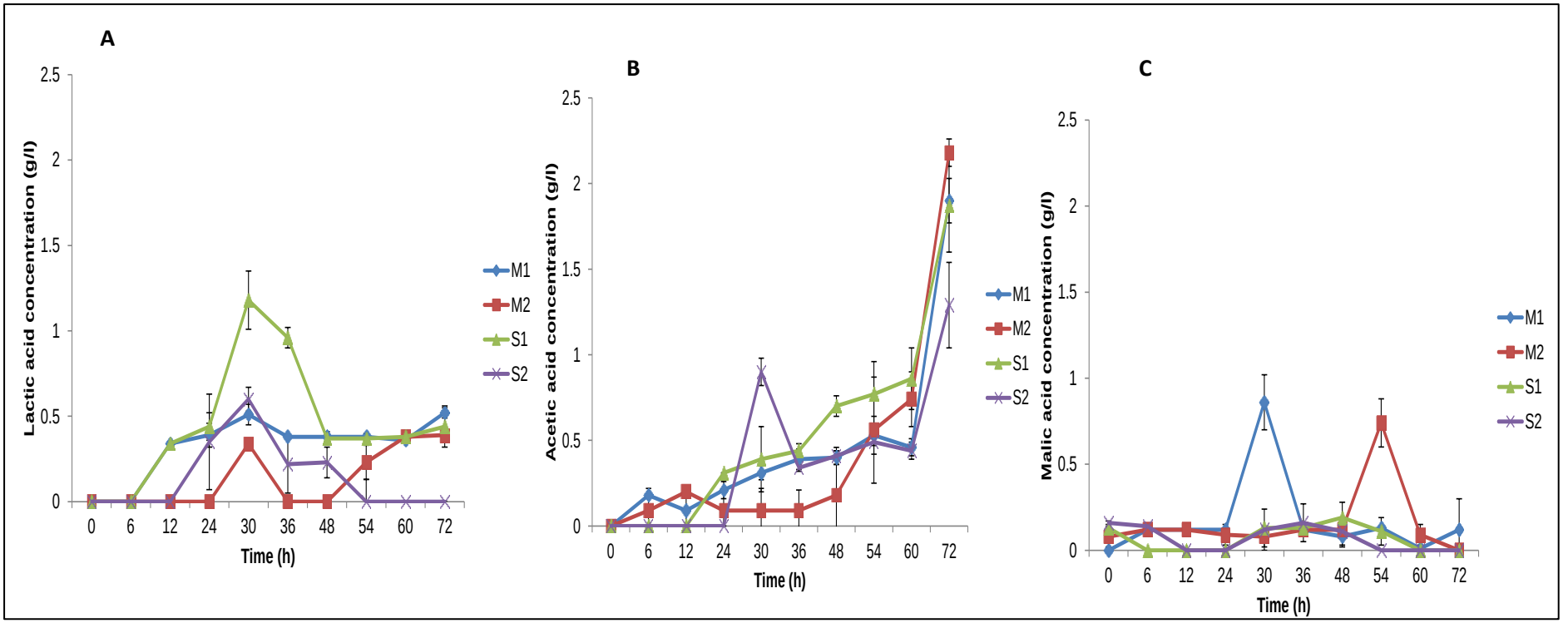



**Figure 5.8:** HPLC analysis of organic acids in steep water (g/l) during natural fermentation of *ogi*

Two sources of grains 1 and 2; M = maize, S = sorghum. Data are means ± standard deviation (n=4). Means with same superscript in the same row are not significantly different ($p>0.05$). Retention times (min); (A) Lactic acid, 15.20, (B) Acetic acid, 18.20 and (C) Malic acid, 12.20; separated on Rezex™ ROA-Organic Acid $H^+$ column of 5 µm particle size and 300 x 7.8 mm dimension (Phenomenex, UK) with 0.005 N $H_2SO_4$ as mobile phase at flow rate, 0.5 ml/min and signal detection by a UV detector at 220 nm wavelength. Injection volume was 20 µl.

All seven alcohols were identified in all four natural fermentations. Ethanol and butanol gave the highest signal intensities. Although accurate quantifications were not carried out, this may reflect that these alcohols are present at the highest concentrations. There was an apparent trend of high levels of 2-propanol in maize while all other alcohols were found at varying levels with no discernible trend between maize and sorghum.

Two ketones (2- propanone and 2-heptanone) and one aldehyde (2-methyl butanal) were identified in all the four *ogi*. Acetone (2-propanone) was relatively more abundant than 2-heptanone in maize and sorghum *ogi* (SO1 and SO2). However 2-heptanone was not found in SO2.

Esters were the largest group of volatile compounds identified in this study. Four out of eleven identified esters were mostly found in maize and sorghum *ogi* and include butyl butanoate, butanoic acid ethyl ester, and methyl thiobutanoate and butyl acetate. These esters correspond to their fatty acid and alcohol precursors as identified (Table 5.4) suggesting the formation of these esters via esterification by yeast, filamentous fungi and bacteria such as *Lactobacillus*, *Aspergillus* and *Schizosaccharomyces* species identified in this study

Three volatile carboxylic acids, acetic, pentanoic and butanoic acids, were identified in these fermentations with the latter showing more abundance in sorghum *ogi* than maize. Carboxylic acids are formed by oxidation of alcohols and the relative abundance of butanoic acid showed positive correlation with the abundance of the alcohol precursor (butanol). Butanoic acid is known to have an unpleasant smell while acetic acid gives a vinegar and acid flavours thus the abundance of these fatty acids in sorghum *ogi* suggests a more pleasant flavour in maize than sorghum *ogi*.

Other volatile components identified in *ogi* include sulphur containing compounds **(**dimethyl disulphide, dimethyl trisulphide**)** and benzene derivatives (phenyl ethylene, 1,3,-ditert-butylbezene**)**.

Table 5.4: Mean signal intensities (‡Peak areas (AU) X $10^4$) and standard deviations (SD) of volatile compounds detected by SPME GC-MS headspace analysis of naturally fermented maize and sorghum *ogi.*

| Volatile compounds | Ion (m/z) | MO1 | | MO2 | | SO1 | | SO2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | mean | SD | mean | SD | mean | SD | mean | SD |
| **Alcohols** | | | | | | | | | |
| Ethanol | 31 | 2429 | 295 | 265 | 6 | 232 | 2 | 1680 | 32 |
| 2-propanol | 45 | 124 | 37 | 87 | 2 | 0.16 | 0.06 | 184 | 0.63 |
| Butanol | 56 | 1966 | 858 | 1229 | 254 | 3661 | 1591 | 2664 | 471 |
| Isoamyl_alcohol | 55 | 76 | 59 | 246 | 34 | 0.92 | 0.24 | 23 | 1.7 |
| 1-Pentanol | 55 | 15 | 1 | 35 | 0.09 | 18 | 0.97 | 8 | 0.38 |
| Hexanol | 56 | 161 | 23 | 155 | 12 | 126 | 4 | 24 | 2 |
| Ethylhexanol | 57 | 4 | 0.39 | 2 | 0.45 | 3 | 0.60 | 1 | 0.62 |
| **Ketones and aldehyde** | | | | | | | | | |
| 2-propanone | 43 | 164 | 5.8 | 75 | 0.05 | 337 | 75 | 11 | 5.3 |
| 2-Heptanone | 58 | 12 | 5.4 | 23 | 2.9 | 7 | 2 | 0 | 0 |
| 2-Methyl butanal | 57 | 2 | 1.5 | 1.2 | 0.14 | 3 | 0.4 | 2 | 0.3 |
| **Esters** | | | | | | | | | |
| Butyric acid propyl ester | 101 | 0.23 | 0.03 | 0.17 | 0.03 | 0.37 | 0.06 | 0.75 | 0.15 |
| Pentanoic acid ethyl ester | 85 | 0.23 | 0.59 | 1.57 | 0.65 | 1.40 | 0.24 | 0.36 | 0.06 |
| Ethyl acetate | 43 | 52 | 42 | 14 | 6 | nd | nd | 76 | 7 |
| Butyl acetate | 73 | 80 | 0.08 | 76 | 0.61 | 79 | 5 | 81 | 7.2 |
| Butyl butanoate | 71 | 17 | 3.3 | 167 | 18 | 17 | 3.31 | 0.56 | 0.55 |
| Ethyl hexanoate | 88 | 10 | 0.4 | 12 | 1 | nd | nd | 5 | 2 |
| Butyl hexanoate | 99 | 1 | 0.04 | 5 | 1 | 332 | 63 | 0.48 | 0.45 |
| Cyclobutanol-3-ethyl acetate | 86 | 41 | 12 | 32 | 5 | 44 | 15 | 9.5 | 0.26 |
| Butanoic acid ethyl ester | 71 | 1594 | 66 | 56 | 0.97 | 142 | 21 | 395 | 17 |
| Methyl thiobutanoate | 43 | nd | nd | 12 | 2 | 12 | 2 | 26 | 2 |
| Butyl isobutyrate | 71 | 1.14 | 0.27 | 3 | 2 | 2 | 0.09 | 0.94 | 1 |
| Isopropylacetic acid | 60 | 0.47 | 0.28 | 44 | 1 | 14 | 2 | 1 | 0.40 |
| **Carboxylic acids** | | | | | | | | | |
| Ethanoic acid (acetic acid ) | 45 | 6 | 9 | 9 | 0.43 | 57 | 9 | 24 | 2 |
| Butanoic acid | 60 | 430 | 101 | 371 | 35 | 1669 | 115 | 1018 | 6 |
| Pentanoic acid | 85 | 1 | 0.24 | 0.36 | 0.07 | 2 | 0.59 | 2 | 0.7 |
| **Other compounds** | | | | | | | | | |
| Dimethyl disulphide | 94 | 6 | 0.25 | 49 | 0.89 | 3.4 | 0.39 | 3.5 | 0.18 |
| Dimethyl trisulphide | 126 | 0.16 | 0.01 | 1 | 0.02 | 0.02 | 0.01 | 0.07 | 0.03 |
| 1-Propene-1-thiol | 73 | 80 | 0.25 | 75 | 0.80 | 80 | 6 | 81 | 7 |
| Phenyl ethylene | 104 | 53 | 1.8 | 30 | 2 | nd | nd | 13 | 1 |
| 1,3,-Ditert-butylbezene | 175 | 64 | 0.06 | 8 | 3 | nd | nd | 3 | 5 |

‡values represent the relative abundance. Two sources of grains 1 and 2; MO=maize *ogi*, SO=sorghum *ogi*. Data are means of four replicates and standard deviation (n=4).

#### 5.3.6.2.2 Principal component analysis

The data obtained from the volatile analysis described above was used to carry out a principal component analysis (PCA) to ascertain whether or not the aroma profile of the four fermentations was significantly different. The PCA biplot of volatile organic compounds of *ogi* is shown in Fig.5.9.

Principal component (PC1) showed the majority of the volatiles and was strongly influenced by all the ketones, carboxylic acids, sulphur-containing volatiles, benzene derivatives and the fusel alcohols apart from butanol with 2-propanone, ethanoic acid (acetic acid), ethyl acetate, ethyl butanoate, ethyl hexanol and dimethyl trisulphide showing high positive values. The second principal component (PC2) accounted for 17% of the volatiles and was distinguished by major levels of aldehyde (2-methyl butanal), benzene derivative (phenyl ethylene), ester (butyric acid propyl ester) and fusel alcohol (butanol). Ethyl hexanoate was an outlier and did not seem to have much influence on both PCs.

Although there was no clear discrimination in the distances between the PCA score plots for the different groups of volatile compounds (Fig.not shown) the two principal components (PC1 and PC2) were able to distinguish between the volatiles of maize and sorghum *ogi*, as shown by the clear separation of the samples on the PCA biplot (Fig. 5.9) which explains 83% of the total variance by combination of two principal components PC1 (59%) and PC2 (24%).

Two groups (1 and 2) were observed with high variations in volatile profiles of both maize *ogi* (MO1 and MO2) and similarities in sorghum *ogi* (SO1 and SO2). The variation observed with MO1 and MO2 may be related to the changes in the microbial population evident at 30-36 h of fermentation (Fig 5.1). The decline in the viable cell populations of yeast and mould in MO1 at the stationary phase may have reduced the complexity in metabolic activities during the log phase thus favouring the build-up of metabolites from other viable populations resulting in the few volatiles associated with MO1. Maize *ogi* (MO1) was mostly associated with PC2 while SO1 and SO2 were

associated with PC1 indicating higher substrate utilization and syntheses of volatiles in sorghum than maize *ogi*.

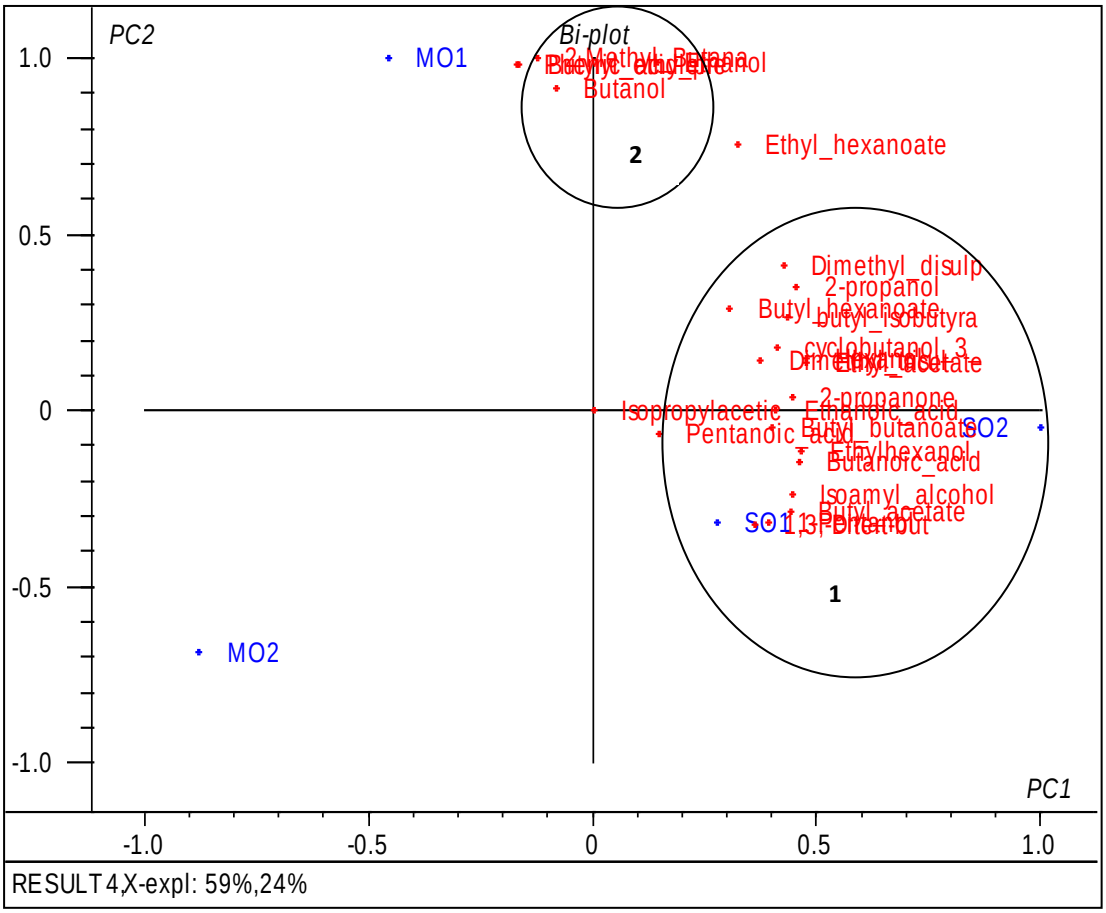



**Figure 5.9:** Principal component analysis biplot of volatile organic compounds from naturally fermented maize (MO1; MO2) and sorghum (SO1; SO2) *ogi* obtained by SPME-GC-MS headspace analysis.

### 5.4 Discussion

Microbial populations increased within the first 24 h in all fermentations. A similar trend has been reported in *ogi* and other related traditionally fermented foods such as *masa* - a fermented maize product (Wakil *et al*., 2008). Variations observed in the viable microbial populations of maize and sorghum during natural fermentation may be associated with the original microbial load in the grains. The presence of LAB is in

agreement with earlier findings from natural fermentation of *ogi* and other cereal products (Odunfa and Adeyele, 1985; Nout *et al*., 1989; Chavan and Kadam, 1989). The high count obtained for enterobacteriaceae in M2 fermentation may be attributed to minimal acidity (pH 5.04) observed in this fermentation. This effect correlated with the low count of LAB on MRS in M2 fermentation. It is expected that increase in LAB population would decrease the pH resulting in death of enterobacteriaceae and other non-acid tolerant species (Nout *et al*., 1989). This was not the case in the S1 fermentation suggesting that acid-producing homofermentative LAB may not have been the major population in S1 fermentation. This result is supported by the actual identification of microbes in these fermentations in Chapter 4 (§4.2.1.1.3) which also showed the presence other groups such as of *Enterobacter* and *Pantoea* (Table 4.8) in S1 fermentation. However the inhibition and total disappearance of enteric bacteria in natural fermentation of cereal grains have been reported to occur at pH < 4.5 (Nout *et al*., 1989; Wakil *et al*., 2008) which is lower than the pH values observed in all the fermentations in this study.

The average starch content was 80.35% and 93.12% in maize and sorghum grains, respectively. These concentrations were greater than the values reported for maize - 62-69% (Bach Knudsen, 1997; Jaworski *et al*., 2015) and sorghum - 69% (Jaworski *et al*., 2015). However, different researchers reported values of 73–82.5% in maze and 70.5–78.6% in sorghum of different varieties from Mali (Barikmo *et al*., 2004). These values were nearer to those obtained in the present study in maize but still lower than in the sorghum.

Starch content only varied significantly in the two sources of sorghum with a very high level observed in the eastern grain (S2). Overall recovery of starch in maize and sorghum *ogi* (§5.3.2) followed a similar trend. The results from maize and sorghum fermentations, show average starch losses of 21% and 23% in both fermentations and suggests similar hydrolytic activities probably by enzymes or the microorganisms. Nevertheless the low amount lost suggests good carbohydrate retention during *ogi* fermentation. Hydrolysis of starch depends on pH, duration of process, substrate concentration and accessibility to enzymes (Jaurena and Pichard, 2001). The result of

pH measurement (Table 5.1) showed significant differences ($p>0.05$) between similar grains at 0-12 h and was then fairly similar until the end of the fermentation. This was also true comparing maize and sorghum fermentations except M1. Since starch loss was similar in all four fermentations this suggests that acidity may not have been a key factor for starch hydrolysis.

Non-starch polysaccharides are present as soluble (SDF) and insoluble (IDF) dietary fibres. Ways of analysing the NSP include the crude fibre method and the detergent method measuring the neutral detergent fibre (NDF) and acid detergent fibre (ADF). The crude fibre assay solubilizes some of the structural polysaccharide and lignin and only measures some components of the fibre. Neutral detergent assay (NDA) directly measures the cellulose, lignin and hemicellulose while the acid detergent measures only cellulose and lignin with the calculation of hemicellulose by difference. The calculation method has limitations of not allowing accurate measurement given that the water soluble and water insoluble NSP could be lost while starch and protein may contaminate the residue. The NDF and ADF do not measure the water soluble NSP which often comprises a high proportion of the NSP (Van Soest, 1963). These assays apart from the limitations are laborious and more precise enzymatic-chemical method and non-enzymatic gravimetric methods have successfully been used (Englyst *et al*., 1982, Prosky *et al*., 1985). In this study the enzymatic-chemical method was chosen owing to its quick and reliable procedure. In this method the starch is solubilized and completely removed enzymatically and NSP is measured as the sum of the constituent sugars released by acid hydrolysis (Englyst *et al*., 1982). Non-starch polysaccharides (NSPs) are degraded by several microbes (Pérez *et al*., 2002). The NSP content of maize in this study is similar to the value of 8.1% reported in the literature but greater than in sorghum at 6.6% (Jaworski *et al*., 2015). The presence of arabinoxylans (49% and 43%) in maize and sorghum (Barikmo *et al*., 2004) predominantly in the bran fraction (which forms the pomace) may probably be the reason for the higher level of xylose and arabinose in the pomace than in the *ogi* at the end of fermentation. The low levels of xylose and arabinose in *ogi* suggests low fibre content. Apart from keeping the digestive system healthy, dietary fibre (NSP)

helps to stabilize glucose and cholesterol levels (Lafiandra *et al*., 2014) therefore the loss of fibre in *ogi* is non-beneficial. On the other hand, while some authors have associated a low fibre diet with constipation (Cummings, 1973) others were of the opinion that ingestion of large amounts of fibre, especially those associated with high viscosity (Lafiandra *et al*., 2014) or encapsulation of digestive enzymes by NSP, (Căpriţă *et al*., 2010) could delay emptying of the gastrointestinal tract resulting in constipation. However, Ho *et al*. (2012), reported that reduction or stopping of dietary fibre intake improved constipation. Traditionally in Nigeria, consumption of raw (uncooked) *ogi* is employed in the management of diarrhoea which could be explained by the probiotic properties conferred by *ogi* (Aderiye and Laleye, 2003). Although the understanding of the chemistry of NSP in relation to indigestion seem to be controversial and inconclusive, low NSP values in *ogi* observed in the present study reveals a second reason for its effectiveness as an anti-diarrhoea remedy which agrees with the opinion of Cummings (1973).

Cereals are rich sources of phenolics (Dykes and Rooney, 2006) and as such the fate of these compounds during fermentation was investigated. A first attempt was made to determine the total levels and fate of the phenolics during the fermentation. Secondly since phenolics occur as free or bound forms which have nutritional significance, an attempt was also made to quantify the relative proportions of these fractions during fermentation. Finally efforts were made to try to identify the causal relationships between the microbial population and the changes in phenolics observed during fermentation.

The determination of total phenolics pose several experimental challenges as a significant proportion of the phenolics are bound to the food matrix and must be solubilised. Since optimal release can vary with the food matrix the use of alkaline hydrolysis is usually applied as reported in the literature to achieve this for periods ranging between 2-24 h (Khoddami *et al*., 2013). A saponification time of 2 h was selected in this study as this has been successfully used in the literature for cereals (Montilla *et al*., 2011). Conventionally phenolics are then partitioned into an organic

solvent such as ethyl ether or ethyl acetate (Lu *et al*., 2014) to facilitate concentration for a subsequent HPLC analysis and also help in the specificity of the assay. This partitioning step was not employed in this comparative study rather the TPC was measured directly on the extract. However, the TPC assay is not completely specific and may overestimate the phenolic content. This assay is considered as an antioxidant assay and thus can also give an indication of the levels and recoveries of antioxidants activity during fermentation. In this study, the total phenolic content in maize grain gave 410–437mg/100g GAE equating to 24.10 µmoles GAE/g which is similar to the value of 15.55 µmoles GAE/g reported by Adom and Liu (2002). In sorghum, the TPC observed (221-247 mg/100g) in both grains varied from values in the previous studies and was lower than 450–554 mg GAE /100g and 306.65–445.84 mg GAE/100g GAE reported by El Hag *et al.* (2013) and Abdelrahman *et al*. (2005), respectively. However the sorghum TPC was within the range of 270–2450 mg GAE/100g reported by Dlamini *et al*. (2007) and higher than 109.21-110.52 mg GAE/100g as shown by Afify *et al.* (2012). The apparent inconsistencies observed with the overall recovery of total phenolics may have arisen from the fact that whilst the Folin assay can be used to indicate phenolic content, it could be confounded by other non-phenolic compounds (Kamath *et al*., 2004). Thus in this study it would appear that overall only about 37-59% of the original total phenolics in the grains were recovered in the *ogi* in all cases, the major loss being due to retention in the pomace (Fig. 5.4). This is perhaps not surprising as the pomace comprises the greater part of the original grain that stores phenolic compounds (Hahn *et al*., 1983, Troszyńska and Ciska, 2002, Butsat *et al*., 2009). The TPC in the steep water increased with the time during fermentation and this exceeded the rate of leaching in an uninoculated control. Given the constraints of the TPC assay this could reflect esterase activity acting to release bound phenolics or represent the synthesis of confounding compounds by the microorganisms. Thus decreasing the fermentation time may enhance the retention of soluble phenolics in *ogi*. Sorghum showed greater losses through the steep water than the maize and this may reflect the relatively higher soluble phenolic content in the original grain.

The determination of soluble phenolics is relatively straightforward and methanol extraction was applied in this study having been reported as the best extraction solvent (Kamath *et al*., 2004). However, a similar experimental limitation as explained with the TPC also applies to the bound fraction. This is the first observation on *ogi* and as such no information is available in the literature on the recovery of soluble and bound phenolics following the fermentation of *ogi*. However, Cui *et al*. (2012) reported an increase of 21.6%-23.4% in the total phenolic acid of yeast fermented maize-wheat porridge.

Further investigation of the leaching of phenolics into the steep water to show the impact of each group of organisms on hydrolysis of phenolic acid during fermentation showed that the increase in MRS count mirrored the increase in phenolic levels. The correlation between increase in MRS counts and phenolic levels suggests that members of LAB could be responsible for this activity. This result may explain the lower recovery of total phenolics in the maize than in sorghum fermentations since LAB were not the major population in the maize. Esterases are known to be produced by members of LAB such as *Lactobacillus* and *Pediococcus* (Matthews *et al*., 2007). The fact that M17 counts did not correlate with phenolic leaching again may suggest that it is a specific subgroup within the lactic acid bacteria which is responsible for the esterase activity. Again most of the population identified on the M17 plate were not LAB. This agreed with the identification results which showed that, apart from *Enterococcus,* LAB were not the major population in the maize fermentations. The presence of *P. pentosaceus* and *Lb. rossiae* in addition to *Enterococcus* spp in sorghum (S1) as seen in Chapter 4 further explains the release of higher level soluble phenolics in the fermentation. However, it was notable that after 36 h the microbial populations declined in all fermentations. This result corresponds with the minimal growth shown by the LAB species which may be attributed to nutrient availability. Again phenolic compounds have been reported to inhibit microbial growth (Gutiérrez-Fernandez *et al*., 2013).

Although population counts on RBCA (for yeast and moulds) did not show any correlation with the release of soluble phenolics, mould species such as *Aspergillus niger* have also been reported to produce esterases (Crepin *et al*., 2004). Thus *Aspergillus* may not have been the dominant population or possibly its activity was inhibited in the fermentations. *Trametes hirsuta* isolated in this study (Fig. 4.8) from sorghum and which showed phytase activity has also been reported to produce laccase, an extracellular enzyme found to oxidize phenolic and aromatic compounds (Nagai *et al*., 2002; Rodríguez Couto *et al*., 2005). However, the activities of this fungus may also have been reduced by other members of the population such as LAB which inhibit fungal growth due to acid production. It is notable that the phenolic leaching observed for S1, from which the isolate of *Trametes hirsuta* was obtained, was not greater than that for S2 (Fig. 5.3).

Calorimetric analysis by iron precipitation and ion-exchange methods form the two major quantitative methods of analysis of phytate. However, the purification and measurement of phytate (myo-inositol hexakisphosphate IP6) seem quite complex with the anion-exchange purification in which the lower myo-inositol phosphates ($IP_3$.$IP_4$ $IP_5$) may also elute resulting in overestimation of phytic acid content (Kasim and Edwards, 1998). Given this limitation in direct measurement of phytic acid from foods, a quantitative method involving the measurement of total available phosphorus released from the samples by acid extraction and alkaline hydrolysis was applied in this study (K-PHYT, Megazyme). Since no single method of phytic acid analysis is completely perfect, the release of small quantities of other phosphorus from monophosphate esters by alkaline phosphatase used in the present approach is also anticipated (Megazyme).

The majority of the fermentations had minimal phytate detectable in the waste products (steep water or pomace). Grain and microbial phytases contribute to phytate reduction during fermentation (Dhankher and Chauhan, 1987, Reddy and Pierson, 1994). Reduced phytate levels during fermentation would result in improved nutrient bioavailability in the *ogi*. The lower levels recovered in maize than sorghum *ogi* may

mean more nutrient bioavailability in the former. Fermentation has been reported to significantly reduce phytate levels in cereal grains. Towo *et al*. (2006) reported 72-88% decrease in the phytate level of fermented sorghum gruel with the addition of phytase enzyme. This decrease is similar to the result obtained in this study (74-82% in maize and 52–60% in sorghum). Variations in phytate contents of maize and sorghum *ogi* may be due to the initial wider microbial community population in M2 and S2 which both included an initial higher yeast and mould population, which are groups known for phytase production. This difference in phytate levels of *ogi* may be attributed to regional effect since phytate in M2 was also higher than in M1 although this was not statistically significant. Several species of bacteria and moulds have been used for commercial phytase production (Haefner *et al*., 2005), thus the presence of phytate hydrolysing moulds such as *Aspergillus* in the fermentations may have contributed to phytate reduction. Phytate reduction was significantly higher in maize than in the sorghum. This result is in accordance with the observation of (Lestienne *et al*., 2005). Again leaching of phytate was observed in this study as also observed by these researchers who reported 21% and 4.6% leaching in maize and sorghum after 24 h soaking in water. However, contrary to their result phytate was detected in M1, S1, and S2 steep waters while the absence of phytate in M2 steep water agreed with their findings. Other factors such as starch (carbon source) and pH which affect microbial growth and activities may also have influenced fermentation and hydrolysis of phytate.

The presence of organic acids contributes to taste and flavour in *ogi*. The three major acids identified in natural fermentation of *ogi* were lactic, acetic and malic acids. Lactic acid is known to be mostly produced by LAB however the absence of lactic acid in M2 up to 24 h even with high counts on M17 and MRS may probably be due to conversion of lactic acid to acetic acid by LAB. This could further explain why acetic acid was detected early in maize fermentations. Again previous findings from DGGE analysis of microbial diversity by bulk cells in this study (§4.2.1.2.5), showed overall similarity in microbial groups present in both maize fermentations, which consisted mostly of yeast species on MRS agar as seen in Chapter 4 (Table 4.6, 4.7 and 4.8). Therefore

metabolic activities by yeast would also be expected to contribute to the absence of lactic acid observed in M2 at 12 – 24 h as this might have been used up by the yeasts as carbon source for growth. Similarities observed with lactic acid levels in M1 and S1 fermentations relate to the microbial population (Fig 5.1) which seemed similar over the fermentation period thus suggesting similar levels of substrate utilization in both fermentations. Again higher lactic content in MO1 and SO1 suggests that these may have a more sour taste and improved shelf stability than MO2 and SO2.

Malic acid is produced from fermentation of sugar and non-sugar sources and is an intermediate acid in cell metabolism from pyruvate through oxaloacetate (Peleg *et al*., 1988; Battat *et al*., 1991). The ability to accumulate L-malic acid has been identified in mostly yeast and filamentous fungi such as *Aspergillus flavus* and *Rhizopus oryzae* (Battat *et al*., 1991). However, the production of malic acid is mostly associated with yeast–alcohol fermentation and has been studied in wine fermentation (Dakin, 1924; Zelle *et al*., 2008). The efficiency of microbial accumulation of L-malic acid during fermentation has been reported to be low (Battat *et al*., 1991). The early detection of malic acid in sorghum was not surprising as this has been reported to be present in the grain apart from being produced by yeasts and moulds (Drozdowicz and Jones, 1995). Although the reason for low efficiency in production of L-malic acid by microbes is not clearly known, other studies have attributed this to high acidity in the fermentation medium (Zelle *et al*., 2008). Again malic acid is not excreted during fungal mycelial growth (Peleg *et al*., 1988). These studies could explain the changes in the level of malic acid recorded in this present study. However, production of malic acid seemed not to show any correlation with the pH of the fermentation medium in maize but detection of low levels of malate during fungal growth agreed with the observation of Peleg *et al*. (1988), due to reduction in sugar levels and probably high nitrogen. Malic acid in M1 and M2 were similar and higher than in S1 and S2. This result is not surprising since the yeast and mould population in M1 and M2 (Table 4.5-4.12) were mostly dominated by *Aspergillus* species (known malic acid producers) while *Penicillium* and *Cladosporium* species (with no report found on malate production) were found in sorghum. The correlation of the increase in yeast and mould

populations at 54h in M2 with the increased level of malic at this hour further supports the contributions of this group to malic acid production. The presence of malic acid may also contribute to taste and flavour of maize *ogi*.

Although known for acetate production, acetic acid bacteria (AAB) were not responsible for production of acetic acid, since AAB were not identified as part of the microbial population in either fermentation. Facultatively anaerobic enterobacteria such as members of the *Enterobacter*, *Erwinia* and *Serratia* genera have been associated with acetate production from glucose in mixed acid fermentations (Müller, 2001). Again lactic acid bacteria are capable of metabolising citrate to form acetate through the Krebs cycle with or without oxygen (Starrenburg and Hugenholtz, 1991; Oude-Elferink *et al*., 2001). Therefore acetic acid might have been produced through the above pathways considering the dominance of *Enterobacter* and *Enterococcus* species in these fermentations as identified in Chapter 4.

During fermentation, the presence of other reducing compounds apart from pyruvate and their electrode potential levels play a major role in determination of the products of fermentation. Hydrogen atoms from reduced NAD are usually diverted to high electrode potential alternatives to pyruvate resulting in various other products such as acetate in addition to lactate (Moat *et al*., 2002c). This dismutation of pyruvate could further explain the presence of acetic acid in natural fermentation of *ogi* without the presence of acetic acid bacteria as observed in this study. Again other enzyme mediated hydrolysis of plant material such as hemicellulose may also contribute to this effect. Xylan has been reported as a major carbohydrate polymer of hemicelluloses and several bacterial and fungal species are capable of producing xylan esterases, degrading hemicellulose into sugars and acetate (Pérez *et al*., 2002). The diversity of the fermentation organisms isolated in this study as discussed in § 4.2.1 and the end pH of the fermentation media which ranged from 4.8–5.04 in both maize and sorghum fermentations further supports this suggestion and agreed with the optimal pH range (4.5-5.5) of most fungal xylanases as reported by Jeffries (1994) and the ability of bacterial species such as *Bacillus* and *Streptomyces* sp. to produce xylanases (Blanco

*et al.,* 1999). Bacterial xylanases are activated at acid to neutral pH 6.0-7.0 (Jeffries, 1994). Production of acetate was observed after 6 h in both fermentations. Based on the pH of all the fermentation systems (Table 5.1) which ranged between 5.86-7.40 at 6-24 h and 4.80–5.98 at 30–72 h, acetic acid produced at these times could be attributed to hydrolysis by bacterial and fungal xylanases, respectively. The greater levels of acetic acid than the lactic and malic acid in all the fermentation suggests significant contribution of this acid to the unique sour taste of *ogi.* Acetate has also been reported to reduce yeast sporulation (Codón *et al*., 1995) thus reducing the viable population of yeast during fermentation.

Headspace analysis of volatile organic compounds (VOCs) by solid-phase microextraction and gas chromatography-mass spectrophotometry (SPME GC-MS) was demonstrated to be an effective technique for analysis of volatiles in *ogi*. Volatile compounds may be produced via microbial metabolism (Silva *et al*., 2011), degradation of fermentation products (Gallais *et al*., 1990) or Maillard reaction products (Salter *et al*., 1988). Several of the identified VOCs in *ogi* are associated with microbial metabolism and degradation products.

Esters, alcohols, aldehydes, organic acids and only a few ketones were mostly present during natural fermentation of *ogi*. Although no report was found on volatile aroma components of *ogi*, studies on other related fermented cereal foods and beverages such as Ghanaian maize dough, *Fen-Daqu* (fermented Chinese liquor from sorghum) and *Bushera* (Ugandan fermented dough) reported similar groups of volatile compounds as identified in this study in addition to phenolic compounds, Maillard products: pyrazines and heterocyclic compounds (furanones and pyrones) with ethanol, lactic and acetic acids as the dominant alcohol and carboxylic acids respectively (Annan *et al*., 2003; Muyanja *et al*., 2012; Le *et al*., 2012).

The major alcohols detected in *ogi* were ethanol, butanol and isoamyl alcohol. These may be produced by fermentation of glucose or amino acids under aerobic or anaerobic conditions by yeasts or by decomposition of secondary hydroperoxides of fatty acids-aldehydes and hydrocarbons (Annan *et al*., 2003; Le *et al*., 2012; Liu *et al*.,

2012). However the source of butanol in these fermentations seemed not be clear and may not be associated with the key microbe (*Clostridium* sp) usually considered responsible for butanol production (Campos *et al*., 2002) since this was not dectected and identified by the characterization techniques used in this study (§4.2.1). The abundance of ethanol in maize *ogi* is not surprising since yeast species were mostly detected in maize. It is expected that favourable growth conditions would enhance metabolic activities. Arroyo-López *et al*. (2009) reported the highest yeast response at 34.1$^{o}$C and pH 4.76. Serra *et al*. (2005) studied the influence of temperature and pH on yeast growth and observed a positive correlation between temperature and pH during yeast fermentation with temperature having a major influence on yeast response and pH being less significant. Again Charoenchai *et al*. (1998) reported an increase in yeast growth rate up to the maximum temperature of 25$^{o}$C with no significant impact of pH on growth rate when varied between pH 3.0-4.0. However, based on growth conditions the reason for the higher relative abundance of ethanol in MO1 compared to MO2 is not clearly evident since the pH and temperature of both fermentations did not differ significantly although yeast growth conditions in MO1 (pH 4.80 and temp. 25$^{0}$C) seemed more favourable and showed more relatedness to those reported in the literature. However, it is notable that two different genera of yeast capable of producing ethanol were detected in MO1 (*Meyerozyma caribbica*) and MO2 (*Schizosaccharomyces pombe*). The use of *Meyerozyma caribbica* in production of alcoholic beverages such as *tequila* and *tchapalo* (a traditionally fermented sorghum drink) has been reported (Saucedo-Luna *et al*., 2011; N'guessan *et al*., 2011). Abubaker *et al*. (2012) in their study reported the highest production of ethanol during fermentation of molasses by *Schizosaccharomyces pombe* at 32 $^{o}$C and pH 4.8 and a decrease in ethanol yield when the sugar concentration in the medium was increased above 20%. This decrease in fermentation efficiency indicated by low ethanol yield was attributed to reduction in sugar conversion to ethanol (Abubaker, *et al*., 2012). The production of ethanol in sorghum *ogi* might have been mostly through chemical reduction of corresponding esters since yeasts were not a major part of the population in the fermentations. Production of alcohol from sugars

during fermentation leads to decrease in carbohydrate levels (Kohajdová and Karovičová, 2007).

Surprisingly butanol and 2-propanone (acetone), along with ethanol, are products of anaerobic fermentation of *Clostridium acetobutylicum* or *Clostridium beijerinckii* (Campos *et al*., 2002) and were detected despite *Clostridium* sp not being present in any of the fermentations when assessed both by cultural and molecular identifications. Again fermentation of *ogi* is not strictly under anaerobic conditions and since other microbes such as *Bacillus* and yeasts (facultative anaerobes) are capable of reducing aldehydes to produce corresponding alcohols (Le *et al*., 2012), the presence of yeasts in these fermentations may be a possible source with 2-methyl butanal (precursor of butanol) detected in all fermentations. Ketones are products of autoxidation of fatty acids (Liu *et al*., 2012). It has also been reported that certain *Bacillus* species such as *Bacillus macerans, B. acetyoethylicum* and *B. granulobacter pectinovorum* are capable of breaking down carbohydrates to produce 2-propanone (acetone) (Petersan *et al*., 1921). This could explain why acetone was also found in these fermentations although different species of *Bacillus* (*B. cereus*, *B. tequilensis* and *B. subtilis*) were identified in this study.

*Schizosaccharomyces pombe* identified in maize (M2) may be significant in ester production in *ogi* fermentation as yeasts of the *Saccharomyces* sp. are well known for ester production (Saerens *et al*., 2010). Esters mostly identified were ethyl acetate, butanoic acid ethyl ester, butyl butanoate, and butyl acetate. Ethyl acetate and butanoic acid ethyl ester have flavour thresholds ranging from 300-6600 ppm and 450 ppm while butyl butanoate and butyl acetate have odour thresholds of 100 ppm and 66 ppm respectively (Patton, 1964; Takeoka *et al*., 1990), thus the low odour threshold compounds may have a more significant contribution to the smell of *ogi* especially MO1 and SO2 in which they are relatively less abundant but mostly present. On the other hand, volatiles with high flavour thresholds which are present in large amounts in the samples such as butanoic acid ethyl ester may also be important in the overall flavour characteristics of *ogi*. Other volatile components such as dimethyl- disulphide

and –trisulphide, although present at very low levels, may also play significant roles in the flavour of *ogi* considering their very low flavour thresholds which ranged from 0.16 -12 ppm and 0.005-0.01 ppm respectively. Volatile sulphur compounds may be formed by oxidation of methionine resulting in off-flavours in foods as identified with dimethyl disulphide which results in off-flavour in milk (Yung *et al*., 1998). The organo-sulphur compound 1-propene-1-thiol belongs to the group of thiols mostly produced by yeasts and has a garlic-like odour which is often strong and repulsive (characteristic of spoilt *ogi*) and could be significant in *ogi* characteristics. Volatile benzene derivatives are odour active components which contribute to flavour of cereals mostly millet (Lui *et al*., 2012). These compounds showed higher relative abundance in maize *ogi* than sorghum. Interference of these compounds with the identified pleasant aroma components could result to an overall complexity in the aroma profile of maize *ogi* leaving sorghum *ogi* with more pleasant aroma notes than the maize.

### 5.5 Conclusions

The changes in the microbial population and chemical composition of *ogi* were studied. Significant variations in microbial population changes during maize and sorghum grain fermentation were observed. Recovery of starch was similar in both maize and sorghum fermentations. The release of phenolics and reduction of phytate during fermentation of both maize and sorghum provided possible evidence of esterase and phytase activities. However, greater loss of phenolics was observed with sorghum and this was associated with increased leaching into the steep water and wash water but mostly by retention in the residue. The reduction in phytic acid content of maize and sorghum during fermentation varied significantly between the different grains. The potential of esterase and phytase activities by other members of lactic acid bacteria such as *Lactobacillus* and two phytate hydrolysing fungi isolated from maize (*Acremonium zeae*) and sorghum (*Trametes hirsuta*) observed in this study suggests more nutrient may be made available in maize and sorghum *ogi* through fermentation.

A total of 29 volatile compounds were identified in *ogi*. Esters were the major group of volatile compounds identified, being a product of carboxylic acid and alcohol which are characteristic of natural fermentations as seen in this study. The dominance of esters in these fermentations may mean high flavoured *ogi,* however, the high flavour threshold values exhibited by esters may also make them less important depending on the amount present. The presence of different volatile compounds in maize *ogi* could be related to the differences in the microbial population, species and metabolic activities during fermentation. Production of fusel alcohols and their aldehydes are characteristic of fermentative yeasts (Damiani *et al*., 1996) and this was clearly evident in this study especially in maize fermentations. The relative abundance of ethanol and isoamylalcohol were higher in maize *ogi* and suggests more characteristic fruity flavour in MO1. The association of maize *ogi* with fewer volatile organic compounds as seen with the PCA analysis suggest a more simple flavour characteristics in maize than sorghum *ogi*.

Overall this study has demonstrated that differences in both microbial consortia and substrate during natural fermentation may influence the phenolic, phytate and aroma profiles of *ogi.* Although natural fermentation presented improved available nutrient and flavoured products, the complexity of microbial metabolism resulting in the inhibition of activities of desired microbes and production of undesirable metabolites was evident in this study. Thus the influence of starter cultures of LAB (*Pediococcus pentosaceus*) and phytate hydrolysing fungi (*Trametes hirsuta* and *Acremonium zeae*) on quality characteristics of *ogi* was investigated in chapter 6 and the overall microbial effect on the compositional changes are discussed further in the final chapter.

# CHAPTER 6

# STARTER CULTURE FERMENTATION

## 6.1 Introduction

Microorganisms are capable of producing enzymes which can hydrolyze bioactive substances in the grain and which also produce aroma compounds. Esterases and phytases are synthesised by several microorganisms such as lactic acid bacteria (LAB) and some mould species (Yoon *et al*., 1996, Pérez-Martin *et al*., 2013). While there is no record of esterase activity in LAB isolated from *ogi* some researchers have studied the esterase activities of wine and dairy related yeasts and LAB (Holland *et al*., 2005, Matthews *et al*., 2007) of similar genera as found in *ogi*. A decrease in the phenolic compound (*p*-NP-octanoate) facilitated by *Pediococcus* (*P.*) 4 *parvulus* has been reported (Pérez-Martin *et al*., 2013). Similarly Chatonnet *et al*. (1997) reported the ability of *Pediococcus pentosaceus* to decarboxylate p-coumaric acid to 4-vinylphenol in wine. Again the use of *Lactobacillus* species as starter cultures of *ogi* have mostly been studied while *Pediococcus pentosaceus* has only been assessed for its ability to synthesis antimicrobial compounds (Banwo *et al*., 2013) thus making this LAB a good candidate for investigation as a starter culture in *ogi* fermentation.

Phytase activity has been reported in fungal fermentations of soybean involving *Rhizopus oligosporus* and *Aspergillus oryzae*. Although fungi form part of the major group of microbes and play significant roles in the natural fermentation of *ogi*, the use of this group in a starter cultures has not been studied. *Trametes hirsuta* and *Acremonium zeae,* having shown phytate degrading ability in the present study, would be employed in starter cultures in *ogi* fermentation.

Knowledge of the interaction of the *Pediococcus pentosaceus*, *Trametes hirsuta* and *Acremonium zeae* is important to improve the nutritional quality of *ogi* and facilitate the application of these microbes in starter cultures among other genera known to hydrolyse phenolics and phytate. The aim of this study was to examine the influence

of these selected microorganisms as single or mixed starter cultures on phytate, phenolics and aroma components during fermentation of *ogi*

## 6.2 Methods

### 6.2.2 Preparation of starter cultures

Bacterial and fungal isolates were removed from frozen storage and resuscitated aseptically by transferring one micro-bead from the microbank$^{Tm}$ tube into appropriate liquid medium MRS broth (Oxoid) for LAB and phytate specific media (§3.2.1.2) for fungi.

*Pediococcus pentosaceus* was resuscitated in 10 ml of MRS broth at 30$^{o}$C for 24 h in a shaking incubator and then sub-cultured twice on MRS agar at 30$^{o}$C for 48 h to obtain pure colonies. A well isolated colony was then inoculated into 100 ml MRS broth and incubated in a shaking incubator at 100 rpm and 30$^{o}$C for 24 h. Twenty millilitres of the broth culture was transferred into a 30 ml sterile universal tube and centrifuged at 4000 xg for 10 min. Harvested cells were washed twice in 5 ml of 0.1% sterile buffered peptone water (Oxoid) and then resuspended in 0.1% buffered peptone water of equal volume to the original MRS broth (20 ml). The cell suspension was further diluted aseptically with the same diluent and adjusted to OD 1.0 at 620 nm to obtain the stock bacterial suspension of 9 log cfu ml$^{-1}$.

*Trametes hirsuta* and *Acremonium zeae* were resuscitated in 100 ml of PSML (§3.2.1.2) at 30$^{o}$C with shaking (150 rpm) for 5 days. These isolates were then inoculated twice on PSMS (§3.2.1.2) to obtain pure colonies. Spores were harvested from the culture plate by washing the colonies with 20 ml of sterile purified water and filtering into a sterilized 250 ml beaker through sterile cheesecloth to remove the filaments. Purity of spore suspension was checked by viewing a wet mount under a microscope. Spore suspensions with filaments were refiltered and then centrifuged at 4000 xg for 10 min. Harvested spores were resuspended in sterile purified water and

adjusted to OD 1.0. Spores were then counted on a haemocytometer to determine the cell density of the stock fungal spore suspension (3.0 X $10^5$ spores/ml).

**Table 6.1**: List of starter cultures used in this study

| Treatment | Culture ID | †Isolates | source |
|---|---|---|---|
| **1** | C1 | *Pediococcus pentosaceus* | Sorghum *ogi* |
| **2** | C2 | *Trametes hirsuta* | Sorghum grain |
| **3** | C3 | *Pediococcus pentosaceus* + *Trametes hirsuta* | Sorghum *ogi* and grain |
| **4** | C4 | *Pediococcus pentosaceus* + *Acremonium zeae* | Sorghum *ogi* and maize grain |
| **5** | C5 | *Acremonium zeae* | Maize grain |

†All strains were isolated from this study

### 6.2.3 *Ogi* preparation using starter cultures

Maize and sorghum grains (maize, M2 and sorghum, S2) obtained from location 2 (Eastern region of Nigeria) were used for the starter experiment. Maize and sorghum were autoclaved at 121$^o$C for 15 min (dry heat) to eliminate the original microbes before inoculation with the starter microorganisms. Both grains were fermented using single and mixed cultures of LAB (*Pediococcus pentosaceus*) and phytate hydrolysing fungi (PHF) (*Trametes hirsuta*) isolated from sorghum and *Acremonium zeae* isolated from maize as shown in Table 6.1.

Four 1000 ml flasks containing 200g of each grain and 400 ml of sterile tap water were set up. Two flasks each were inoculated to obtain $10^6$ cells/ml of LAB by adding 0.1 ml of the bacterial cell suspension and $10^3$ spores/ml of PHF with 1.3 ml of the standardized fungal isolates as applicable to each treatment (Table 6.1). Two uninoculated flasks were set up as controls. Samples were fermented at 30$^o$C for 72 h and sample collection was as described in §3.1.2.

## 6.3 Results

### 6.3.1 Effect of autoclaving on nutrient content of grains

Since in this experiment the grain was sterilised by autoclaving prior to fermentation it was hypothesized that this may affect the content of phytochemicals such as phytate or phenolics. Therefore in order to establish if there were changes in levels of phytate or phenolics in the original grain after heat treatment, and to be able to make inference on the impact of the starter organisms during fermentation, the phenolic and phytate contents of the heat treated grains were both assessed. In this instance the phenolic content was assayed by the sequential extraction method only.

The effect of autoclaving on phenolic content of maize and sorghum grains is presented in Fig. 6.1. In maize the total phenolic acid content decreased significantly ($p<0.05$) from 317 to 270 mgGAE/100g of grain after heat treatment. Since the TPC assay is based on antioxidant capacity this may reflect the fact that there has been some oxidation occurring during the autoclaving. The bound and soluble fractions in the heat treated maize (MHT) were 207 and 63 mgGAE/100 g of grain, respectively. Bound phenolics had decreased by 21% while the soluble fraction increased by 12.5%, although the soluble phenolics in MHT was at a similar level ($p>0.05$) to the untreated maize (M). This observation may indicate that autoclaving has resulted in the solubilisation of bound phenolics.

In sorghum, total phenolic acid content increased from 174 to 234 mgGAE/100g following the heat treatment. This was in contrast to the effect in maize and in this instance may reflect the fact that autoclaving has increased the extractability of the phenolics or other antioxidant compounds. The bound fraction and soluble phenolics in the heat treated sorghum (SHT) gave 119 and 115 mg GAE/100g of grain showing an reduction of 3% in the bound and a 22% increase in the soluble fraction. Although not statistically different, the increase in the soluble phenolics of heated sorghum may again be due to a solubilization effect of the heat treatment on the bound fraction.

The effect of heat treatment on phytate content of maize and sorghum is shown in Fig. 6.2. In maize, heat treatment did not change the phytate content ($p<0.05$). In sorghum,

phytate content varied significantly ($p>0.05$) between the heat treated and untreated grains and increased by 43% in the treated grain. This increase again could suggest that changes in the grain matrix brought about by the heat treatment may have made the phytate more accessible to extraction as may have also been observed with the phenolics.

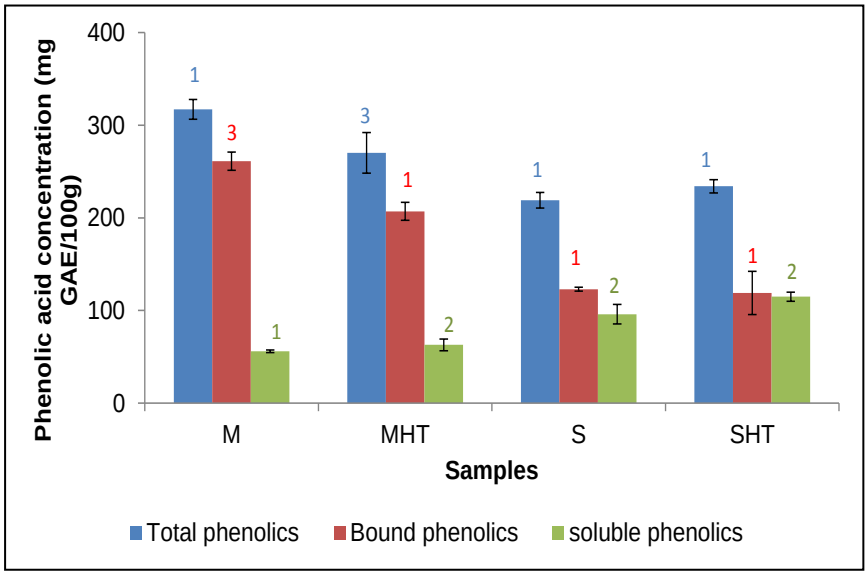



**Figure 6.1:** Effect of heat treatment on phenolic acids of maize and sorghum Maize grain (M), Heat treated maize (MHT), sorghum grain (S), Heat treated sorghum (SHT). Data are means ± standard deviation of six replicates from three determinations (n=6). Similar numbers on same coloured bars are not significantly different ($p>0.05$). Values are the total amount of each phenolic acid fraction in 100g of grains.

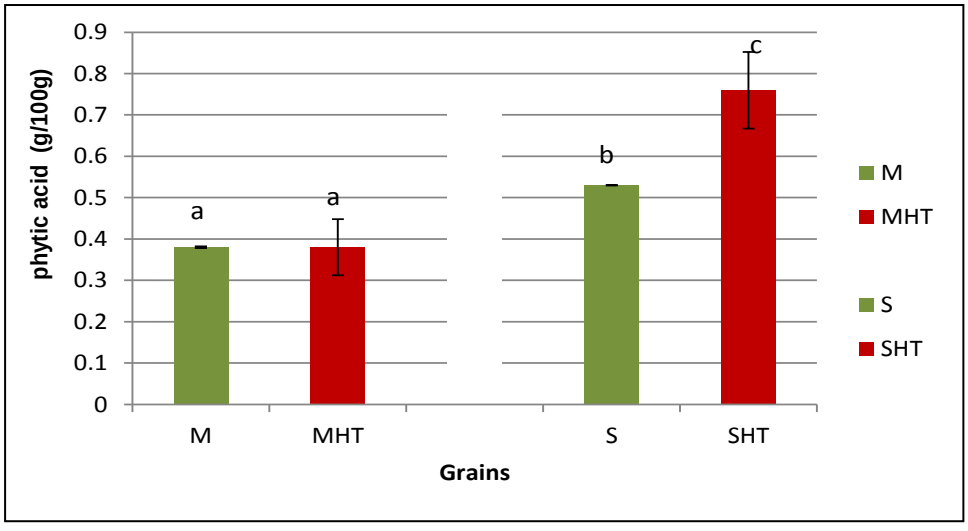



**Figure 6.2:** Phytate levels of maize and sorghum grains with and without heat treatment. Data are means ± SD of six replicates from three determinations (n=6) and are calculated based on 100g of grains. M: maize untreated; MHT: heat treated maize, S: sorghum not heat treated; SHT: heat treated sorghum.

### 6.3.2 Microbial count and pH

The result of the viable population counts for the starter organisms during maize and sorghum fermentations are presented in Fig. 6.3. The growth of *Pediococcus pentosaceus* followed similar trends both in pure culture and in mixed culture fermentations with *Trametes hirsuta* and *Acremonium zeae* in both maize and sorghum. A significant drop in viable cell count for *P. pentosaceus* was observed after 12 h in both mixed fermentations with the fungi Fig 6.3b and Fig 6.3c. Although this effect was particularly evident in the sorghum fermentation with *T. hirsuta* it seemed not to be related to the fungal population. The reason being that a similar drop in viable count was also observed at 12 h in the *P. pentosaceus* pure culture fermentation in sorghum. Therefore, this decrease probably may have been due to metabolic stress resulting in the death of the cells. However, it was notable that the viable population of *P. pentosaceus* in the mixed fermentation with *T. hirsuta* (Fig. 6.3b) varied between maize and sorghum at 12 h. The increase in the cell density of *P. pentosaceus* (Fig 6.3a) correlated to the drop in the viable population of *T. hirsuta* at 24 h in maize and 36h in sorghum as shown in Fig. 6.3e suggesting a possible antagonistic effect on the mould by the lactic acid bacteria.

The pH of the fermentations also showed similar trends in both maize and sorghum fermentations with a significant drop at 24 h except in the mixed culture of *P. pentosaceus* and *A. zeae* (C4) which dropped earlier at 12 h in sorghum fermentation (Fig. 6.4b).

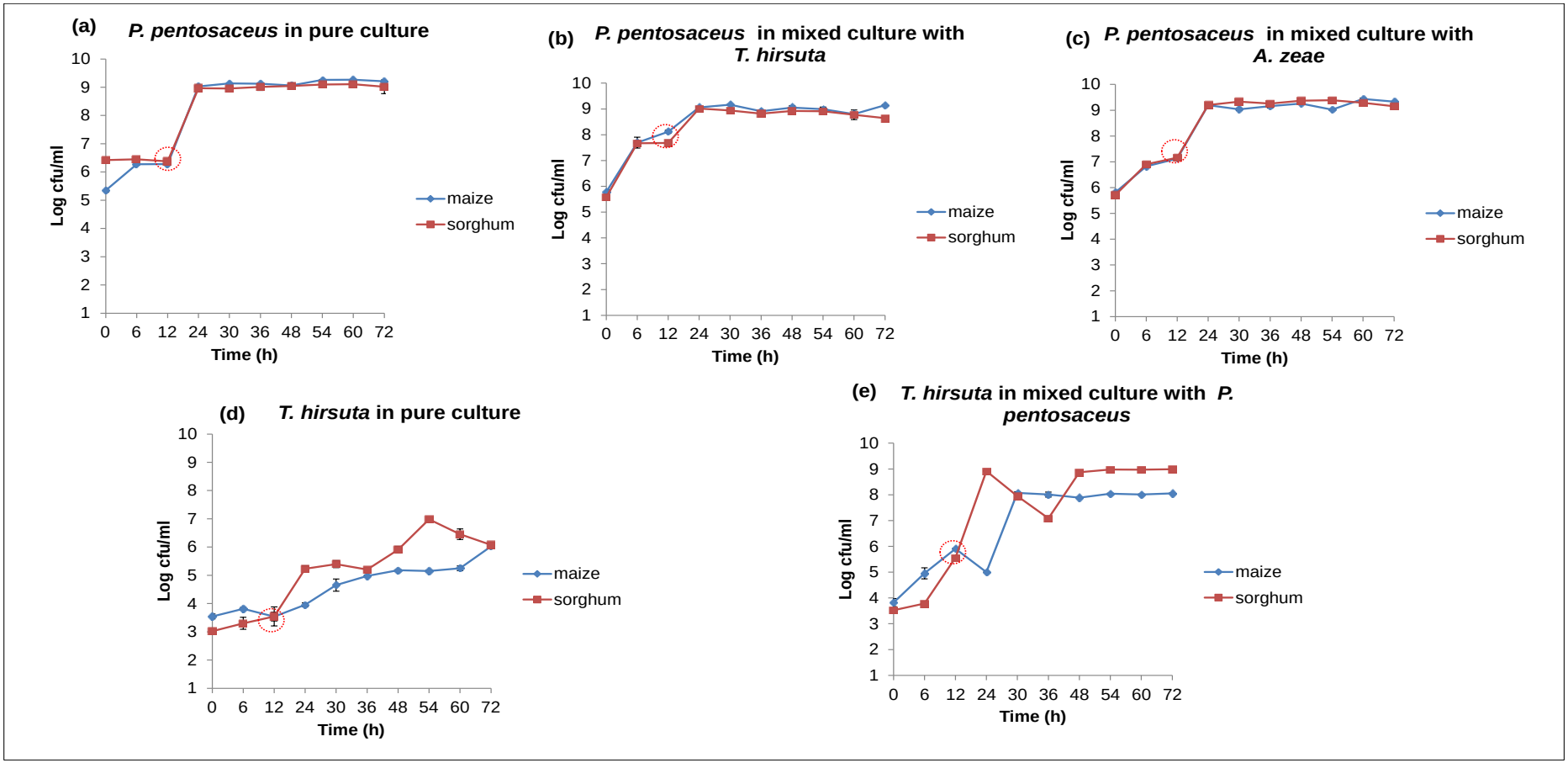



**Figure 6.3:** Viable population of pure culture and mixed cultures of *Pediococcus pentosaceus, Trametes hirsuta* and *Acremonium zeae* in steep water during fermentation of *ogi*. Data are means ± standard deviation of four replicates from two determinations (n=4).

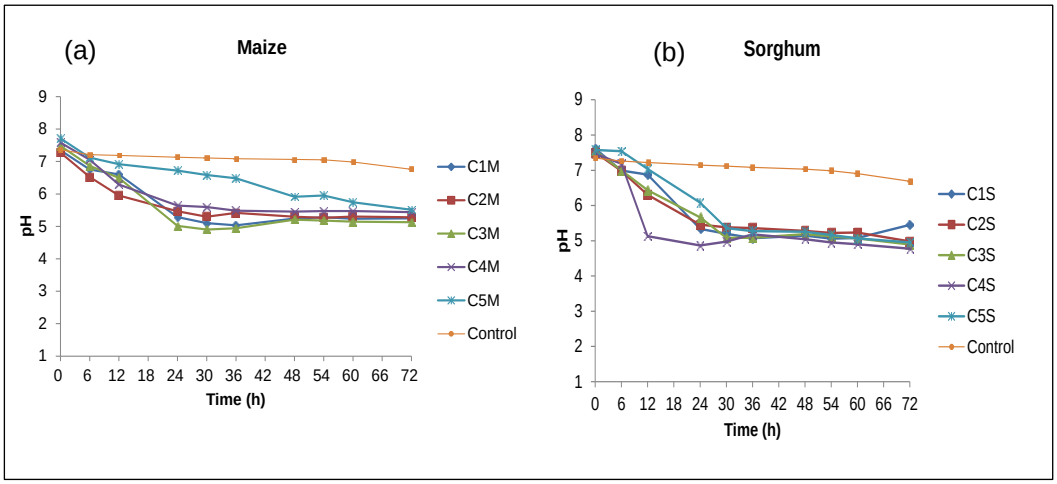



**Figure 6.4**: pH changes in steep water during *ogi* fermentations with pure and mixed starter cultures. M: maize, S: sorghum, C1: *Pediococcus pentosaceus,* C2*: Trametes hirsuta,* C3: *Pediococcus pentosaceus* + *Trametes hirsuta:* C4: *Pediococcus pentosaceus* + *Acremonium zeae,* and C5: *Acremonium zeae.* Data are means of four replicates from two determinations (n=4)

### 6.3.3 The influence of selected microorganisms on recovery of phenolics in *ogi.*

In this study the total phenolic content (TPC) is presented as mg GAE and represents the total actual recovery in each fraction from the fermentation of 200g of grain in all cases.

#### 6.3.3.1 Maize fermentation

The TPC of the steep water during maize fermentation is shown in Fig. 6.5. In all cases the TPC measured in the steep water was greater in inoculated samples than in uninoculated controls. *Trametes hirsuta* (C2) and *Acremonium zeae* (C5) significantly increased the TPC. Mixed cultures of *P. pentosaceus* with *T. hirsuta* (C3) and *P. pentosaceus* with *A. zeae* (C4) showed similar trends and the increases were relatively more consistent all through the fermentation period. In the first 24 h, TPC levels were similar in C1 and C3. A higher level was observed in C2 (*T. hirsuta*) and C5 (*A. zeae*). However, TPC level was reduced in the co-culture fermentations with these moulds (C3 and C4) although the level was still greater than in the pure culture

of the LAB (C1). Higher levels were observed in C2 and C5 than in C1 which suggests possible inhibition of the mould activity by *P. pentosaceus* as was also evident in the growth pattern as demonstrated in §6.3.1. Overall the TPC in steep water in maize reached a maximum level after 24-30 h.

Total phenolic content (TPC) in original grain (heat treated) and recovery in *ogi*, pomace, steep and wash waters during pure and mixed culture fermentations in maize are presented in Fig.6.6 while the percentage recoveries in each fraction are presented in Table 6.2. The total TPC recovery in the uninoculated control was only 77%. Again as the TPC is based on antioxidant capacity this may reflect the fact that a degree of oxidation has occurred during this fermentation. Total recovery was significantly greater ($p<0.05$) in all five cultures suggesting that the fermentation conditions may have served to stabilise the antioxidant activity (possibly by lowering the pH) or that some antioxidant compounds are being synthesised by the microorganisms. Comparing the five cultures in the *ogi* TPC levels in C1 were significantly higher than in C2 and declined even further in the co-culture with these two organisms (C3). In contrast levels in the pomace were significantly higher in C2 than in C1 and increased further in the C3 co-culture. In the other combination TPC in the *ogi* from the C1 and C5 fermentations were statistically identical and there was no change in the co-culture of these two organisms (C4). In the pomace the TPC was significantly higher in C5 compared to C1 but in the co-culture the TPC remained identical to that with the C5 monoculture.

The distribution of TPC between the fermentation fractions remained generally similar although there were some statistically significant differences. C1 appears to be identical to the uninoculated control except for the significantly greater increase of TPC in the steep water. Similarly leaching into the waste water was significantly higher in all fermentations than the control. C2 and C3 both showed a significant decline in the *ogi* fraction and a corresponding increase in the pomace fraction. C4 and C5 also demonstrated a significant increase in the pomace but in this instance there was no corresponding change in the *ogi* fraction.

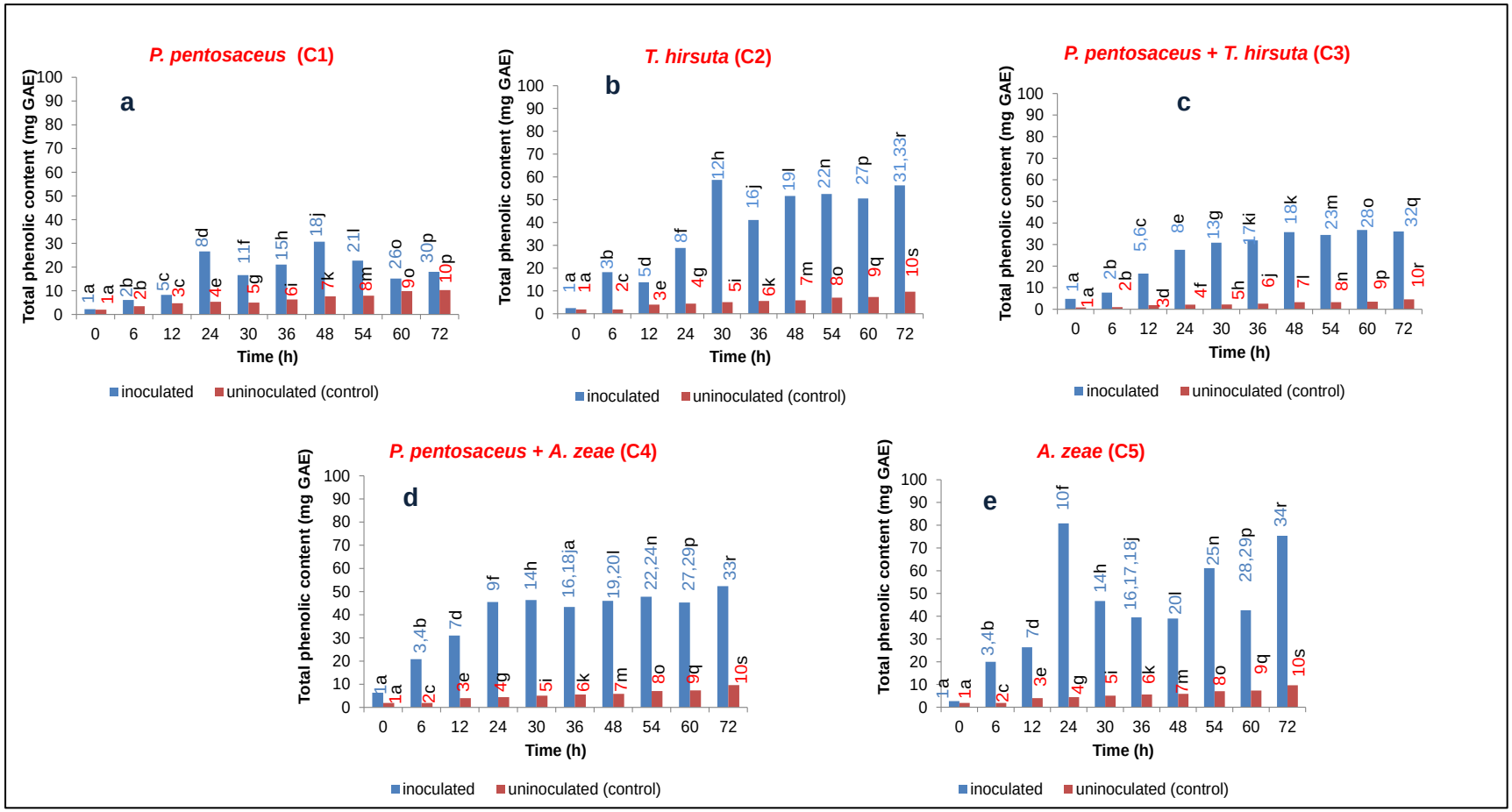



**Figure 6.5:** TPC levels in steep water during maize fermentation. Data are means ± SD of six replicates from three determinations (n=6). Data are the actual amounts recovered from 200g of maize. Columns with the same number of the same colour are not significantly different p>0.05. Letters compare the test (inoculated) and control at each time point separately for each fermentation.

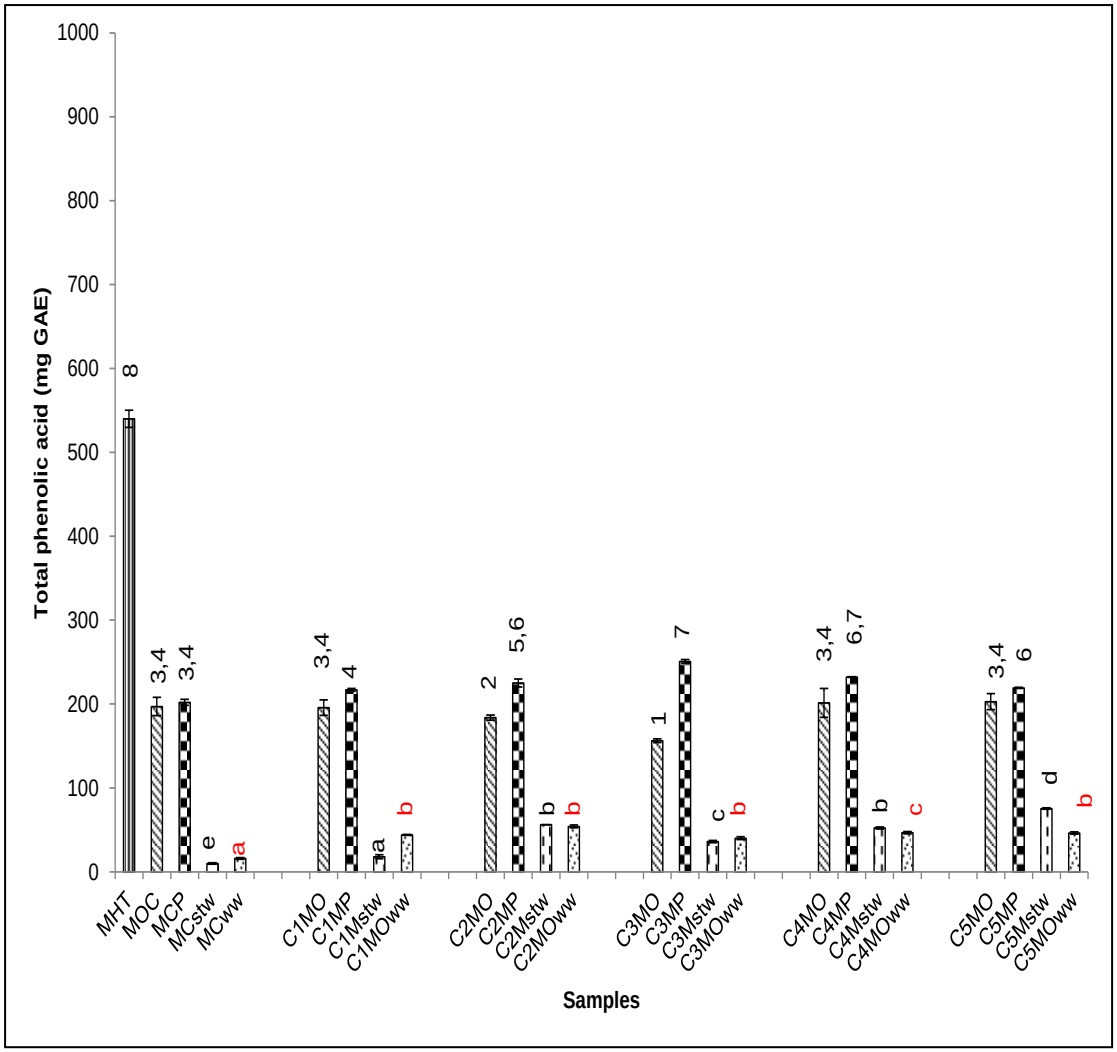



**Figure 6.6**: Total phenolic acid content of maize grain and fractions recovered during fermentation of *ogi* using variable starter cultures.

Autoclaved maize grain was fermented either uninoculated as a control (MHT), or following inoculation with *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures. Samples of *ogi* (MOC), steep water (MCstw), wash water (MCww) or pomace (MCP) were collected from the fermentation and analysed for total phenolic content, Data represent the actual total phenolic content in each fraction and are presented as the mean $\pm$ standard deviation of six replicates from three determinations (n=6).

The bound and soluble phenolics in *ogi* were further monitored to determine if there was any evidence of esterase activity in the selected microorganisms (*Pediococcus pentosaceus*, *Trametes hirsuta* and *Acremonium zeae*) (Fig. 6.7). The bound phenolic acid originally present in the heated maize grain accounted for 76% of the total phenolics in the grain. The recovery of bound phenolics in *ogi* and pomace was

assessed as the percentage of this initial amount of the bound fraction in maize grain (Table 6.2). The total recovered bound TPC values for all five cultures were lower than the control and apart from C4 the total soluble recovery was always greater than the control. This could indicate either the presence of esterase activity releasing bound compounds (probably phenolics) or the loss of other bound antioxidants and the synthesis of soluble antioxidants by the microorganisms.

**Table 6.2:** Percentage recoveries of phenolic acid fractions in maize *ogi*

| Cultures | ***Total phenolic content (TPC)** | | | |
|---|---|---|---|---|
| | *ogi* | pomace | Waste water | Total |
| C1 | 36 | 40 | 11 | 87 |
| C2 | 34 | 42 | 20 | 96 |
| C3 | 29 | 46 | 14 | 89 |
| C4 | 37 | 43 | 18 | 99 |
| C5 | 38 | 41 | 22 | 101 |
| control | 35 | 37 | 5 | 77 |
| | **†Bound phenolics** | | | |
| Cultures | *ogi* | Pomace | Waste water | Total |
| C1 | 26 | 42 | N/A | 68 |
| C2 | 31 | 41 | N/A | 71 |
| C3 | 27 | 34 | N/A | 60 |
| C4 | 29 | 40 | N/A | 68 |
| C5 | 29 | 40 | N/A | 69 |
| control | 32 | 42 | N/A | 74 |
| | **†Soluble phenolics** | | | |
| Culture**s** | *ogi* | Pomace | Waste water | Total |
| C1 | 69 | 33 | 14 | 116 |
| C2 | 76 | 30 | 44 | 150 |
| C3 | 49 | 33 | 29 | 111 |
| C4 | 30 | 15 | 41 | 86 |
| C5 | 46 | 19 | 60 | 125 |
| control | 50 | 23 | 21 | 94 |

*Total phenolics recoveries are based on the total phenolic content in 200g of the grain. †Bound and soluble values are percentages of the original amount of each fraction in the grain. Data are presented as percentages of the mean of six replicates from three determinations (n=6). Starter cultures: *Pediococcus pentosaceus* (C1),

*Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures.

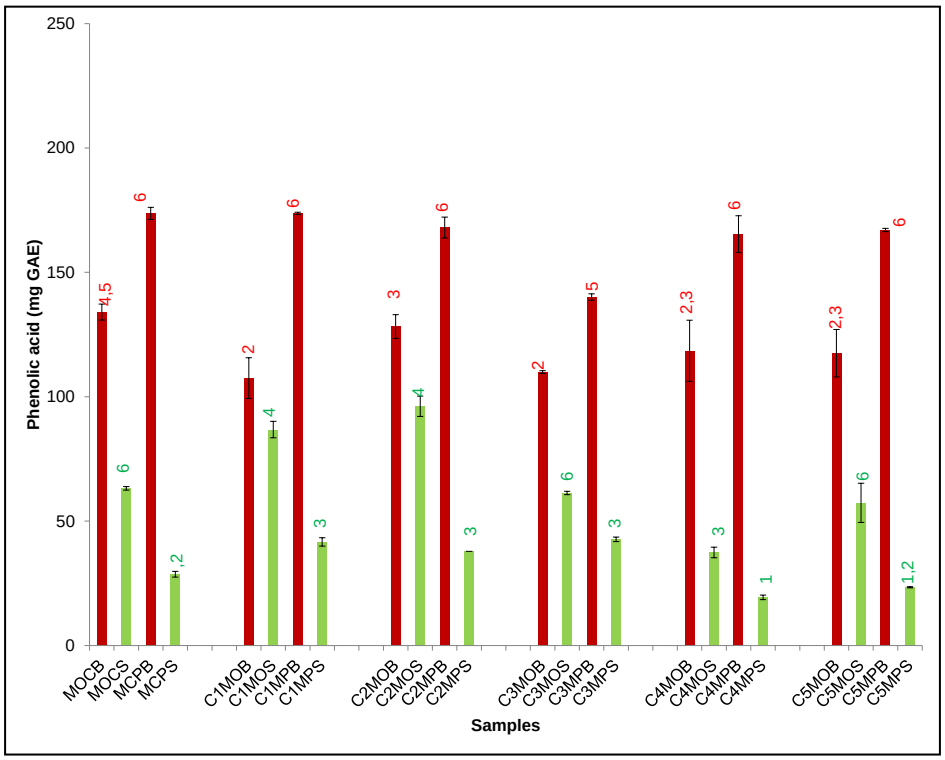



**Figure 6.7:** Bound and soluble TPC recovered during fermentation of maize *ogi* using variable starter cultures.

Autoclaved maize grain was fermented either uninoculated as a control, or following inoculation with *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures. Samples of *ogi* (MOC, MO) or pomace (MCP, MP) were collected from the fermentation and analysed for bound phenolic content, Data represent the actual bound phenolic content in the fraction and are presented as the mean ± standard deviation from six replicates from three determinations (n=6). Bound phenolics: B (red columns), Soluble phenolics: S (green columns).

Looking at the individual fractions it was evident that TPC values for the bound fraction were lower than the control in all five fermentations. Comparisons between the fermentations demonstrated that all were statistically similar apart from C2 which, although still lower than the control, was significantly higher than the other fermentations. For the pomace the levels of bound TPC were all statistically similar to the control. Looking at the soluble TPC values in these two fractions it was seen that the impact of the five cultures was quite variable. In the *ogi* fraction soluble TPC levels

were increased, compared to control, in C1 and C2, decreased in C4 and remained the same in C3 and C5. Variation was less in the pomace when compared to the control. Soluble TPC levels were increased in C1, C2 and C3 but remained constant in C4 and C5.

Comparing the five cultures it can be seen that for the bound TPC in the *ogi* C2 gave a significantly higher value than C1 and that in the co-culture of these two organisms (C3) the TPC was statistically similar to that from the C1 monoculture. In the other combination the two mono cultures C1 gave a similar TPC with C5 and in the co-culture (C4) of these organisms the TPC level although not statistically different from either of the two monocultures was higher than C1.

In the pomace the two monocultures C1 and C2 gave similar bound TPC values but this was significantly lower in the co-culture (C3) of these organisms. The third monoculture (C5) was also similar to C1 but in this instance the co-culture did not result in any significant difference in the pomace bound TPC. In terms of soluble TPC again the two mono cultures C1 and C2 were identical and the co-culture showed a significant decrease in soluble TPC compared to the two monocultures. The C1 monoculture gave a significantly lower soluble TPC compared to C5 and this was further reduced in the co-culture (C4) of these two organisms.

#### 6.3.3.2 Sorghum fermentation

TPC level in steep water during fermentation of sorghum *ogi* with selected microorganisms is presented in Fig. 6.8. *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2) and *Acremonium zeae* (C5) in pure and mixed fermentations significantly increased TPC in the steep water. High levels were seen which was mostly similar ($p>0.05$) in C1, C2 and C3 (*P. pentosaceus* with *T. hirsuta*) and differed significantly from C4 (*P. pentosaceus* with *A. zeae*) and C5. The maximum TPC was attained after 24 h in C1 and C3 and at 30 h in C2, C4, and C5 with values of 77, 77, 74, 97 and 97 mg GAE in C1, C2, C3, C4 and C5 ($p>0.05$). Although TPC in C4 and C5 did not show

a consistent increase or decrease, high levels obtained in the mixed fermentation (C4) suggest that *A. zeae* may have played a major role in the increase since this mould alone (C5) also showed higher levels compared to *P. pentosaceus* (C1).

The recovery of TPC during fermentation of sorghum with the pure and mixed starter cultures (C1-C5) is presented in Fig. 6.9 while the percentage recovery of phenolic acid fractions is presented in Table 6.3. The total recovery in the control was 95% suggesting that in this instance the antioxidant capacity and presumably phenolics were relatively stable during the fermentation. Recovery in all five fermentations was higher than the control and exceeded 100%. This again might suggest that there has been some synthesis of antioxidant capacity by the microorganisms. Looking at the distribution between the fermentation fractions it can be seen that TPC in the *ogi* has significantly increased, compared to the control, in C1, C3 and C4 but has been unchanged in the other two fermentations. In the pomace there has been a significant decline in all five cultures, compared to the control, but there was no significant difference between them. As with the maize there was a very significant increase in the TPC value for the waste water fraction.

Comparing the five culture regimes it was found that the TPC in the pomace was consistent across all five cultures with no statistically significant differences identified. In contrast there were statistically significant differences observed in the *ogi* fraction. In this case C1 gave higher TPC than C2 and in the co-culture of these two organisms (C3) the levels were equivalent to that in C1. A similar pattern was found in the other combination where TPC levels in the *ogi* were significantly higher in C1 than in C5 and were similar to the levels with C1 alone in the co-culture (C4) of these two organisms.

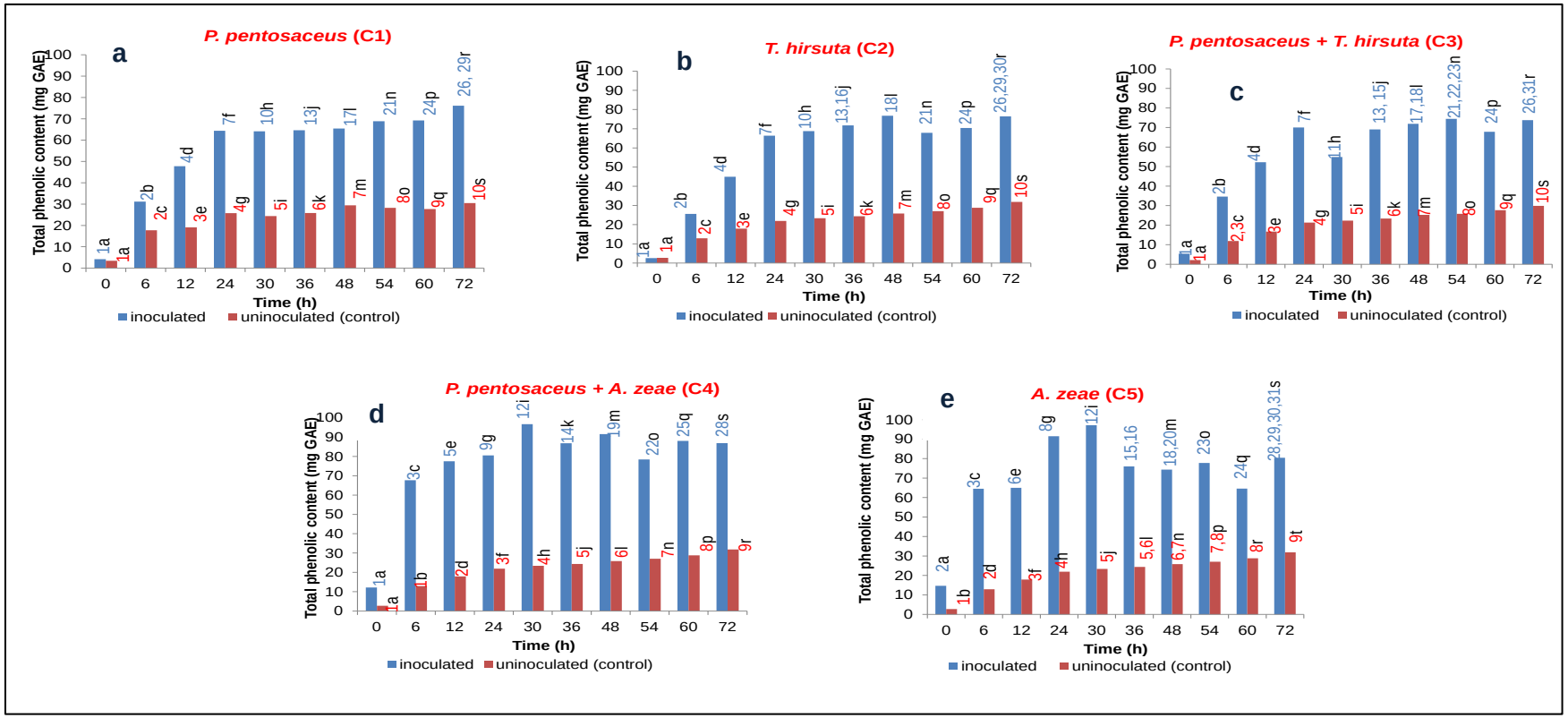



**Figure 6.8:** TPC values in steep water during **sorghum** fermentation. Data are means ± SD of six replicates from three determinations (n=6). Data are the actual amounts recovered in 200g of sorghum. Columns with the same number of the same colour are not significantly different p>0.05. Letters compare the test (inoculated) and control at each time point separately for each fermentation.

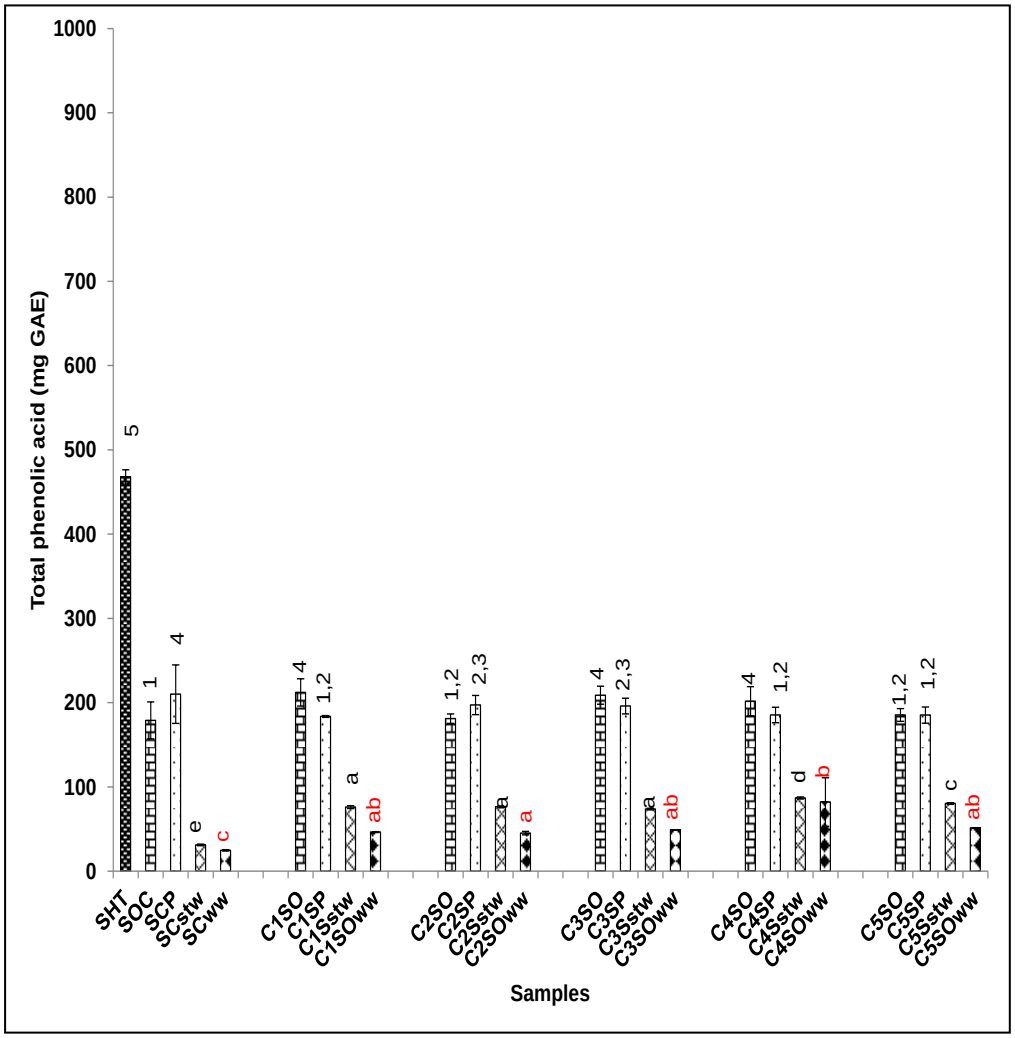



**Figure 6.9**: Total phenolic acid content of sorghum grain and fractions recovered during fermentation of *ogi* using variable starter cultures.

Autoclaved sorghum grain was fermented either uninoculated as a control (SHT), or following inoculation with *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures. Samples of *ogi* (SOC), steep water SCstw), wash water (SCww) or pomace (SCP) were collected from the fermentation and analysed for total phenolic content, Data represent the actual total phenolic content in the fraction and are presented as the mean $\pm$ standard deviation of six replicates from three determinations (n=6).

**Table 6 3:** Percentage recoveries of phenolic acid fractions in sorghum *ogi*

| **Cultures** | **Total phenolics content (TPC)** | | | |
|---|---|---|---|---|
| | *ogi* | pomace | Waste waters | Total |
| C1 | 45 | 39 | 26 | 110 |
| C2 | 39 | 42 | 26 | 107 |
| C3 | 45 | 42 | 26 | 113 |
| C4 | 43 | 40 | 36 | 119 |
| C5 | 40 | 40 | 28 | 107 |
| control | 38 | 45 | 12 | 95 |
| | **Bound phenolics** | | | |
| **Cultures** | *ogi* | pomace | Waste waters | Total |
| C1 | 55 | 43 | N/A | 98 |
| C2 | 38 | 54 | N/A | 92 |
| C3 | 40 | 58 | N/A | 98 |
| C4 | 42 | 39 | N/A | 81 |
| C5 | 66 | 71 | N/A | 137 |
| control | 43 | 61 | N/A | 104 |
| | **Soluble phenolics** | | | |
| **Cultures** | *ogi* | pomace | Waste waters | Total |
| C1 | 45 | 31 | 53 | 129 |
| C2 | 43 | 16 | 53 | 112 |
| C3 | 38 | 19 | 53 | 110 |
| C4 | 31 | 16 | 73 | 120 |
| C5 | 16 | 25 | 57 | 98 |
| control | 26 | 12 | 24 | 62 |

*Total phenolics recoveries are based on the total phenolic content in 200g of the grain. †Bound and soluble values are percentages of the original amount of each fraction in the grain. Data are presented as percentages of the mean of six replicates from three determinations (n=6). Starter cultures: *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures.

The bound and soluble TPC in sorghum *ogi* and pomace are shown in Fig. 6.10 and the percentage recovery in Table 6.3. Recovery of bound TPC in the control was 104% and this was lower in all five fermentations but only in C4 was this much below 100%. In contrast recovery of soluble TPC in the control was only 62%. However there seemed to be an overestimation in the bound and soluble TPC in the control resulting in the observed higher values than those of direct TPC extraction. Recovery was higher in all five fermentations and apart from C4 was in excess of 100%.

Looking at the distribution between *ogi* and pomace it can be seen that in the *ogi* bound TPC was significantly higher than the control in C5 but was similar in all the other four fermentations. In contrast levels of bound TPC in the pomace were lower than the control in C1 and C4 but similar in the other three fermentations. In terms of soluble TPC in the *ogi* C1, C2 and C3 all showed significantly higher levels, whilst C5 was lower and C4 unchanged. In the pomace soluble TPC was again increased in C1, C2 and C3 and remained constant in the other two fermentations.

Comparing the five cultures for the bound TPC in the *ogi* it was seen that C1 gave significantly higher TPC than C2 and that the co-culture (C3) gave an intermediate value that was statistically similar to both C1 and C2. In contrast the bound TPC in the *ogi* using C5 was significantly higher than with C1 but that in a co-culture of these two organisms (C4) the TPC was similar to the lower value obtained with C1 alone. The bound TPC in the pomace was lower in C1 than in C2 and again in the co-culture (C3) the level was similar to that with C1. As in the *ogi* the bound TPC in C1 was significantly lower than with C5 and again the co-culture (C4) gave TPC values similar to the C1 monoculture. For soluble TPC in the *ogi* the two monocultures C1 and C2 gave similar levels whilst the co-culture (C3) gave a slightly lower, but statistically significant, value. Levels of soluble TPC in the *ogi* were higher in C1 than in C5 and in this instance a co-culture (C4) gave an intermediate value. In the pomace C1 gave higher TPC values than either of the other two mono cultures (C2 and C5) whilst a co-culture of C1 and C2 gave values similar to C2 and that between C1 and C5 gave a TPC even lower than the two constituent mono cultures.

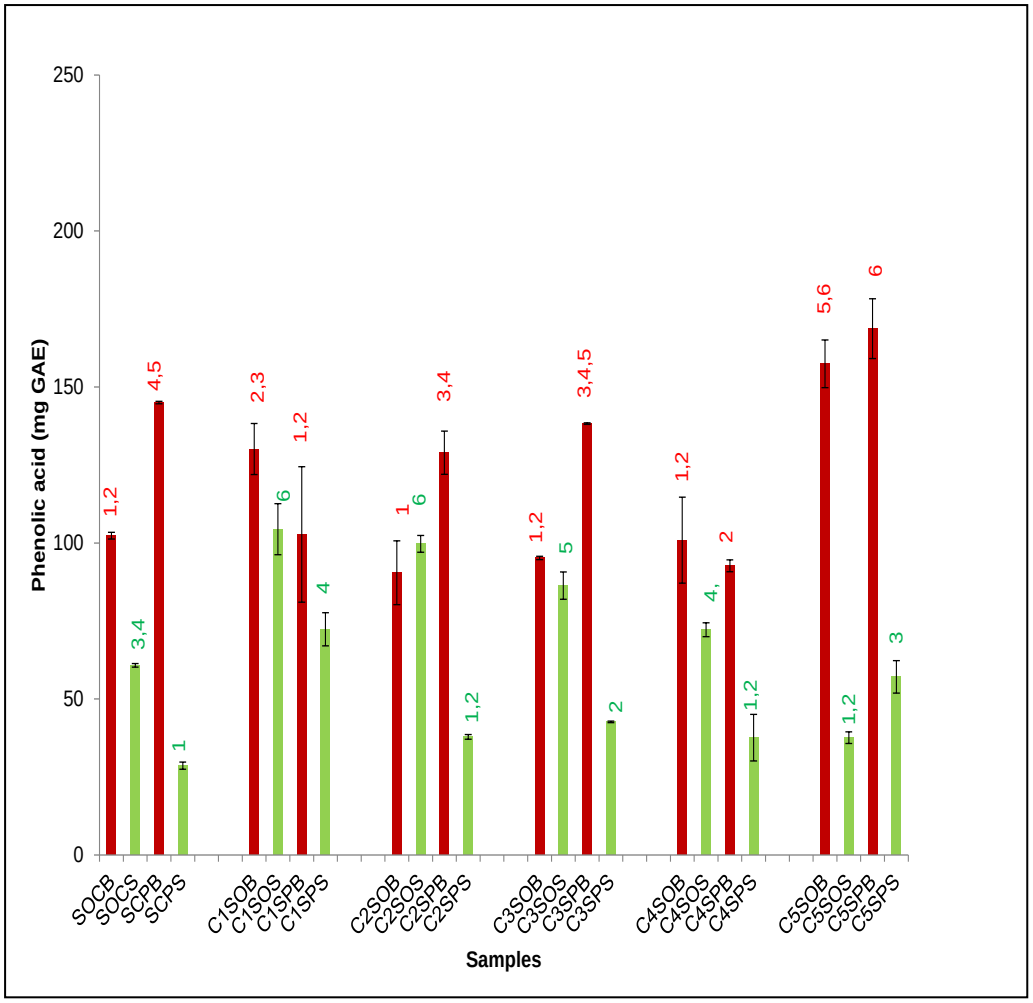



**Figure 6.10:** Bound and soluble phenolic acid content recovered during fermentation of sorghum *ogi* using variable starter cultures

Autoclaved sorghum grain was fermented either uninoculated as a control, or following inoculation with *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures. Samples of *ogi* (SOC, SO) or pomace (SCP, SP) were collected from the fermentation and analysed for bound phenolic content, Data represent the actual bound phenolic content in the fraction and are presented as the mean ± standard deviation of six replicates from three determinations (n=6). Bound phenolics: B (red columns), Soluble phenolics: S (green columns).

### 6.3.4 The influence of selected microorganisms on phytate content of *ogi*

Phytate levels in the fermentations were calculated and are presented in the figures 6.11 and 6.12 as the actual total amount of phytate (g) per total weight of sample recovered from the fermentation of 200g of grain in all cases.

#### 6.3.4.1 Phytate in maize fermentation

The phytic acid content in the original grain (heat treated), and recovery in *ogi*, pomace (residue) and steep water during pure and mixed culture fermentations of maize with *P.pentosaceus*, *T. hirsuta* and *A. zeae* is shown in Fig.6.11.

Total recovery of phytate in *ogi*, pomace and steep water (combined) was 116% of the original grain content in the uninoculated control. This suggests that the phytate is stable during the fermentation in the absence of any fermentative organisms. Total phytate recovery in the five fermentations was near to that for the control at C1:89%, C3:96%, C4:84% and C5:95% apart from C2 which at 28% was significantly lower than the amount recovered in the control. The low level of phytate recovered in C2 may have been due to the degradation of phytate and uptake of inorganic phosphorus in *T. hirsuta* fermentation.

In the *ogi* the levels of phytate obtained in the culture fermentations were all significantly identical to the control apart from C2 which was significantly lower. Levels in the pomace varied between cultures but in all cases were statistically similar to the control. Again the lowest value was observed in the C2 monoculture. The steep water showed different levels ($p<0.05$) of phytate which was again found to be lowest in C2 suggesting greater phytase activity in the pure culture fermentation with *T. hirsuta*. Therefore pure cultures of *T. hirsuta* followed by and co-culture of *P. pentosaceus* with *T. hirsuta* showed the highest phytate hydrolysis at 48% and 43% respectively.

In the mixed fermentations of *P. pentosaceus* with *T. hirsuta* (C3) and *A. zeae* (C4) respectively, the level of phytate in the *ogi* were similar ($p>0.05$). However C4 showed values lower than in the respective pure cultures (C1 and C5) suggesting synergistic interaction in the activities of the LAB (*P. pentosaceus*) and fungi (*A. zeae*) in the co-culture fermentations. The lower level of phytate in C2 than in C3 *ogi* suggest that *T. hirsuta* may have contributed significantly to phytate hydrolysis in the co-culture fermentation of *P. pentosaceus* with *T. hirsuta*. In the pomace, phytate recovered in the uninoculated control varied significantly from C2 which showed a lower amount than the control. The recovery of phytate in steep water showed a similar trend in the *ogi* and pomace. Leaching was more apparent in the control than in the culture

fermentations. The low recovery of phytate in the steep waters may be attributed to degradation and utilization of phytate phosphorus by microbes in the inoculated samples.

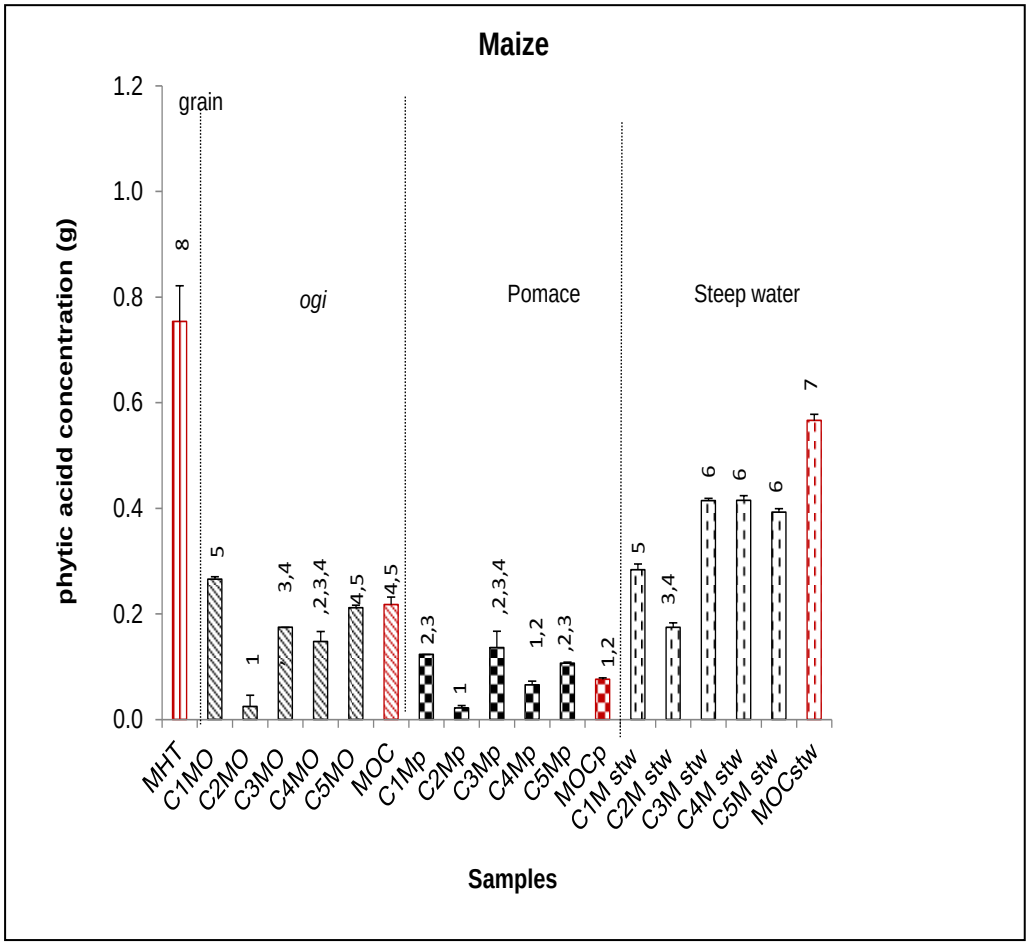



**Figure 6.11:** Total phytic acid during starter culture fermentation of maize *ogi*.

Autoclaved maize grain was fermented either uninoculated as a control - red columns (MHT), or following inoculation with *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures. Samples of *ogi* (MOC), steep water (MCstw), wash water (MCww) or pomace (MCP) were collected from the fermentation and analysed for phytate content, Data represent the actual phytate content in the fraction and are presented as the mean $\pm$ standard deviation of four replicates from duplicate determinations (n=4).

### 6.3.4.2 Phytate in sorghum fermentation

The level of phytate originally in the heat treated grain and recovery in sorghum *ogi*, pomace and steep water during pure and mixed starter culture fermentation with *P.*

*pentosaceus*, *T. hirsuta* and *A. zeae* is shown in Fig. 6.12. The total recovery of phytate was 95% of the original grain (in *ogi*, pomace and steep water) in the uninoculated control. Again this demonstrates good stability during the fermentation in the absence of any fermentative organisms. Significant phytate reductions were observed in all culture fermentations. A recovery of phytate (in *ogi*, pomace and steep water) observed in C2 (18%) and C4 (16%) were much lower than in the control and may suggest possible degradation and usage by the microbes during the fermentations.

In the pure culture fermentations (C1, C2 and C5) phytate levels in *ogi* were similar in C2 and C5 ($p>0.05$) and significantly different in C1 ($p<0.05$). Highest recovery was in C1 and the lowest in C5 suggesting higher phytase activity. Although the levels of phytate in the pure culture fermentations were not statistically different in the pomace, the amount recovered in C2 was lower than in C1, and C5. Leaching of phytate into steep water was also observed in sorghum fermentation with the least recovery observed in the pure culture fermentation of *T. hirsuta*.

In the mixed fermentations of *P. pentosaceus* with *T. hirsuta* or *A. zeae* (C3 and C4), phytate in *ogi* were similar ($p>0.05$) with C4 having values lower than in C3. Phytate in C3 *ogi* was found to be at similar level with C2 *ogi* (*T. hirsuta*) varied significantly ($p<0.05$) and lower than in C1 *ogi* (*P. pentosaceus*). Phytate recovered in C3 pomace and steep water was significantly higher than in those of C4 and may indicate higher phytase activity in the latter fermentation and suggests synergistic effect in the fermentation of *P. pentosaceus* with *A. zeae.*

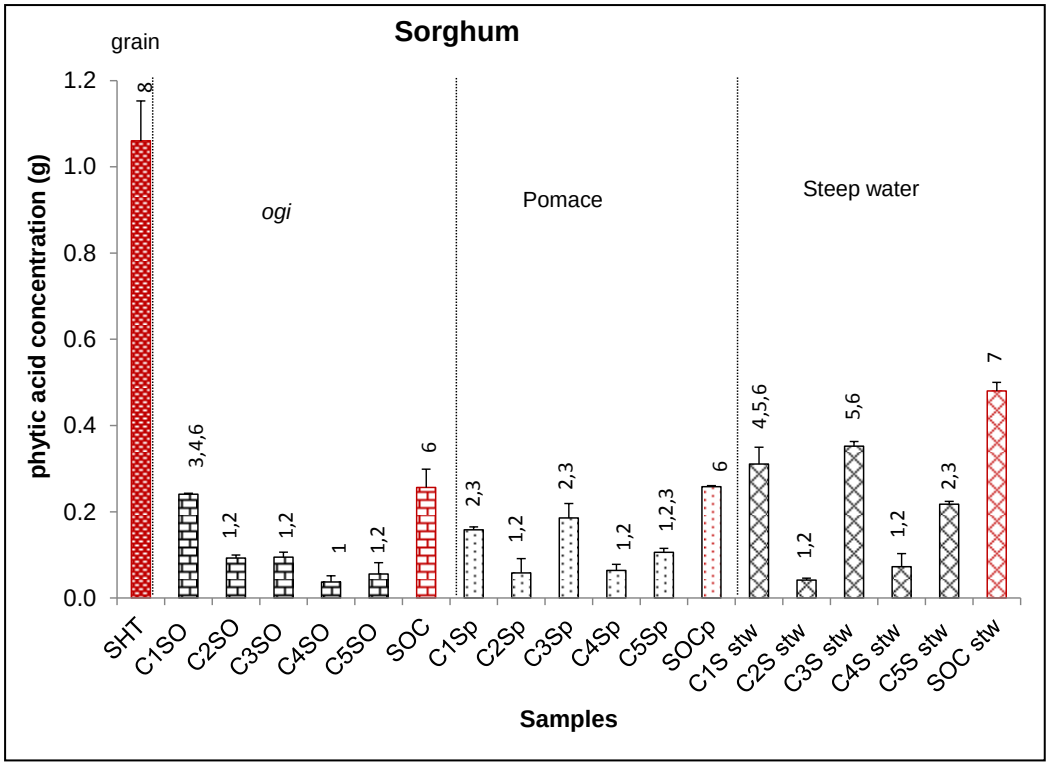



**Figure 6.12:** Total phytic acid during starter culture fermentation of sorghum *ogi*.

Autoclaved sorghum grain was fermented either uninoculated as a control - red columns (SHT), or following inoculation with *Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P.pentosaceus* and *T. hirsuta* (C3), *P.pentosaceus* and *Acremonium zeae* (C4) or *A. zeae* (C5) as starter cultures. Samples of *ogi* (SOC), steep water (SCstw), wash water (SCww) or pomace (SCP) were collected from the fermentation and analysed for phytate content, Data represent the actual phytate content in the fraction and are presented as the mean ± standard deviation of four replicates from duplicate determinations (n=4).

### 6.3.5. Analysis of volatile organic (VOCs) compounds by gas chromatography mass spectrophotometry (GC-MS)

The relative abundance of the VOCs was measured in this study by SPME GC-MS headspace analysis. The area of the peak was the measure by which relative abundance of these volatile components in the samples was measured. Although this does not give the actual concentrations it forms a good tool for effective comparison of the relative levels of these compounds in *ogi*.

#### 6.3.5.1. Volatile compounds in starter culture fermentation of maize *ogi*

Volatile compounds identified by SPME GC-MS headspace analysis of maize *ogi* fermented with pure and mixed cultures of *Pediococcus pentosaceus, Trametes hirsuta* and *Acremonium zeae* are listed in Table 6.4. A total of 29 major volatile compounds were identified in the different *ogi* and classified as alcohols (7), ketones (3), aldehyde (1), esters (10), carboxylic acids (2), sulphur containing - (3), benzene based (2) compounds and phenol (1). The total of the major volatile compounds identified in each sample were: C1MO (26), C2MO (25), C3MO (24), C4MO (24) and C5MO (23) and MHT (12).

Alcohols were found in *ogi* with apparently high signal intensities in the co-culture fermentations of *A. zeae* with *P. pentosaceus* (C4) and in the pure culture fermentation of *A. zeae* (C5). Ethanol showed the highest signal intensity in the fermentations with *A. zeae* alone (C5). However the majority of the alcohols were found at low levels in the pure culture and co-culture fermentations of *T. hirsuta* (C2 and C3). Alcohols dectected in the control (MHT) were relatively less abundant.

Of the three identified ketones, 2-propanone was found in the control and all culture fermentations except *P. pentosaceus* with *T. hirsuta* (C3) showing greater relative abundance in the fermentations with *A. zeae* (C4 and C5). The ketone, diacetyl was only present in C1, C2 and C3 with the highest level in C1.

Esters were the major volatile components identified in all the culture fermentations in maize but these were found at relatively low levels across all samples except in the pure and co-culture fermentations of *A. zeae* with *P. pentosaceus* (C4 and C5) which were relatively more abundant. However ethyl acetate and butyl acetate were apparently high in all the samples. Two carboxylic acids (butanoic and acetic acids) were present and mostly abundant in pure culture of *P. pentosaceus* (C1) and *T. hirsuta* (C2) and co-culture fermentation of this LAB with the fungi (C3).

**Table 6.4**: Mean signal intensities (‡Peak areas (AU) X $10^4$) and standard deviations (SD)[4] of volatile compounds detected by SPME GC-MS headspace analysis of **maize** *ogi* fermented with pure and mixed cultures of †LAB and fungi.

| Volatile compounds | Ion (m/z) | Starter culture fermented *ogi* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C1MO | | C2MO | | C3MO | | C4MO | | C5MO | | HMT | |
| | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **Alcohols** | | | | | | | | | | | | | |
| Ethanol | 31 | 55 | 2 | 43 | 5 | 33 | 0.10 | 668 | 27 | 17943 | 1257 | nd | nd |
| 2-propanol | 45 | 228 | 22 | 182 | 40 | 1 | 1 | 106 | 3 | 98 | 59 | 20 | 4 |
| Butanol | 56 | 19 | 5 | 45 | 3 | 4 | 3 | 0.72 | 0.20 | nd | nd | nd | nd |
| Isoamyl_alcohol | 55 | 2 | 0.50 | 3 | 1 | 0.27 | 0.09 | 66 | 5 | 4 | 0.77 | nd | nd |
| 1-Pentanol | 55 | 0.14 | 0.00 | 0.08 | 0.02 | 0.27 | 0.11 | 38 | 5 | 66 | 6 | 35 | 20 |
| Hexanol | 56 | 0.15 | 0.04 | 0.33 | 0.22 | 0.78 | 0.64 | 156 | 108 | 659 | 219 | 0.30 | 0.12 |
| Ethylhexanol | 57 | nd | nd | nd | nd | nd | nd | 145 | 72 | 59 | 2 | nd | nd |
| **Ketones and aldehyde** | | | | | | | | | | | | | |
| 2-propanone | 43 | 0.02 | 0.00 | 0.01 | 0.00 | nd | nd | 885 | 67 | 16860 | 5075 | 436 | 37 |
| 2-Heptanone | 58 | nd | nd | nd | nd | nd | nd | 711 | 63 | nd | nd | nd | nd |
| Diacetyl | 43 | 852 | 22 | 30 | 2 | 21 | 1 | nd | nd | nd | nd | nd | nd |
| 2-Methyl butanal | 57 | 0.12 | 0.11 | 0.13 | 0.05 | 0.06 | 0.03 | 2 | 0.45 | 21 | 2 | 191 | 84 |
| **Esters** | | | | | | | | | | | | | |
| Butyric acid propyl-ester | 101 | 1 | 0.75 | 2 | 0.53 | 1 | 0.87 | 0.24 | 0.10 | 0.28 | 0.04 | nd | nd |
| Pentanoic acid ethyl- ester | 85 | 2 | 0.16 | 2 | 0.26 | 0.05 | 0.01 | 4 | 0.49 | 5 | 0.12 | 0.13 | 0.07 |
| Ethyl acetate | 43 | nd | nd | nd | nd | nd | nd | 77 | 0.26 | 5923 | 565 | nd | nd |
| Butyl acetate | 73 | 11 | 0.30 | 7 | 0.29 | 7 | 0.42 | 929 | 52 | 883 | 26 | nd | nd |
| Butyl butanoate | 71 | 16 | 3 | 21 | 8 | 0.10 | 0.01 | 61 | 10 | 40 | 0.13 | nd | nd |
| Ethyl hexanoate | 88 | 0.09 | 0.02 | 0.17 | 0.05 | 0.10 | 0.06 | 59 | 2 | 5.20 | 0.60 | 0.10 | 0.06 |
| Butyl hexanoate | 99 | 0.11 | 0.01 | 0.09 | 0.03 | 0.19 | 0.03 | 1.62 | 0.18 | 2 | 0.70 | 0.18 | 0.01 |
| Cyclobutanol-3-ethyl- acetate | 86 | 203 | 8 | 0.06 | 0.04 | 0.06 | 0.01 | 1166 | 136 | 422 | 100 | nd | nd |
| Butyl isobutyrate | 71 | 28 | 6 | nd | nd | nd | nd | 123 | 0.40 | 3 | 0.36 | 0.70 | 0.56 |
| Isopropylacetic acid | 60 | 0.83 | 0.43 | 0.37 | 0.01 | 0.52 | 0.10 | 2 | 0.47 | 3 | 1 | 0.81 | 0.34 |
| **Carboxylic acids** | | | | | | | | | | | | | |
| Ethanoic acid (acetic acid) | 45 | 128 | 50 | 39 | 18 | 9 | 9 | nd | nd | 38 | 20 | nd | nd |
| Butanoic acid | 60 | 437 | 210 | 174 | 7 | 33 | 10 | nd | nd | nd | nd | nd | nd |
| **Other compounds** | | | | | | | | | | | | | |
| Dimethyl disulphide | 94 | 0.43 | 0.40 | 38 | 0.53 | 0.12 | 0.02 | 1 | 1 | 49 | 0.89 | 0.11 | 0.07 |
| Dimethyl-trisulphide | 126 | 0.11 | 0.00 | 0.10 | 0.03 | 0.07 | 0.07 | 0.64 | 0.11 | 0.34 | 0.25 | 0.27 | 0.09 |
| 1-propene-1-thiol | 73 | 11 | 0.35 | 7 | 0.06 | 7 | 0.57 | nd | nd | nd | nd | nd | nd |
| Phenyl ethylene | 104 | 12 | 0.07 | 0.66 | 0.43 | 1 | 0.17 | 254 | 24 | 145 | 56 | 93 | 42 |
| 1,3,-Ditert-butylbezene | 175 | 0.06 | 0.04 | 0.17 | 0.10 | 0.07 | 0.02 | 0.16 | 0.04 | 0.19 | 0.02 | nd | nd |
| 4-ethyl-2 - methoxyphenol | 33 | 101 | 0.95 | 37 | 1 | 10 | 0.42 | nd | nd | nd | nd | 264 | 11 |

‡values represent the relative abundance. Data are means of four replicates from duplicate determinations (n=4) and standard deviation (SD).MO: maize *ogi*, MHT: uninoculated control. †*Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P. pentosaceus* and *T. hirsuta* (C3), *P. pentosaceus* and *Acremonium zeae* (C4), *A. zeae* (C5), nd: not detected.

The benzene, phenol and sulphur-containing compounds were present at varying levels but all are low. However, phenyl ethylene was discernibly at high levels in the pure culture and co-culture fermentations of *A. zeae*. The phenol 4 ethyl-2-methoxyphenol was unique and relatively abundant in maize and seemed to be characteristic of maize as seen from the apparent high values in the control (MHT).

#### 6.3.5.1.1 Principal component analysis (PCA)

Headspace SPME analysis of maize *ogi* displayed a wide range of varying levels of volatile components as seen in the previous section. The principal component analysis was performed on the data obtained from the GC-MS analysis of the starter culture fermented *ogi* and the naturally fermented *ogi* to be able to assess the differences in their aroma characteristics in relation to the volatile profiles. The PCA biplot of maize *ogi* fermented with pure and mixed cultures of *P. pentosaceus*, *T. hirsuta* and *A. zeae* is shown in Fig. 6.13. All *ogi* samples were clearly separated in the biplot by the two principal components PC1 and PC2 that account for 45 and 18% of the total variance, respectively.

Three clusters were evident and showed variations in profile of the volatile components in the *ogi*. The majority of the compounds were clustered into one group, cluster 3, comprising mostly of the alkyl esters and ketones. The control MHT showed closeness to cluster 3 and was mostly distinguished by the high intensity of the phenolic compound-4-ethyl-2-methyoxyphenol. Cluster 1 was distinguished by the alcohol (2-propanol), the sulphur containing compound (dimethyl disulphide), the aldehyde (diacetyl) and ethanoic acid (acetic acid). Cluster 2 comprised mostly of 2 alcohols, 1 ketone (2-heptanone) and few esters. However, none of the *ogi* showed very strong association with the clustered groups.

The volatile profile of *ogi* from *P. pentosaceus* in co-culture fermentation with *T. hirsuta* (C3MO) was mostly related to the naturally fermented *ogi* (MO2) on the PC2. *Ogi* from pure cultures of *P. pentosaceus* and *T. hirsuta* were relatively similar and mostly associated with synthesis of alcohol, ketone and carboxylic acid. *Ogi* from pure

(C5MO) and mixed cultures of *A. zeae* with *P. pentosaceus* (C4MO) were clearly distinguished on the PCA plot as outliers and were respectively associated to cluster 2 with the $C_6$–$C_8$ alcohol and cluster 3 having mostly the alkyl esters.

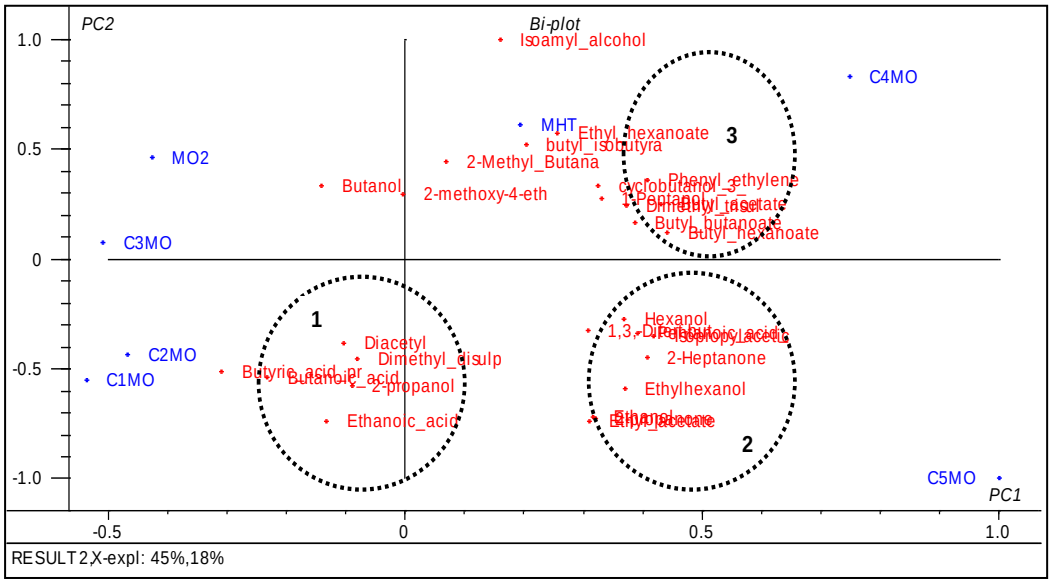



**Figure 6.13**: Principal component analysis biplot of maize *ogi* fermented with pure and mixed cultures of *P. pentosaceus*, *T. hirsuta* and *A. zeae.* MO2: naturally fermented *ogi*, starter culture fermented *ogi* (C1MO: *P. pentosaceus,* C2MO: *T. hirsuta*, C3MO: *P. pentosaceus* in co-culture with *T. hirsuta,* C4MO: *P. pentosaceus* in co-culture with *A. zeae*, and C5MO: *A. zeae* in pure culture, MHT: uninoculated control.

### 6.3.5.2. Volatile compounds in starter culture fermentation of sorghum *ogi*

Volatile compounds identified in sorghum *ogi* fermented with pure and mixed cultures of *Pediococcus pentosaceus, Trametes hirsuta* and *Acremonium zeae* are listed in Table 6.5. A total of 30 volatile compounds were identified in sorghum *ogi* and classified as alcohols (7), ketones (3), aldehyde (1) esters (11), carboxylic acids (2), sulphur containing - (3) benzene based (2) compounds and phenol (1). The total of the major volatile component identified in each sample were: C1SO (18), C2SO (20), C3SO (22), C4SO (24), C5SO (22) and SHT (9).

Alcohols were found at low levels in all sorghum *ogi* except 2-propanol which was relatively abundant in C1 (*P. pentosaceus* in pure culture), C2 (co-culture with *T. hirsuta*) and C4 (co-culture with *A. zeae*), and in the pure culture of *A. zeae* (C5). It was noticeable that highest levels of all the alcohols were produced in the pure and

mixed cultures of *A. zeae* (C5 and C4). However, only 2-propanol, 1-pentanol and ethyl hexanol were detected at very minimal levels in the control (SHT). Ketones were not a major component of this fermentation. However, varying levels of 2-propanone were found in pure culture fermentations of *T. hirsuta* (C2) and co-culture fermentation of *P. pentosaceus* with *A. zeae* (C4). *P. pentosaceus* in pure culture and in mixed culture fermentation with *T. hirsuta* was identified with production of diacetyl. Esters were the major components in all the culture fermentations.

The active aroma ester components in sorghum include ethyl acetate, butyl acetate and butyl hexanoate. Butyl acetate was the most abundant ester in all the fermentations. High levels of esters were characteristic of fermentations with *P. pentosaceus* in pure culture (C1) and co-culture with *A. zeae (*C4), and *A. zeae* in pure culture (C5). Two carboxylic acids were identified and these were present in C3 and C5 *ogi.* Acetic acid was found at a higher level in C5 *ogi*. As observed with the maize, all other compounds were present at low levels in all sorghum culture fermentations. However the phenol (4-ethyl 2-methoxyphenol) and the benzene compounds (1,3-Ditert-butylbenzene and phenyl ethylene) were fairly relatively abundant in the control (SHT).

#### 6.3.5.2.1 Principal component analysis (PCA)

Principal component analysis was also performed on these data to show the pattern of the volatile compound profile in sorghum culture fermentations. The PCA biplot of volatile compounds identified in *ogi* fermented with *P. pentosaceus* in pure culture and in mixed cultures with *T. hirsuta* and *A. zeae* is shown in Fig. 6.14.

The PCA biplot showed minimal separation of the compounds but clearly distinguished between the samples in the score plot generated by PC1 and PC2 which accounted for 21% and 48% of the total variance, respectively. PC1 was greatly influenced by the esters (butyl isobutyrate, butyl acetate and butyl hexanoate), benzene derivative (phenyl ethylene), ketone (diacetyl) and alcohol (1-pentanol and hexanol). PC2 was mostly influenced by the ketone (2- heptanone), sulphur-containing compounds (dimethyl sulphides) and alcohol (2-propanol).

**Table 6.5**: Mean signal intensities ([‡]Peak areas (AU) X $10^4$) and standard deviations (SD)[4] of volatile compounds detected by SPME GC-MS headspace analysis of **sorghum** *ogi* fermented with pure and mixed cultures of [†]LAB and fungi.

| Volatile compounds | Ion (m/z) | Starter culture fermented *ogi* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C1SO | | C2SO | | C3SO | | C4SO | | C5SO | | SHT | |
| | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **Alcohols** | | | | | | | | | | | | | |
| Ethanol | 31 | 4 | 3 | 127 | 10 | 10 | 0.40 | 823 | 98 | 494 | 30 | nd | nd |
| 2-propanol | 45 | 8563 | 1 | 464 | 2 | 3 | 1 | 3083 | 627 | 4905 | 423 | 0.46 | 0.35 |
| Butanol | 56 | nd | nd | 194 | 30 | 7 | 6 | 2 | 2 | 3 | 0 | nd | nd |
| Isoamyl_alcohol | 55 | 0.07 | 0.05 | 3 | 0.27 | 0.46 | 0.23 | 400 | 56 | 3990 | 4 | nd | nd |
| 1-Pentanol | 55 | 0.41 | 0.26 | 1 | 0.31 | 0.23 | 0.21 | 55 | 8 | 48 | 9 | 0.45 | 0.35 |
| Hexanol | 56 | 0.15 | 0.02 | 0.11 | 0.06 | 0.41 | 0.12 | 743 | 143 | 524 | 17 | | |
| Ethylhexanol | 57 | nd | nd | nd | nd | nd | nd | 147 | 26 | 90 | 7 | 0.27 | 0.14 |
| **Ketones and aldehyde** | | | | | | | | | | | | | |
| 2-propanone | 43 | nd | nd | 199 | 24 | nd | nd | 5569 | 849 | nd | nd | nd | nd |
| 2-Heptanone | 58 | nd | nd | nd | nd | nd | nd | 142 | 15 | nd | nd | nd | nd |
| Diacetyl | 43 | 22 | 3 | nd | nd | 16 | 3 | nd | nd | nd | nd | nd | nd |
| 2-Methyl butanal | 57 | 0.21 | 0.11 | 0.17 | 0.04 | 0.20 | 0.02 | 10 | 1 | 3 | 0.39 | 0.85 | 0.45 |
| **Esters** | | | | | | | | | | | | | |
| Butyric acid propyl ester | 101 | 1 | 1 | 3 | 0.32 | 2 | 1 | 1 | 1 | 0.28 | 0.07 | nd | nd |
| Pentanoic acid ethyl ester | 85 | 3 | 2 | nd | nd | 1 | 1 | 0.47 | 0.10 | 1 | 0.38 | 1.40 | 0.78 |
| Ethyl acetate | 43 | nd | nd | nd | nd | nd | nd | 67 | 2 | 21 | 0.14 | nd | nd |
| Butyl acetate | 73 | 9 | 1 | 4 | 5 | 7 | 0.25 | 952 | 387 | 1046 | 456 | nd | nd |
| Butyl butanoate | 71 | 0.16 | 0.08 | 19 | 3 | 0.18 | 0.03 | 42 | 9 | 36 | 2 | nd | nd |
| Ethyl hexanoate | 88 | nd | nd | 0.08 | 0.05 | 0.12 | 0.00 | 38 | 7 | 1538 | 317 | nd | nd |
| Butyl hexanoate | 99 | 0.44 | 0.22 | 0.15 | 0.01 | 0.07 | 0.01 | 1 | 0 | 1 | 2 | 0.11 | 0.01 |
| Cyclobutanol-3-ethyl acetate | 86 | 3 | 0.42 | 2 | 0.28 | 0.04 | 0.01 | 132 | 75 | 957 | 147 | nd | nd |
| Butanoic acid ethyl ester | 71 | nd | nd | 631 | 287 | 69 | 28 | 652 | 46 | 1697 | 7 | nd | nd |
| Butyl isobutyrate | 71 | 17 | 4 | nd | nd | nd | nd | 53 | 28 | 10 | 1 | 0.23 | 0.02 |
| Isopropylacetic acid | 60 | nd | nd | 1 | 1 | 0.49 | 0.30 | 2 | 0.37 | 5 | 1 | nd | nd |
| **Carboxylic acids** | | | | | | | | | | | | | |
| Ethanoic acid (acetic acid ) | 45 | nd | nd | 45 | 10 | 7 | 1 | nd | nd | 6310 | 100 | nd | nd |
| Butanoic acid | 60 | nd | nd | 631 | 287 | nd | nd | 69 | 28 | nd | nd | nd | nd |
| **Other compounds** | | | | | | | | | | | | | |
| Dimethyl disulphide | 94 | 0.21 | 0.13 | 0.09 | 0.04 | 0.23 | 0.02 | 2 | 1 | 2 | 0.10 | nd | nd |
| Dimethyl trisulphide | 126 | 0.15 | 0.10 | nd | nd | nd | nd | nd | nd | nd | nd | 0.48 | 0.07 |
| 1-Propene-1-thiol | 73 | 9 | 0.64 | 6 | 0.12 | 7 | 0.11 | nd | nd | nd | nd | nd | nd |
| Phenyl ethylene | 104 | 62 | 5 | nd | nd | 1 | 0.06 | 59 | 6 | 61 | 1 | 16 | 3 |
| 1,3,-Ditert-butylbezene | 175 | nd | nd | nd | nd | 0.19 | 0.20 | 1 | 0.06 | 0.14 | 0.09 | 0.56 | 0.38 |
| 4-ethyl-2- methoxyphenol | 33 | 4 | 2 | 20 | 8 | nd | nd | nd | nd | nd | nd | 3.3 | 1.1 |

[‡]Values represent the relative abundance. Data are means of four replicates from duplicate determinations (n=4) and standard deviation (SD). SO: sorghum *ogi*, SHT: uninoculated control. [†]*Pediococcus pentosaceus* (C1), *Trametes hirsuta* (C2), *P. pentosaceus* and *T. hirsuta* (C3), *P. pentosaceus* and *Acremonium zeae* (C4), *A. zeae* (C5), nd: not detected.

Four major clusters were clearly distinguished on the PCA plot. The volatile profiles of C1SO, C2SO and C3SO were associated with group 1. These were also similar to the naturally fermented sorghum *ogi* and were characterized by production of esters and alcohol by *P. pentosaceus* and *T. hirsuta*. This may suggest similar metabolic activities of these microbes in the pure and co-culture fermentations. Again C4SO and

C5SO were related to group 2 on the PC2 with the majority of the flavour compounds comprising mostly of alkyl esters. The PCA showed clear separation of the control (SHT) uniquely distinguished by the alkyl carboxylic acid, phenol and benzene compound associated with cluster 3 on PC1.

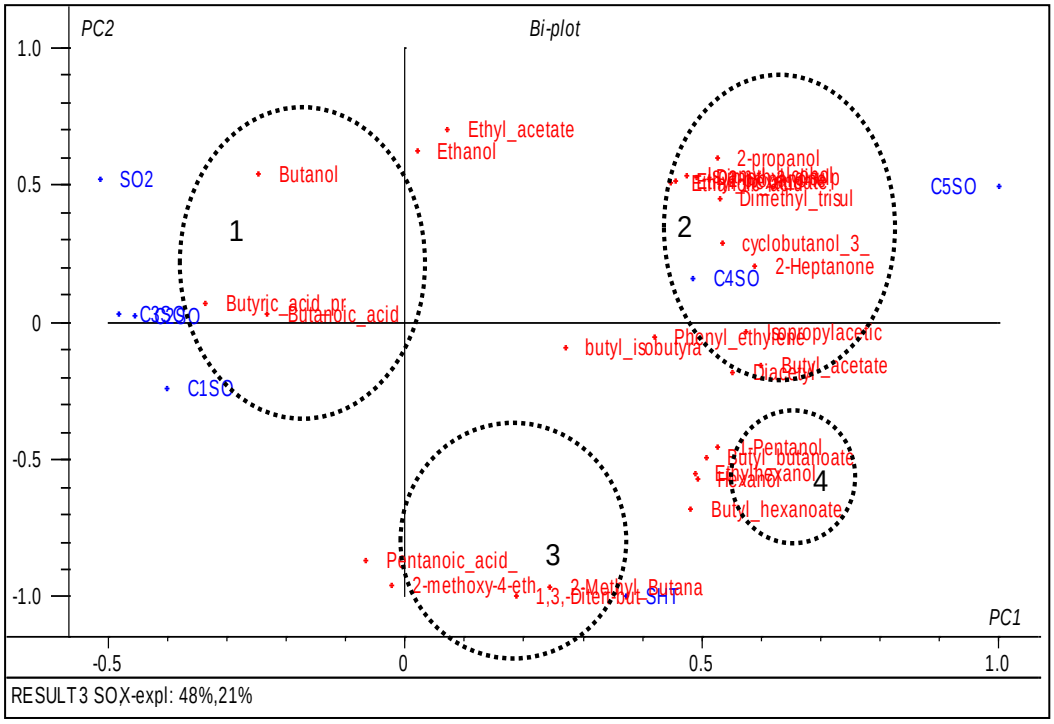



**Figure 6.14**: Principal component analysis biplot of sorghum *ogi* fermented with pure and mixed cultures of *P. pentosaceus*, *T. hirsuta* and *A. zeae.* SO2: naturally fermented *ogi*, starter culture fermented *ogi* (C1SO: *P. pentosaceus,* C2SO: *T. hirsuta*, C3SO: *P. pentosaceus* in co-culture with *T. hirsuta,* C4SO: *P. pentosaceus* in co-culture with *A. zeae*, and C5SO: *A. zeae* in pure culture, SHT: uninoculated control.

## 6.4 Discussion

### 6.4.1 Effect of autoclaving on nutrient content of grains

Controlled fermentation by starter cultures requires sterilisation of the starting grain. This could either be achieved by chemical or heat treatment. In this study dry heat treatment by autoclaving was applied. However, given that heat may change the chemical composition of the grains, the TPC and phytate levels were compared between heat-treated and untreated grains. Heat treatment was found to reduce the TPC in maize grain but had little or no effect in sorghum. Heat treatment has little or no effect on phytate levels in maize and actually increased these in sorghum. The

analysis of the TPC results is confounded by the fact that whilst this is a traditionally used method to measure total phenolics it is based on an antioxidant principle and may be influenced by other compounds. However, for the purposes of this discussion it will be assumed that the TPC is a relatively accurate measure of the phenolics in the samples.

Heat treatment may decrease the phenolic content of plant materials. Xu *et al*., (2007) demonstrated a decrease of up to 7.61 mg/g in polyphenol (flavanone glycoside) in citrus peels after heating for 90 min at 120$^{o}$C while (Asami *et al*., 2003) recorded a decrease of 30.1 % in total phenolics of organic corn air dried at >60$^{o}$C. This study has demonstrated a decline in the bound phenolics of heat treated maize (MHT) with a 22% increase in the soluble phenolics. This suggests that some bound phenolics may have been solubilised by heat. A similar effect was reported by Wataniyakul *et al*. (2012) who observed a 55% increase in recovery of total phenolics from defatted rice bran preheated at 60–100$^{o}$C. Although heat treated sorghum (SHT) did not show a decrease in the total phenolics, the increase in the soluble phenolics observed may have resulted from the solubilization of the bound fraction by heat.

Heat treatment is known to inactivate plant phytase and degrade or solubilize phytate (Blaabjerg *et al*., 2010). However, the extent of phytate degradation may depend on the level of enzyme inactivation. Although maize phytate was not affected by heat an increase of 45% observed in sorghum phytate may also suggest a solubilization effect, making it more accessible and resulting in the increase observed in the heat-treated grain. Although this result agreed with the work of (Brejnholt *et al*., 2011) which demonstrated that heat treatment made wheat phytate more accessible, it may not be definitive and as such further confirmatory analysis is recommended to check the extent of enzyme inactivation in order to support this present finding in sorghum. Overall maize phytate was not affected by heat treatment and the significant variation between phytate content in heat-treated maize and sorghum suggests differences in the phytate-matrix interactions (Brejnholt *et al*., 2011) which may determine accessibility to dephosphorylation.

### 6.4.2 Microbial population dynamics

The complexity of the succession of microbes during natural fermentation of *ogi* as demonstrated in Chapter 4 may lead to products of unpredictable and undesirable quality characteristics. The use of starter cultures of *Lactobacillus brevis* and *Saccharomyces cerevisiae* in *ogi* fermentation has been found to present a more predictable and acceptable product (Teniola and Odunfa, 2001). Although these researchers focused only on the influence of these microbes on lysine, methionine and organoleptic qualities of *ogi* they also thought that growth dynamics of these microbes on the substrate may have contributed to the qualities observed in the *ogi*. Of all the microorganisms selected in this study only *P. pentosaceus* has been identified previously in *ogi* (Oguntoyinbo and Narbad, 2012). However, no record was found on the use of these microbes as starter culture in *ogi* and their interaction during fermentation was not known. Therefore, in this study the growth and interaction of *Pediococcus pentosaceus*, *Trametes hirsuta* and *Acremonium zeae* in relation to pH in pure and co-culture *ogi* fermentation was investigated. This will help in determining how microbial interactions and resultant changes in growth will influence of phenolics and phytate levels and the aroma profile in *ogi*.

The changes in viable populations of *P. pentosaceus* in this study during steeping seem to differ slightly in the pure and co-culture fermentations with the fungi. However, in the *P. pentosaceus* pure culture fermentation, the viable population recorded at 24h was log 8.96 cfu/ml, which increased to a maximum of log 9 cfu/ml after 36 h, and remained stable until the end of the fermentation at 72 h. Although no record was found on the use of *P. pentosaceus* as a starter culture in *ogi* fermentation and given that microbial density may depend on the initial starting level, this result varied slightly from the initial succession of this microbe in accelerated (back slopping) maize *ogi* fermentation (Teniola *et al*., 2005). In their work the population of *P. pentosaceus* among other LAB present increased from log 7.6 to 7.9 and 9.2 log cfu/ ml at 24 h, 48 h, and 96 h of fermentation which is not far from the trend observed in this study.

In the co-culture fermentation of *P. pentosaceus* with *T. hirsuta,* the maximum population of log 9 cfu/ml was reached earlier at 24 h but decreased up to log 8.64 cfu/ml at 72 h and suggests competitive interaction or possible amensalistic effect which may have repressed the growth of the LAB. *T hirsuta* is known to secrete laccase (oxidoreductase) - a multi-copper enzyme which is capable of degrading lignin (Mayer and Staples, 2002; Couto *et al*., 2006). It may be possible that the presence of this enzyme could interfere with LAB growth. This may have resulted in further increase in the viable population of *T. hirsuta* after the significant drop in cell density which occurred earlier in maize fermentation (Fig 6.3e). Microbial growth succession may also depend on the available nutrients and their utilization by the microbes (Cho *et al*., 2006) which tend to decrease towards the late stage of fermentation. The interaction of *P. pentosaceus* in co-culture with *A. zeae* showed a more consistent growth pattern similar to that observed in pure cultures. Although LAB are known to exhibit antimicrobial effects, this may not have been significant in this fermentation since *A. zeae* is also known to produce pyrrocidine antibiotics capable of inhibiting other fungi (Wicklow et al., 2005, Poling *et al*., 2008, Wicklow and Poling, 2009) in addition to production of hydrolytic enzymes capable of hemicellulolytic activities (Bischoff *et al*., 2009).

The pH values followed similar trends in all culture fermentations. This is not surprising since *P. pentosaceus* although an acidophilic microaerophilic homofermentative LAB are able to grow at pH 4.5–8.0 (Lv *et al*., 2014) which is similar to those of the fungi, at pH 3–13 (optimal 5-7) (Dhakar and Pandey, 2013). Overall, the pH at 72 h in all cases ranged from 5.1-5.5 in maize and 4.78-4.95 in sorghum. Although near to those obtained in the natural fermentation in Chapter 5 (4.80-5.04), the pH values in this study were much higher than those recorded in literature for spontaneous (3.9), back slopping (3.8) or starter cultures of *Lactobacillus* and yeast (3.7) fermentations (Teniola *et al*., 2005). Apart from acid production by LAB, the level of acid produced in these fermentations could also depend on the amount of freely metabolisable carbohydrate sources (available as carbon source for microbial growth) in the substrate which may have been affected in the heat-treated grains. Thus higher

pH levels as observed in this study seem undesirable since most pathogens are capable of growing at similar levels.

### 6.4.3 Phenolics in starter culture fermentations

Again interpretation of the TPC data is difficult as this may not be an accurate reflection of phenolics content and could be influenced by other antioxidant compounds. The TPC recovered in the uninoculated controls was 77% for the maize and 95% for the sorghum. This demonstrates a very good recovery in the case of sorghum but suggests a loss of TPC in the maize. This may reflect an oxidative process during the fermentation but why this should have occurred only in maize is unclear. In all cases the recovery from the culture fermentations was higher than the controls and for sorghum actually exceeded 100%. It may be that the conditions during these fermentations (low pH) may have served to stabilise the phenolics, or other antioxidants, thus increasing the recovery. Alternatively, the microorganisms themselves may be synthesising antioxidants that are interfering with the TPC assay.

An increase in TPC in the steep water was clearly evident in all culture fermentations for both maize and sorghum. If this is a true reflection of phenolic content it would suggest esterase activity. It could however, be the result of the synthesis of soluble confounding antioxidants by the microorganisms. It was beyond the scope of this present investigation to attempt to measure individual phenolics directly but this would be an obvious next step in the study. This leaching effect was higher in sorghum than in maize and also higher in cultures containing fungi suggesting that esterase activity if present is more prevalent in fungi.

Changes in the distribution of the bound and soluble phenolics were observed in all maize fermentations. The lower bound phenolic values and higher values in the soluble fraction in *P. pentosaceus* (C1) and *T. hirsuta* (C2) pure culture fermentations than in the control might indicate esterase activity. Given the original amount of soluble and bound phenolics in the maize and the overall recoveries of these fractions

as well as the phenolics solubilized from the bound fraction during steeping, additional release of phenolics through esterase activity was also evident. However, this effect was found to be higher in the fungal co-cultures with the LAB (C4 and C3) and in the pure cultures of *T. hirsuta* (C2) and *A. zeae* (C5) again suggesting that a higher esterase activity was associated with the fungi rather than the LAB. This effect seemed contrary to the observation in the natural fermentation in which the only LAB population showed a higher correlation to the release of phenolics into the steep water than the yeasts and moulds (§5.3.4- Fig. 5.6). The low efficiency of mould to release of phenolics in the natural fermentation may have resulted from antagonistic effects of the other microbial populations such as the LAB or Enterobacteriaceae during the natural fermentation. The recovery of total phenolics in maize *ogi* was lower in C3 than in the control and other culture fermented *ogi* as measured by the original grain total. This may be attributed to the leaching effect as demonstrated by the losses observed in the steep water. It is notable that the free and bound phenolics recovered in maize *ogi* from all the culture fermentations were all lower than originally present in the grain indicating possible release of phenolics and subsequent losses through leaching. Nevertheless, maize seemed to show good retention of soluble phenolics in the *ogi.*

A similar result was seen in the sorghum fermentations where there was a general increase in the soluble fraction. This was clearly noticeable in the *ogi* from the C1, C2, and C3, fermentations again suggesting esterase activity.

Although no studies could be found on the leaching of phenolics in sorghum during fermentation, a significant decrease in the total polyphenol content of sorghum slurry following traditional lactic acid starter culture (back slopping) fermentation has been reported (Dlamini *et al*., 2007, Avila *et al*., 2009). The degradation of phenolics has been attributed to phenolic acid decarboxylase and reductase (Svensson *et al*., 2010). Phenolic acid metabolism by LAB species such as *L. fermentum and L plantarum* resulting in lower levels or below detectable limits in sorghum slurry fermentation has been reported (Svensson *et al*., 2010). However *P. pentosaceus* showed reasonable recovery of soluble phenolics in the *ogi*. Overall in sorghum fermentation, although *T.*

*hirsuta* (C2) seemed to have shown the highest solubilization of phenolics from the bound fraction in *ogi*, given by the lower recovery of the bound phenolics than in the other *ogi*, the highest levels of soluble phenolics was recovered from C1 *ogi*. Thus *P. pentosaceus* in pure culture fermentation may be showing the highest esterase activity and produced *ogi* with the highest antioxidant potential in sorghum fermentation. Phenolic acid was mostly lost through leaching thus sorghum *ogi* showed less retention of soluble phenolics than the maize.

### 6.4.5 Phytate hydrolysis in maize starter culture fermentation

In the pure culture fermentations phytase activity was mostly evident in C2 (*T. hirsuta*). Only 3% of the initial phytate content in the grain was recovered in C2 *ogi* and this was found to be much lower than the 18% recovery in the naturally fermented maize *ogi* as seen in Chapter 5. In the mixed culture fermentation, *P.pentosaceus* with *T. hirsuta* (C3) showed higher phytase activity with slightly lower levels of phytate than in the co-culture of *P. pentosaceus* with *A. zeae* (C4). However, the decrease in phytate in both pure (64-97%) and mixed culture (72–77%) fermentations is in agreement with the range observed by Sokrab *et al*., 2014, who showed a 65–80% reduction in phytate during natural fermentation of corn flour within 2–14 h. In their study, phytate reduction showed a positive correlation to the time of fermentation. This supports the high phytate reduction of 97% observed in the present study after 72 h of fermentation in C2.

Although lactic acid bacteria (LAB) are known to be able to hydrolyse phytate, not all extracellular phytase from LAB seem to show significant phytase activity during fermentation (De Angelis *et al.,* 2001, Fredrikson *et al*., 2002). However, only *Lactobacillus plantarum* and *Lactobacillus amylovorus* have been reported to show significant phytase activity in submerged culture (Sreeramulu *et al*., 1996) but phytase activity by *Lactobacillus plantarum* was attributed to the presence of non-specific acid phosphatase (Zamudio *et al.,* 2001). However, phytate reduction could also be related to leaching since phytate is soluble in water. In their work, Lestienne *et al*. (2005)

reported a 21% loss of phytate through leaching in maize after 24 h of soaking at 30$^{o}$C. A similar result was observed in this study with leaching of phytate in steep water which was detected as 24% (72 h; 30 $^{o}$C) of the original grain phytate content. Thus the low level of phytate recovered in C2 *ogi* may also be partly attributed to a leaching effect and loss through the pomace and only about 70% reduction may have been due to phytase activity (Fig 6.11). Overall 24–70% of phytate reduction in maize *ogi* may be attributed to microbial hydrolysis.

*Trametes hirsuta* was found to show greater phytase activity in both the pure and co-culture fermentation with *P. pentosaceus* than *A. zeae.* Although no record was found on phytase activity of *Trametes hirsuta, a* different species of the same genus, *T. pubescens,* was observed to produce phytase identified with the *phyA* gene which showed specificity to 3- and 6-positions of Ins(1,2,3,4,5,6) phosphates (Lassen *et al*., 2001). This activity could be applicable to *T. hirsuta* and may explain the high phytate reduction in C2 *ogi*. Therefore further studies on the identification of *phyA* gene in *T. hirsuta* may be useful to validate the phytase activity of this fungus during *ogi* fermentation.

Phytase activity was also evident in *Acremonium zeae* fermentations. Similarly no record is available in the literature on phytase activity of *A. zeae.* However, *A. zeae* is a maize protective endophyte known for antibiotic production (Wicklow and Poling, 2009). The activity of this mould with *P. pentosaceus* resulting in lower phytate level in *ogi* than in the pure culture fermentations (C1 and C5) may suggest synergistic interaction in the activities of the LAB (*P. pentosaceus*) and fungi (*A. zeae*) in the co-culture fermentations.

### 6.4.5 Phytate hydrolysis in sorghum starter culture fermentation

*Trametes hirsuta* and *A. zeae* were found to show greater phytase activity than *P. pentosaceus* in both the pure and co-culture fermentations in sorghum fermentation although *A. zeae* showed the greatest phytase activity and higher phytate reduction in the *ogi*. The recovery of phytate in the pure culture fermentation of sorghum *ogi* showed lower levels in C2 and C5 and was least in *A. zeae* (C5) showing only 4%

recovery of the original phytate content in the grain. This value was found to be much lower than the amount recovered in the naturally fermented sorghum *ogi* (48%) as demonstrated in the previous chapter. Recovery of phytate in C2 and C5 fermentations were at similar low levels and suggest higher phytase activity and better nutrient bioavailability in the *ogi*. Overall phytate reduction observed in sorghum ranged from 75-92% and 92-96% in the pure and mixed culture fermentations. These values are higher than the reported values of 51.90-52.90% in traditionally fermented (28-32$^{o}$C: 14 h) sorghum dough from three cultivars (El Hag *et al*., 2013), although phytate reduction has been reported to increase with time during fermentation (Sokrab *et al*., 2014).

Similarly phytate levels in *ogi* in the mixed culture fermentations (C3 and C4) did not show significant differences from each other and this may suggest similar levels of phytase activity in both fermentations. Considering the individual pure culture fermentations, *P. pentosaceus* (C1) seems to show less phytase activity having shown a higher level of phytate than in C2 and C5 and thus is likely to make a smaller contribution to phytate reduction in the mixed fermentations C3 and C4. Reale *et al*. (2007) reported that phytate degradation was independent of LAB species but dependent on the endogenous phytases present in the grain. This may partly explain the low phytase activity observed in the *P. pentosaceus* fermentation in this study, since the grain endogenous phytase may have been inactivated by autoclaving as evident in the control. This effect is evident in the control with high levels of phytate dectected in the *ogi* and steep water. Although *P. pentosaceus* CFR R123 was found to show phytate degrading ability in an in-vitro culture study (Raghavendra *et al*., 2010) the *P. pentosaceus* strain examined here did not seem to show a significant contribution to phytate hydrolysis in sorghum fermentation.

In sorghum, phytate was mostly lost through the steep water and the pomace. Losses in the steep water may be attributed to a leaching effect which was mostly evident in the control. Lestienne *et al*. (2005) reported 4% leaching of phytate in sorghum as a result of soaking. Their result did not correspond with the result in the present study which showed that 45% of the original grain total leached into the steep water. The

high leaching effect may be attributed to heat treatment of the grains which may have made phytate more accessible. The low level of phytate in C4 *ogi* may also have been partly as a result of leaching and loss through the pomace and as such only about 36% may be attributed to a microbial effect. Overall 8–36% of phytate hydrolysis in sorghum may have been due to microbial hydrolysis.

### 6.4.6 Volatile organic compounds

This study has demonstrated the presence of alcohols, aldehydes, ketones, carboxylic acids, esters, benzene derivatives, thiol, phenol and sulphur-containing compounds during fermentation of maize and sorghum *ogi* with the selected starter cultures of LAB and fungi. The volatile organic profile of *ogi* made from the pure cultures suggests differences in the relative abundance of the compounds. The production of microbial volatile organic compounds would depend on the substrate, species, growth phase and environmental conditions (Larsen and Frisvad, 1994). *Pediococcus pentosaceus* is an acidophilic homofermentative lactic acid bacterium and primarily produces lactic acids from glucose fermentation. However, *P. Pentosaceus* is capable of producing other compounds (heterofermentative) through alternative pathways following changes in the metabolic conditions and may also exhibit homolactic fermentation in a rich carbohydrate medium or through glycerol metabolism to produce compounds such as acetoin, acetate, diacetyl and $CO_2$ (Dobrogosz and Stone, 1962b). This may explain the presence of various volatile components in the pure culture fermentations with *P. Pentosaceus*. Similarly filamentous moulds are also known to produce various volatile compounds, mostly ketones, esters, sulphur-containing compounds, phenols, hydrocarbons and alcohols (Korpi *et al*., 2009). Thus the profiles of volatile compounds obtained in this study are characteristic of bacterial and fungal fermentations although this is the first study on aroma characteristics of *ogi* with *P. Pentosaceus, T. hirsuta* and *A.zeae* as starter cultures.

#### 6.4.6.1 Alcohols

In maize *ogi* the alcohols identified were similar to those found in maize dough fermented with *S. cerevisiae* and *Lactobacillus fermentum* (Annan *et al*., 2003). These were mostly ethanol and the fusel alcohols including 2-propanol and 1-pentanol. Alcohols are products of fatty acid esters associated with lipid oxidation and are metabolised in both bacterial and fungal species. Ethanol was the most abundant alcohol in the pure culture fermentation of maize with *A. zeae* (C5). Although filamentous fungi are known for low to moderate production of ethanol, the high relative abundance of ethanol and hexanol in C5MO may suggest the presence of more efficient biological enzymes (amylase, xylanase, and cellulase) and higher metabolic activities which enhanced alcohol fermentation in *A. zeae* (Skory *et al*., 1997). The production of 2-propanol was consistent in all maize fermentations with the highest abundance in C1MO.

In sorghum *ogi*, similar alcohols as identified in the maize were evident but available in slightly lower levels than in maize except 2-propanol. This result reveals the compatibility of the starter cultures on both substrates although the production of these compounds may depend on the metabolic activities of the microbial species used in the fermentation. The alcohol profile in this study was slightly different from those identified in sorghum beer fermented with pure and co-cultures of *S. cerevisiae*, *L. fermentum* and *Issatchenkia orientalis* (Lyumugabe *et al*., 2014). These researchers in addition to those identified in this study showed the abundance of more of fusel alcohols (2-methylpropan-1-ol, 3-methylbutan-1-ol and 2-methylbutan-1-ol) in sorghum fermentation.

Overall in both maize and sorghum, it is notable that the abundance of majority of the alcohols followed a similar trend in all the culture fermentations suggesting the presence of similar nutrient sources in both substrates. The low levels of ethanol in C1 fermentations than in C4 and C5 suggest that *A. zeae* may also have played a major role in alcohol production in the co-culture fermentation with *P. pentosaceus*. Ethylhexanol was unique to C4 and C5 in maize and sorghum fermentations. Alcohols have high threshold values (Flath *et al*., 1967) and as such those present in relatively

low values may not show significant impact on the aroma characteristics of *ogi*. However the abundance of ethanol in C4MO, C5MO, C4SO, and C5SO and 2-propanol in C1MO, C2MO, C1SO, C2SO, C4SO and C5SO may be responsible for the ethereal and sweet fruity notes in each case, in these *ogi.*

#### 6.4.6.2 Ketones and Aldehydes

In maize 2-propanone was the most abundant ketone in C4MO and C5MO and diacetyl in C1MO. Ketones are formed by oxidation of fatty acids (Dong *et al*., 2014). The high levels of these ketones correspond to the lower levels observed in their respective alcohols and suggests possible conversion of the alcohols to ketones. In sorghum 2-propanone was also abundant in C4SO. The disappearance of this ketone in C5MO may also be attributed to possible conversion to related aldehydes. Diacetyl was also unique to C1SO but showed a much lower level than in the maize. This may suggest the presence of adequate nutrients in the substrate since *P. pentosaceus* as a homofermentative LAB may only be able to produce diacetyl through the alternative pathway of glycerol metabolism in times of nutrient deficiency (Dobrogosz and Stone, 1962a).

Overall the ketones identified in this study were mostly related to those reported in barley fermentation (by LAB and yeast) in the literature (Annan *et al*., 2003). The ketone 2-heptanone is characteristic to moulds and was found uniquely in C4MO and C4SO. This may have been degraded or not made in the pure culture fermentation probably because the intermediate compound in the mixed culture may have originated from the LAB resulting in the absence in both C5MO and C5SO. The lower threshold value (140 ppm) of 2-heptanone than other ketones (Buttery *et al*., 1994) may make its characteristic aroma of cheesy and fruity smell significant in *ogi*. The aldehyde 2-methyl butanal is formed through the isoleucine degradation pathway and is responsible for roasted cocoa aroma and may also be significant in *ogi* given its low threshold value of 3 ppm (Buttery *et al*., 1994).

#### 6.4.6.3 Esters and carboxylic acids

Esters are mostly produced by esterification of carboxylic acids and alcohols (Ansorena *et al*., 2001). In maize ethyl acetate, butyl acetate and ethyl hexanoate were mostly characteristic of C4MO and C5MO. A negative correlation between the esters and their respective alcohols and acids was apparent and may suggest chemical conversions of these compounds. Acetic acid identified in C1MO, C2MO, and C3MO may also have been produced by chemical degradation or an alternative pathway from an intermediate (pyruvate) in glycerol metabolism (Dobrogosz and Stone, 1962a). Acetic acid has been reported as the most abundant organic acid in most heterofermentative LAB fermentations (Halm *et al*., 1993) and is responsible for the sour and vinegar notes in foods (Annan *et al*., 2003). The presence of acetic acid in C1, C2 and C3 *ogi* suggests that the use of the *P. pentosaceus* and *T. hirsuta* as starters could contribute to the sour taste of *ogi* which is a desirable quality in the traditionally fermented product. In sorghum, similar esters as in the maize were unique in addition to butanoic acid ethyl ester (ethyl butanoate) and were relatively abundant. Ethyl acetate, butyl acetate and ethyl hexanoate are respectively, responsible for fruity sweet rum-like, sharp ethereal banana and green sweet juicy aroma (Tgsc, 2015) in the *ogi*. Acetic acid was mostly abundant in C5SO. Overall, the profile of esters identified in maize and sorghum *ogi* as demonstrated in this study were slightly similar to those reported for maize dough (Annan *et al*., 2003) and different to those in sorghum beer (Lyumugabe *et al*., 2014). In addition to the higher levels of these esters, the presence of butanoic acid ethyl ester only in sorghum may be significant in the aroma characteristics of the *ogi* given its unique fruity, pineapple-like smell (Tgsc, 2015). The relatively high abundance of these esters in *A. zeae* fermentations suggests more sweet and fruity smells in C4 and C5 *ogi*.

#### 6.4.6.4 Other volatile compound

Sulphur-containing compounds (dimethyl disulphide and dimethyl trisulphide) were characteristic in C2 and C5 and the phenol 4-ethyl-2- methoxyphenol was mostly

abundant in C1 maize fermentations. The sulphur containing compounds have low odour thresholds ranging from 0.16–12 ppm for dimethyl disulphide and 0.005-0.01 ppm for dimethyl trisulphide (Buttery *et al*., 1994). They are characteristic of several mould species (Fielder *et al*., 2001, Morath *et al*., 2012) and give unpleasant, sulphurous, garlic-like odour. In sorghum these compounds were present at very low levels. The threshold values of some phenolic compounds such as protocatechuic and syringic acid range from 240-300ppm (Velioğlu, 2010). Thus the presence of the phenolic compound 4-ethyl-2- methoxyphenol could also be significant in the taste of *ogi* having sour, bitter, stringent and phenolic-like taste. This may be more significant the maize *ogi* given the relatively high abundant levels in the grain. However despite the high abundance of this phenol in maize grain it was mostly accessible in the *P. pentosaceus* fermentation indicating that it was either used as energy source or not efficiently hydrolysed by the fungi resulting to its absence or minimal levels detected in the fungal fermentations. The PCA showed good discrimination of the compounds and clearly distinguished the *ogi*. *P. pentosaceus* and *T. hirsuta* seemed to produce similar compounds and both the pure and co-culture fermentation *ogi* were more similar to the naturally fermented *ogi*. In both maize and sorghum the majority of the esters and ketones were associated with *A. zeae ogi* (C4MO, C4SO). The activities of C4 and C5 cultures varied between maize and sorghum substrates, thus C4MO and C5MO seemed different while C4SO and C5SO were more similar. Thus the grain used in *ogi* fermentation could impact on the final sensory characteristics.

### 6.6 Conclusion

The changes in viable populations during starter culture fermentation showed slight differences in the *P. pentosaceus* pure culture fermentation and in co-culture with the fungi. *P. pentosaceus* was found to exhibit a more stable population in the earlier stage of fermentation reaching the maximum within 36 h. Competitive growth or amensalistic interaction may have resulted in the decline in the viable population observed in the co-culture fermentation of *P. pentosaceus* with *T. hirsuta*. A more stable interaction was observed in the fermentation of *P. pentosaceus* with *A. zeae*

which may be an equilibrium effect of the antimicrobial properties of both microbes. It is notable that the pH ranges of all the fermentations were similar in both maize and sorghum and seemed not to have correlated to the microbial growth succession during fermentation. This effect could be explained by the relatedness of the growth pH of the LAB and fungal species used in this study. Overall the stability and ability of the microbes to attain higher populations earlier in the fermentation seem desirable in *ogi* fermentation as this could facilitate the reduction of the fermentation time.

Increased leaching of soluble phenolics into the steep and wash waters in both maize and sorghum fermentations was observed. In addition changes in the ratios of bound to soluble phenolics were also observed in both the *ogi* and pomace fractions. It is suggested that esterase activity may have been responsible for these observations. However, synthesis of confounding compounds by the microorganisms cannot be ruled out as possible alternative explanation. The involvement of esterase activity was also suggested by the fact that the overall recovery of soluble phenolics (in *ogi*, pomace, steep and wash waters) showed higher values (29-45%) than the original amount of soluble phenolics (24%) in the grain. A lower amount of soluble phenolics was left in *ogi* (7-18%) and a greater amount was lost with the pomace and wash waters (19–27%). However, ranking the recovery of soluble phenolics in maize fermentation in ascending order gave C2, C5, C1, C3, and C4, suggesting highest esterase activity was present in the pure culture fermentation with *T. hirsuta*. Sorghum showed higher leaching of phenolics than maize. Overall recovery of the soluble phenolics was 53-64 % which was higher than the original total of 49% in the grain. A lower amount remained in the *ogi* (8-22%) and a greater amount (34-47%) was lost with the waste. Similarly, ranking the recovery of the soluble phenolics from the highest gave C1, C2/C5, C3 and C4. Thus, suggesting that *P. pentosaceus* in pure culture showed the highest esterase activity in the sorghum fermentations. The reason for this difference in ranking is unclear but may relate to the composition of the two substrates. This study has demonstrated that the use of starter cultures in fermentation of *ogi* can enhance the release of soluble phenolics and increase the recovery in the *ogi* compared to natural fermentations despite the high level of

leaching. In general the leaching of phenolics from sorghum was at a higher level compared to maize suggesting the dependence of phenolic hydrolysis on grain structure and food matrix.

*T. hirsuta* exhibited the highest probable phytase activity in pure culture fermentation of maize. Leaching of phytate was obvious in the fermentations and in all cases only about 24-70% of the phytate reduction may be attributed to biological activities. *A. zeae* showed the highest phytase activity in pure culture fermentation of sorghum and only about 8–36% of phytate reduction in all cases seemed to have resulted from microbial hydrolysis. Although not originally isolated from maize the high activity of *T. hirsuta* in phenolics and phytate hydrolysis showed good compatibility of this microbe to the maize substrate in addition to enzyme specificity during fermentation. This study has demonstrated that these microbes show reduced activity in the co-culture fermentations which probably may be due to some amensalistic interactions.

A total of 29-30 major volatile compounds were present in maize and sorghum *ogi.* Alcohols and their respective esters were characteristics in both grain fermentations. Only a few ketones, aldehydes, and carboxylic acids were identified. *P. pentosaceus* and *T. hirsuta* in co-culture fermentations produced the least levels of volatile compounds in both *ogi*. Esters were mostly produced in *A. zeae* fermentations (C4 and C5). In general the key aroma active compounds in both maize and sorghum *ogi* were ethyl acetate, butyl acetate and ethyl hexanoate. Butanoic acid ethyl ester found only in sorghum may be significant in the aroma characteristics of the *ogi* given its unique fruity and pineapple aroma. The PCA analysis revealed that *P. pentosaceus* and *T. hirsuta* in pure and in co-culture fermentations produced *ogi* with aroma notes mostly related to the naturally fermented product.

# CHAPTER 7

## GENERAL DISCUSSION AND CONCLUSION

*Ogi* is an important fermented cereal food in Nigeria processed by natural fermentation. Traditional fermentation of *ogi* involves a wide range of microorganisms introduced by chance inoculation, resulting in the inconsistency of quality characteristics and reduced shelf life caused by the spoilage microbes. The safety and nutritional quality of *ogi* are greatly important since this product is consumed by infants and the convalescent. Previous works have looked at the microbial ecology of traditionally fermented *ogi* (Odunfa and Adeyele, 1985) using both cultural and molecular techniques (Ampe *et al*., 1999, Ampe and Miambi, 2000; Oguntoyinbo *et al*., 2011). The diversity of the bacterial community in the fermentation of *ogi* has received greater attention. Only yeast has been studied among the fungi thus leaving a gap in the diversity and significance of the filamentous fungi in addition to yeast during *ogi* fermentation.

The composition of cereal grains and changes in the components during fermentation is nutritionally important. Carbohydrates (starch and non-starch polysaccharides) form the major component of cereal grain. The cell wall polysaccharides are capable of forming linkages with the phenolic components. For instance arabinose residue of the arabinoxylans may be substituted with ferulic acid forming cross links of ferulic acid through oxidation (Lafiandra *et al*., 2014). Thus the hydrolysis of starch and non-starch components would depend on the food matrix in addition to enzyme efficiency and microbial activities. The extent of NSP hydrolysis may also impact on the release of phenolic compounds esterase. Substrate availability may also affect the activities of esterases and release of phenolics (Wheelock *et al*., 2008). While the antioxidant activity of cereal phytochemicals in *ogi* is also nutritionally important, the hydrolysis of some aromatic components in the grains may play a significant role in the aroma characteristics of *ogi*. Therefore the aim of this study has been to characterize the diversity of bacterial and fungal species in the grains and *ogi*, assess the population

dynamics and the role of the key species on the antioxidant and aroma properties of *ogi* targeting the changes in the levels of phytate and phenolics.

This study has demonstrated that microbial consortia during natural fermentation varied with the source of the grain and showed significant impact on the compositional changes during *ogi* fermentation. The levels of phenolics increased and phytate reduced while varying aroma compounds were generated in the naturally fermented *ogi.* For the first time starter cultures of *P. pentosaceus*, *T. hirsuta* and *A. zeae* were used in the pure and co-culture fermentations of *ogi* and interestingly these microbes in all cases improved the reduction of phytate and the release of soluble phenolics from the bound fraction than observed in the natural fermentation. However leaching of soluble phenolic acid into the steep water was apparent in all fermentations with sorghum having the highest losses and may have depended on the food matrix.

**7.1 Microbial diversity in *ogi* fermentation**

This study demonstrated a regional effect on the microbial diversity of similar grains from two different sources showing a wide range of species of the *Micrococcus*, *Staphylococcus*, *Pantoea*, *Bacillus* and yeast with the *Enterobacteriacae* and mould groups predominating in *ogi* fermentations. Lactic acid bacteria (LAB) of the genera *Pediococcus*, *Lactobacillus* and *Enterococcus* were identified but surprisingly, only two strains of *P. pentosaceus* and *Enterococcus* species were recovered by cultivation. *Lactobacillus rossiae* was only detected in sorghum by the PCR-DGGE technique. Apart from LAB, the diversity of microbes in *ogi* reflected the groups originally found in the grains and varied between the different sources of maize and sorghum. Thus in addition to *Pediococcus* and *Lactobacillus* identified in sorghum and *Bifidobacterium* in maize, *Enterococcus, Enterobacter,* yeasts and moulds of the genera *Aspergillus*, *Cladosporium* and *Penicillum* predominated in both sorghum and maize fermentations. The presence and uniqueness of *Lactobacillus rossiae* in sorghum and *Bifidobacterium breve* in maize are of nutritional importance since both microbes are known for their probiotic properties (De Angelis *et al*., 2014). Previous studies have reported LAB as the major part of the microbial population in natural fermentation of

*ogi* (Odunfa and Adeyele, 1985, Teniola and Odunfa, 2002, Oguntoyinbo *et al*., 2011) but this was not the case in the present study. This probably may be due to the variations in the source of the grains, or the dominance of other non-LAB groups early in the fermentation resulting in the shift observed with the identified species compared to those found in the literature. However microbial species are expected to vary in different sources of *ogi*. For example *ogi* produced by using sterile utensils and water as in this present study would show great variations in the species available in the fermentation if sampled with the traditionally made *ogi*. Due to the nutritional importance of phytate in food of plant origin and accumulation of acetic acid in *ogi* fermentation an attempt was made to examine the presence of microbes associated with the hydrolysis of these compounds in *ogi* fermentation. Thus in addition to the classical identification of organisms involved in *ogi* fermentation, the absence of acetic acid bacteria in *ogi* fermentation and the presence of two fungal isolates on maize and sorghum grains (having phytase activity) were demonstrated.

### 7.2 Microbial population and compositional changes in *ogi*

This study has demonstrated that variations in the microbial population diversity in the four grain (M1, M2, S1, S2) fermentations correlated with the changes in starch, NSP, phytate, phenolics and aroma characteristics of *ogi*. To be able to understand the fate of starch and NSP in the natural fermentation of *ogi,* these were tested in the original grains and their respective fermented products. The starch contents of maize and sorghum *ogi* were at similar levels in the two maize grains and differed in the two sorghum grains while NSP were similar between the same grains, giving no clear evidence of overall regional effect except in the sorghum starch. This is also true in the *ogi*. However the reduction observed in the carbohydrate levels of the *ogi* gave clear evidence of microbial effect which could be mostly attributable to the presence of moulds such as *Aspergillus oryzae*, *Penicillum biourgeianum*, *Acremonium zeae* in maize and *Cladosporum macrocarpum* and *T. hirsuta* in sorghum which demonstrated amylolytic properties when examined in this study (§4.2.3). This correlated with the

observation in the diversity of microbes in Chapter 4 which revealed mostly similar species in the maize fermentation and slightly different species in the sorghum thus the recovery of the same level of starch in the maize and different levels in the sorghum. Overall both grains showed good retention of starch making both *ogi* good sources *ogi* energy.

Although some species of LAB are known for phytase activity (*L. plantarum*, *L. acidophilus*, *L. casei* and *L. rhamnosus* (Khodaii *et al*., 2013) and esterase activity (in *L. buchneri*, *L. hilgardii* and *Pediococcus pentosaceus*) (Matthews *et al*., 2007) no record was found in the literature on the activity of other group of microbes (such as *Pantoea*, *Kytococcus*, *Enterobacter*, *Bacillus*, *Aeribacillus, Acinetobacter, Cyanobacterium, Amycolatopsis, Methanoculleus,* yeasts of the *Hyphopichia* and *Pichia* genera) identified in both maize and sorghum fermentations in this study. Interestingly *Bifidobacterium* detected in maize *ogi* and *Enterococcus* sp (although not acceptable in food fermentation) in sorghum *ogi* have been reported to show high phytase activity (Marounek *et al*., 2009, Khodaii *et al*., 2013). Apparently moulds of the genera *Aspergillus*, *Penicillum*, *Cladosporum,* and yeasts *Hyphopichia* and *Pichia* dominated in both maize fermentations (§4.2.1.1.3). These fungal species are similar to those previously isolated from maize grain and characterized for amylase, cellulase, protease and esterase activities (Abe *et al*., 2015). *Enterococcus* species and yeasts were found predominant in the sorghum fermentations. However, sorghum showed higher recovery of soluble phenolics than the maize which may be attributed to the synergistic activity of microbes in the sorghum fermentation as reported for LAB species in cheese (Kongo *et al*., 2007). Thus the presence of *Enterococcus*, *Pediococcus* and *Lactobacillus* in the sorghum fermentations may have contributed to the higher esterase activity and release of soluble phenolics. It is also notable that the microbial population may not have been causal in the reduced enzyme activity in maize given the similarity in the cell counts in both grain fermentations.

There was evidence of additional release of phenolics apart from the leached fraction in both grain fermentations as measured by the level of soluble phenolics recovered

from the original grains and the *ogi*. However, the lower recovery of soluble phenolics in the maize *ogi* may suggest antagonistic activities due to species differences resulting in reduced production and activity of the esterases. Again due to the specificity of these enzymes the phenolic acid matrix of the maize could also contribute to inefficient enzyme activity. However despite the high recovery of phenolics in sorghum fermentations, higher retention of the soluble fraction was observed in the maize *ogi* than in the sorghum. Loss in the soluble phenolics correlated with the high leaching effect observed in sorghum. This effect may have resulted from the differences in the starch matrix of both grains. Since increase in the leaching increased with the time of fermentation, reducing the fermentation time may improve the retention of soluble phenolics in *ogi*. Overall the retention of higher levels of soluble phenolics in maize *ogi* than in the sorghum *ogi* means higher antioxidant properties in the maize.

Phytate was detected at varying levels in maize and sorghum grains. Apparently the levels of phytate in the grains showed a regional effect. In contrast to phenolics the microbial population diversity (predominantly consisting of the mould species of *Aspergillus* and *Penicillum* in M1 and M2) showed positive correlation to phytase activity in the maize fermentations. This effect was also true for S1. As observed with phytate in a previous study, maize fermentation showed significant phytate reduction than sorghum fermentation (Lestienne *et al.*, 2005) but with higher amounts leached into the steep water than in the sorghum. Overall the maize phytate matrix in addition to the microbial activity may have contributed to the lower recovery in the *ogi*. Thus lower phytate in the resultant maize *ogi* means greater bioavailability of nutrient than in the sorghum *ogi.* Food processing methods such as such as fermentation has been reported to reduce phytate and increase the bioavailabilty of zinc (Afify *et al*., 2011).

### 7.3 Starter culture fermentation

The application of *P. pentosaceus* in *ogi* fermentation as a single and mixed starter culture with *Acremonium zeae* and *Trametes hirsuta* was investigated for the first time

in this study. In the first instance it was demonstrated that sterilization of grains by autoclaving prior to fermentation with the starter cultures while being effective in getting rid of the unwanted microbes also solubilized phenolics making it more accessible in both grains as demonstrated by the ratio of the recovery of the bound phenolics to the soluble fraction. This was also true of the phytate in the grains. Apart from the removal of microbes this approach was employed to be able to know whether microbial esterases and phytases showed significant impact on the hydrolysis of phenolics and phytate since heat treatment is known to inactivate endogenous esterases and phytases (Blaabjerg *et al*., 2010).

The starter culture fermentations in all cases showed slightly higher pH levels than observed with natural fermentation. However sorghum fermentations showed lower values than the maize. The pH levels in the pure starter culture fermentations appeared similar to the co-culture fermentation suggesting that there may not be differences in acid level of the fermentations. The cell populations in all starter culture fermentations varied and reached the average maximum of log 6 - 9 cfu/ml. The differences in the viable cell populations influenced the release of phenolics in all starter fermentations. Overall in the maize, the total phenolic content values obtained from direct saponification were near 100% and did not show any increase from the TPC reactive compounds. Fermentation decreased the bound phenolics in all cases and these were lower than in the control indicating esterase activity. Change in the distribution of soluble phenolics was mostly clear in only *P. pentosaceus* (C1) and *T. hirsuta* (C2) pure culture fermentations which showed higher values than in the control (Fig. 6.7). Only *ogi* from *P. pentosaceus* (C1)*, P. pentosaceus* and *A. zeae* (C4) and *A. zeae* alone (C5) fermentations showed similar levels of TPC in *ogi* when compared to the naturally fermented *ogi*. However in all cases the recovery of soluble phenolics in *ogi* as a percentage of the total soluble fraction in the grains was higher in the starter culture fermentations than in the natural fermentation. Thus the use of these microbes as starter cultures in *ogi* fermentation showed significant increase on the recovery of soluble phenolics in maize.

In the sorghum the TPC values were slightly above 100% but seemed not to have been impacted by TPC reactive compounds given the 95% recovered from the control. There were also changes in the distribution of the bound phenolic in the sorghum *ogi*. But in contrast with the maize except in *T. hirsuta* (C2), *P. pentosaceus* with *T. zeae* (C3) and *P. pentosaceus* with *A. zeae* (C4) fermented *ogi* there was no reduction in the bound phenolics based on the percentage recoveries, given the higher values obtained in the culture fermentations than in the control. Soluble phenolics also showed some changes in the distribution following the use of the starter cultures. The increase in the soluble phenolics observed in all cases than in the control except in *A. zeae* (C5) implies esterase activity as reflected in the values of the bound fractions.

Overall based on the TPC only C1, C4 and C5 showed values near the percentage amount recovered in the naturally fermented sorghum *ogi*. However based on recovery from the total soluble fraction in sorghum, only C1 and C2 *ogi* showed significant increase in soluble phenolics than in the naturally fermented *ogi*. Thus the use of *P. pentosaceus* and *T. hirsuta* in pure culture fermentations of *ogi* significantly increased the soluble phenolic content in sorghum *ogi.*

This is the first observation on phenolic acid hydrolysis in *ogi* thus further studies using other known acid producing LAB species such as *Lactobacillus plantarum* starter cultures with these moulds would be useful especially in sorghum, since pH is one of the contributing factors to the release of phenolics. This approach may help in further reduction of the pH and improvement of the phenolic content in *ogi*. Again monitoring the pH of such fermentation would be necessary as very high acid conditions may result to breakdown of soluble phenolics. It is also notable that the assessment of phenolics in *ogi* is associated with experimental problems and the results obtained in this study whilst being suitable for comparative purposes may not be definitive. For this reason more detailed analysis on identification and quantification of individual phenolics would be necessary. This may seem difficult but could be achieved by fractionating the extracts into an organic solvent such as ethyl acetate and analysing the samples by HPLC. The use of phenolic standards to calibrate the HPLC and to evaluate recovery during extraction would enhance the accurate measurements of

phenolics present in the *ogi*. Ferulic and p-coumaric acids may be assessed as these are the major phenolic acids in the grains (Hahn *et al*., 1983; Zhou *et al*., 2004; Mattila *et al*., 2005; Holtekjolen *et al*., 2006). Again since antioxidant activity is of nutritional importance and is also difficult to assess experimentally, the use of other antioxidant assays such as the ferric reducing antioxidant potential (FRAP) to monitor the fermentation may be useful.

In addition to being an anti-nutrient, the chelating property of phytate also confers health benefits to the gastrointestinal tract and other internal organs (Reale *et al*., 2007). The presence of free phosphorus than phytate result to nutrient bioavailability and good health. However a threshold of 25 mg/ 100g or food containing 0.035% of phytate is recommended (Onomi and Katayama, 2004). This study has demonstrated that the use of starter cultures of LAB and fungi reduced the phytate levels in *ogi* by up to 90% in both maize and sorghum. However the levels of phytate recovered in the naturally fermented *ogi* gave higher values than are recommended at 0.07 g/100g (70 mg/100g) and 0.12-0.26 g/100g (120-260 mg/100g) in maize and sorghum respectively. Interestingly *T. hirsuta* fermentations showed reductions in maize *ogi* phytate levels lower than the threshold value at 0.02 g/100g (equivalent to 20 mg/100g) while *A. zeae* gave a fairly similar value at 0.04 g/100g (equating to 40 mg/100g).

Fermentation with a pure culture of *T. hirsuta* showed the highest reduction of phytate (97%) in maize *ogi*. However leaching of phytate was apparent in all fermentations owing to its high solubility in water (Lestienne *et al*., 2005). Thus given the amount of phytate leached into the control steep water, only about 8–48% of phytate reduction in maize fermentations seemed attributable to microbial hydrolysis. Thus pure cultures of *T. hirsuta* and co-culture of *P. pentosaceus* with *T. hirsuta* showed the highest phytate hydrolysis at 48% and 43% respectively. Interestingly the use of starter culture in maize fermentation showed higher phytate reduction in *ogi* made with a pure culture of *T. hirsuta* (C2) and then the co-culture of *P. pentosaceus* with *A. zeae* (C4) than observed in the natural fermentation. It is notable that the pure culture of *T. hirsuta*

showed greater activity than *A. zeae* in the maize fermentation contrary to the observation when tested in the media broth (§4.2.2.2.1).

Similarly in the sorghum *ogi A. zeae* in pure culture showed the highest phytate reduction at 96% of the original total. Again as a result of leaching only about 17–45% of phytate reduction in sorghum fermentations seemed to have resulted from microbial hydrolysis, with *A. zeae* showing the greatest hydrolysis. Thus phytate hydrolysis in sorghum fermentation by the starter cultures showed lower percentage of phytate reduction than in the natural fermentation which could be attributed to the reduction of phytate in the heat treated grain used in the starter culture fermentations The pure culture of *A. zeae* (C5) showed the greatest phytate reduction followed by pure culture of *T. hirsuta* (C2). This is supported by the greater activity exhibited by *A. zeae* than *T. hirsuta* when tested in broth media as observed in §4.2.2.2.1. Overall the minimal level of phytate reduction in *P. pentosaceus* fermentations agrees with the findings of Reale *et al*. (2007) who reported that LAB are not directly involved in phytate degradation but help to provide the optimum conditions such as lowering the pH for phytate hydrolysis. Thus both fungal isolates have great potential of improving the bioavailability of nutrients in *ogi* and should be used with other well-known acid-producing LAB species.

### 7.4 Organic acids and volatile aroma compounds of *ogi*

Organic acids contribute to taste, flavour and shelf-life of *ogi*. Lactic, acetic and malic acids were investigated since these are mostly associated with cereal fermentations and are mostly produced by the LAB and filamentous fungi (Magnuson and Lasure, 2004). These acids were found at minimal levels in all the fermentations. This corresponds to the fairly acid conditions observed even with the homofermentative LAB species (*Enterococcus* and *Pediococcus* spp.) present in both maize and sorghum fermentations. These LAB species seemed to have behaved more like the heterofermentative species and only showed minimal growth at pH 3 for 3h (Figure 4.5). Nevertheless lactic and acetic acids were more abundant while malic acid was the acid produced least in all fermentations. The presence of acetic acid in *ogi*

fermentation was obviously not from acetic acid bacteria (AAB) as these were not isolated. Therefore apart from generation of this acid through alternative pathways such as the glycerol pathway and metabolism of citrate through Krebs cycle, microbial conversion of lactic acid to acetate (Oude-Elferink *et al*., 2001) and enzyme mediated degradation of hemicellulose into sugars and acetate (Pérez *et al*., 2002) could form other possible sources. Although this study was based only on the natural fermentation of *ogi*, acetic acid was also detected in the starter culture fermented *ogi* by the headspace analysis by solid-phase microextraction and gas chromatography-mass spectrophotometry (SPME GC-MS), thus supporting the possible sources of this acid without the presence AAB. Acetic acid may be causal to the unique sour taste of *ogi* owing to its production during the fermentations.

Analysis of volatile organic compounds (VOCs) by solid-phase microextraction and gas chromatography-mass spectrophotometry (SPME GC-MS) demonstrated to be an effective technique for analysis of volatiles in *ogi* having detected and shown good discrimination of volatile compound are significant in *ogi*. Esters, alcohols, aldehydes, organic acids and only a few ketones were generated in both natural and starter culture fermentations of *ogi*. Microbial population diversity of the different fermentations significantly influenced the generation of the volatile organic compounds. Since the fermentation of *ogi* is not strictly anaerobic, some of the compounds may have been formed through oxidation (Yung *et al*., 1998). Overall the key aroma active compounds in both maize and sorghum *ogi* were ethyl acetate, butyl acetate and ethyl hexanoate. The ester, methyl thiobutanoate was found to be unique in the naturally fermented *ogi* but not in the starter fermented products. Thus this compound, although generally characteristic of the fungi, is most likely not produced by any of the species used as starter culture in this study. The presence of this compound in the naturally fermented *ogi* results in a sulphurous, musty, onion, fruity, tropical green notes (Tgsc, 2015) and may be significant in distinguishing between the aroma notes of the non-starter and starter culture *ogi*. Availability of similar compounds in both maize and sorghum starter culture fermentations means similar metabolic activities in both fermentations. However variations in the relative

abundance of the volatile compounds gave a clear indication of dependence on the substrate matrix and availability to the microbes. Thus the higher relative abundance of these flavour volatiles in maize may mean more substrate availability than in the sorghum. This was also notable in the natural fermentations of both grains. However this may present significant and distinguishing effects in both grain fermentations only with compounds having high threshold values such as the alcohols. Butanoic acid ethyl ester found only in all the starter culture fermented sorghum *ogi* may be significant in the aroma characteristics of the *ogi* given its unique fruity and pineapple aroma.

Overall this study has demonstrated that the dominance of LAB and fungal species as seen in the natural fermentation contribute to the generation of the volatile organic compounds in *ogi*. This is further supported by the fact that *P. pentosaceus* and *T. hirsuta* in pure and in co-culture fermentations produced *ogi* with aroma notes mostly related to the naturally fermented product as found on the PCA plot.

This is the first study on *ogi* and was based on a comparative analysis of the samples based on the relative abundance of the compounds measured by the size of the peak areas of the signal intensity from the headspace analysis of the volatiles by SPME GC-MS technique. This does not give the actual concentrations of the compound. Thus there is the need for further studies on quantitative analysis using the internal standards of the compounds to know the concentration of each in *ogi*. Again a parallel study combining the population dynamics with the metabolite target analyses in both the natural and starter culture fermentations would be useful in order to better understand the relationship of the successions of the microbes and the metabolites produced in *ogi* over the fermentation period. This approach may be useful in optimization of *ogi* fermentation to achieve a more consistent product.

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## APPENDICES

**Appendix 4.1: Multiple sequence alignment of *Micrococci* specie isolated from maize.**

| GenBank accession number | Nucleotide sequence | Nucleotide length | Strain ID |
|---|---|---|---|
| FJ229461.1 | -GGCGCAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTC | 59 | M211 |
| FJ229461.1 | --GCGCAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTC | 58 | M213 |
| FJ229461.1 | GGGCGCAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTC | 60 | M212 |
| EU134634.1 | ----------TGCAGCCACGCCGCGTGAATGATGACGGCCTTCGGGTTGTAAA---- | 43 | M214 |
| | ****** ********** ********** | | |
| FJ229461.1 | TTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGT | 119 | M211 |
| FJ229461.1 | TTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGT | 118 | M213 |
| FJ229461.1 | TTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGT | 120 | M212 |
| EU134634.1 | ----------GTAC---------- | | M214 |
| | **** | | |
| FJ229461.1 | GCCAGCAGCCGCGGTAATTCTTC---- | 142 | M211 |
| FJ229461.1 | GCCAGCAGCCGCGGTAAAC-------- | 137 | M213 |
| FJ229461.1 | GCCAGCAGCCGCGGTAATAATACATTA | 147 | M212 |
| EU134634.1 | ---------- | | M214 |

A sequence alignment, produced by ClustalW2, of four nucleotide sequences identified on the left by GenBank accession number.
**Key:** "*": Identical/conserved region. "---": indels (insertion/deletion mutation), "Gap/ mismatches": point mutations

**Appendix 4.2a: Carbohydrate fermentation pattern of MRS isolates from *ogi***

| | Sugar fermentation test (MRS isolates) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Isolates** | Glucose | Fructose | Lactose | Mannitol | Sucrose | Galactose | Mannose | Sorbose | sorbitol | Inositol | Cellobiose | Melibiose | maltose | Raffinose | Rhamnose |
| MO131 | + | + | w+ | w+ | + | w+ | + | - | ww+ | - | + | w+ | + | w+ | - |
| MO132 | + | + | ww+ | w+ | + | w+ | + | - | ww+ | - | + | ww+ | + | ww+ | - |
| MO133* | + | + | w+ | + | + | + | + | + | + | w+ | + | - | + | + | - |
| MO134* | + | + | w+ | w+ | + | ww+ | + | - | ww+ | + | + | + | + | - | - |
| MO135 | + | + | w+ | ww+ | + | + | + | - | ww+ | - | + | + | + | ww+ | - |
| MO231 | + | + | - | - | w+ | - | + | - | - | - | - | - | - | - | - |
| MO232 | + | + | - | - | - | - | + | - | - | - | - | + | - | - | - |
| MO233 | + | + | + | w+ | + | + | w+ | - | w+ | - | + | + | + | + | - |
| MO236 | + | + | + | - | + | + | w+ | - | w+ | - | + | - | + | w+ | - |
| MO237 | + | + | - | ww+ | - | + | + | - | ww+ | - | + | - | + | ww+ | - |
| MO238 | - | - | - | - | - | - | + | - | + | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | |
| SO131 | + | + | w+ | ww+ | + | + | + | - | w+ | ww+ | + | + | + | ww+ | - |
| SO134 | + | + | w+ | w+ | + | w+ | + | - | ww+ | - | + | + | + | ww+ | w+ |
| SO135 | + | + | - | - | + | w+ | + | - | - | - | + | - | + | ww+ | - |
| SO136 | + | + | w+ | w+ | + | - | + | - | - | - | w+ | - | + | w+ | - |
| SO137 | + | + | ww+ | - | + | w+ | + | - | ww+ | - | + | w+ | + | ww+ | - |
| SO231 | + | + | + | - | + | + | + | - | - | - | + | - | + | - | - |
| SO232 | w+ | w+ | - | - | ww+ | ww+ | - | - | - | - | - | - | - | - | - |
| SO233 | + | + | + | - | + | + | + | - | - | - | + | + | + | ww+ | - |

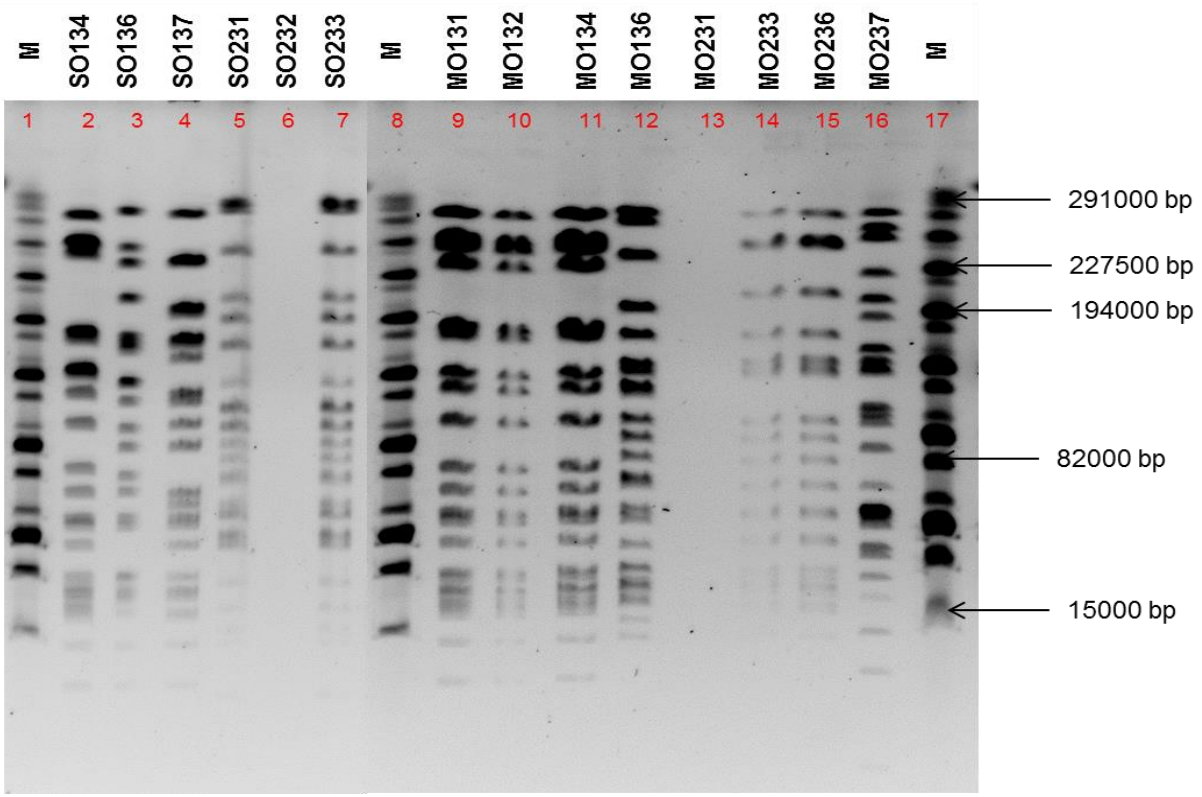
- Gas production

**Appendix 4.2b: Carbohydrate fermentation pattern of M17 isolates from *ogi***

| Isolates | Glucose | Fructose | Lactose | Mannitol | Sucrose | Galactose | Mannose | Sorbose | Sorbitol | Inositol | Cellobiose | Melibiose | Maltose | Raffinose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MO111* | + | + | + | + | + | + | - | + | + | + | + | + | + | + |
| MO112* | + | + | + | + | + | + | + | + | + | w+ | + | + | + | + |
| MO113 | + | + | w+ | - | w+ | w+ | + | - | - | - | + | - | + | - |
| MO114* | + | + | w+ | + | + | + | + | + | + | + | w+ | + | + | + |
| MO115 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MO116* | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MO117 | + | + | + | - | + | + | + | - | - | - | + | w+ | + | - |
| MO118* | + | + | w+ | + | + | + | + | + | + | + | + | - | + | + |
| MO119* | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MO211* | + | + | w+ | + | + | + | + | + | + | + | + | - | + | + |
| MO212 | + | + | + | w+ | + | + | + | - | ww+ | - | + | + | + | w+ |
| MO213 | - | - | - | - | - | - | - | - | - | - | - | w+ | w+ | - |
| MO214 | + | + | + | - | + | + | + | - | - | - | + | + | + | + |
| MO215 | + | + | + | - | + | + | + | - | - | - | + | w+ | w+ | w+ |
| MO216 | + | + | + | - | + | w+ | + | - | - | - | + | w+ | + | w+ |
| SO111 | w+ | w+ | w+ | - | w+ | w+ | ww+ | - | ww+ | - | w+ | - | + | ww+ |
| SO112* | + | + | + | + | + | + | + | + | + | + | + | + | + | ww+ |
| SO113 | + | + | w+ | - | + | + | + | - | ww+ | - | + | - | + | ww+ |
| SO114 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SO115 | w+ | w+ | - | - | - | - | w+ | w+ | - | - | - | - | w+ | - |
| SO116 | - | - | - | - | - | - | - | - | - | - | - | - | ww+ | + |
| SO117* | + | + | w+ | + | + | + | + | + | + | w+ | + | + | + | + |
| SO118 | + | + | w+ | - | + | + | + | - | ww+ | - | + | + | + | ww+ |
| SO119 | ww+ | ww+ | ww+ | - | - | w+ | ww+ | - | - | - | - | w+ | - | w+ |
| SO211* | + | + | + | + | + | + | + | + | + | ww+ | + | + | + | + |
| SO212* | + | + | + | + | + | w+ | + | + | + | + | w+ | + | + | + |
| SO213 | + | + | - | - | + | - | - | - | ww+ | - | + | - | + | - |
| SO214 | w+ | w+ | w+ | - | - | - | + | w+ | - | ww+ | + | - | ww+ | - |
| SO215* | + | + | + | + | + | + | + | ww+ | + | + | + | + | + | + |
| SO216 | w+ | w+ | w+ | - | - | + | - | w+ | - | - | - | w+ | - | - |
| SO217 | w+ | w+ | w+ | - | - | - | + | + | - | ww+ | - | - | - | w+ |
| SO218* | + | + | w+ | + | + | + | + | - | + | ww+ | - | + | + | + |
| SO219 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

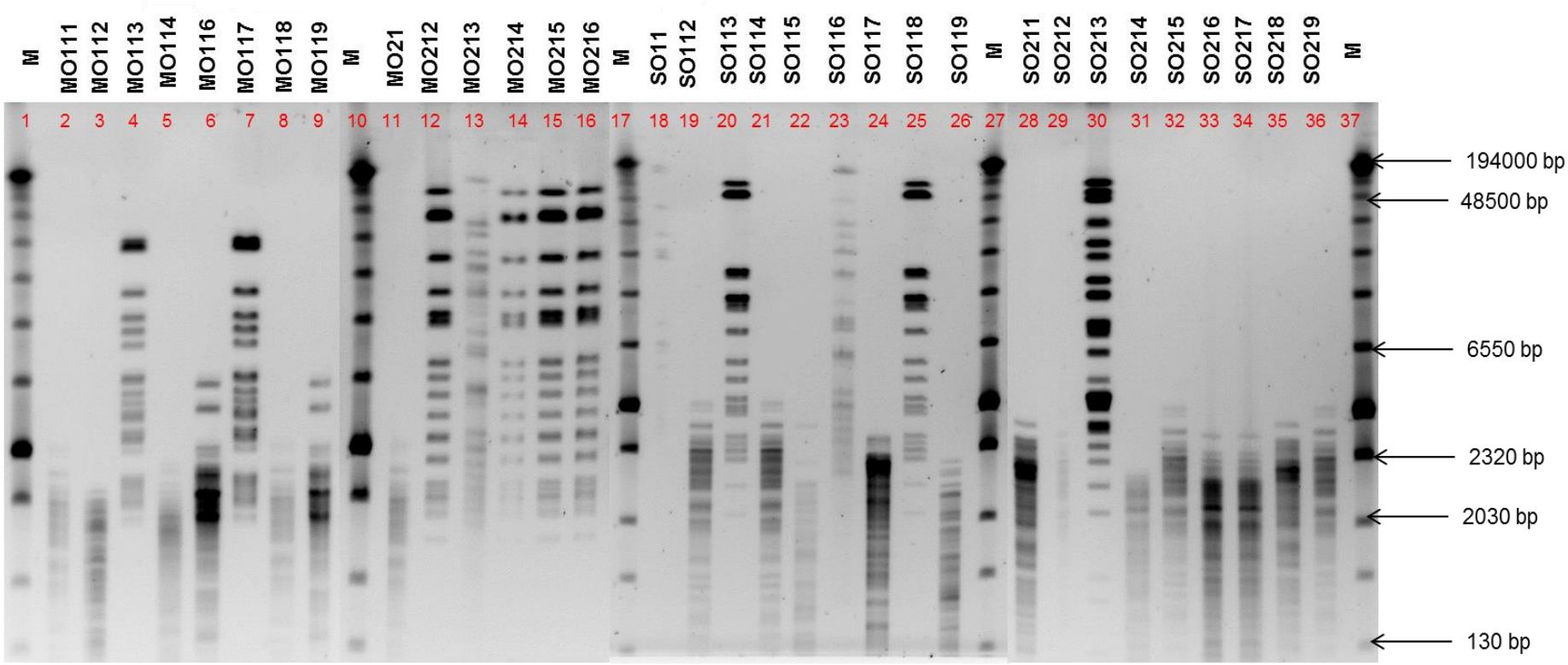
*****Gas production

**Appendix 4.3a: PFGE pulsotypes of SmaI digested genomic DNA of MRS isolates from maize and sorghum *ogi.***




Switch time ramp was 1-20s for 17 h and voltage 6V/cm. M= mid-range marker, MO= isolates from maize *ogi.* SO= isolates from sorghum *ogi.*

**Appendix 4.3b: PFGE pulsotypes of SmaI digested genomic DNA of M17 isolates from maize and sorghum *ogi.***




Switch time ramp was 1-20s for 17 h and voltage 6V/cm. M= Low-range marker, MO= isolates from maize *ogi.* SO= isolates from sorghum *ogi*

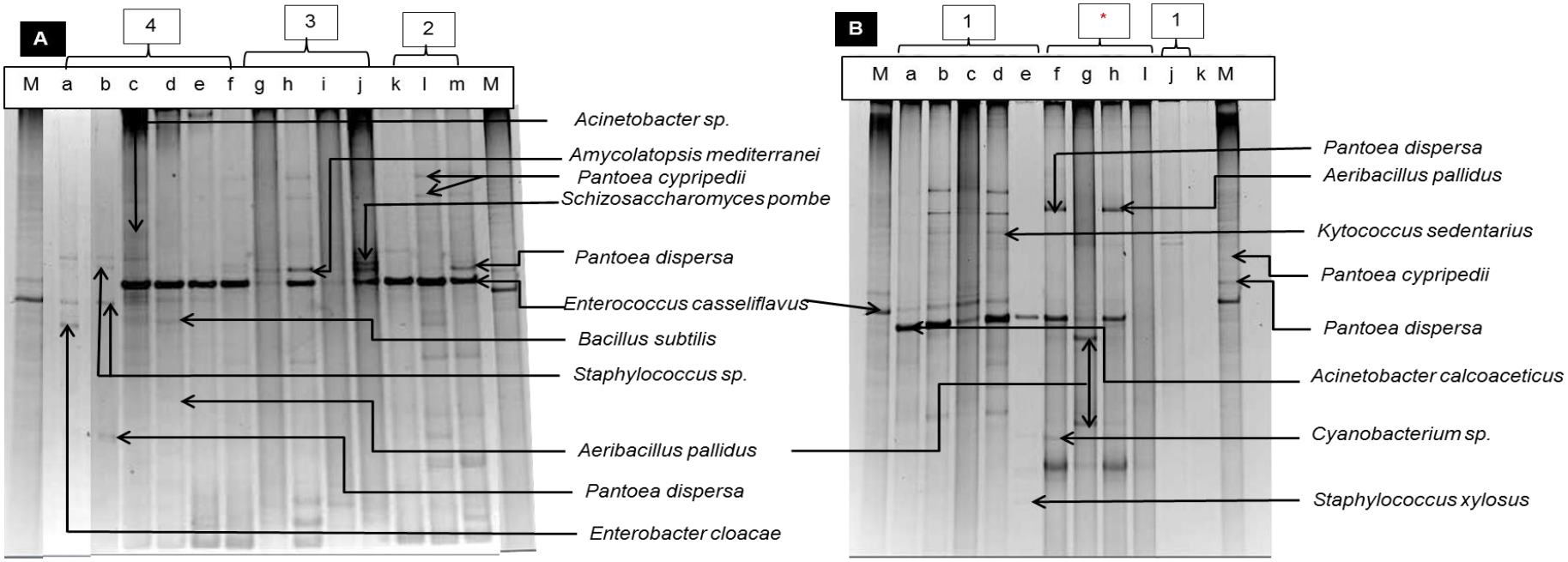
**Appendix 4.4: Summary of AMOVA for testing the significance of ERIC PCR clustering profile of acetic acid bacteria**

| Source of variation | df | SS | MS | Est. Var. | % |
|---|---|---|---|---|---|
| **Among Pops** | 5 | 629.084 | 125.817 | 15.647 | 42% |
| **Within Pops** | 40 | 879.996 | 22.000 | 22.000 | 58% |
| **Total** | 45 | 1509.080 | | 37.647 | 100% |

| Stat | Value | P(rand >= data) |
|---|---|---|
| **PhiPT** | 0.416 | 0.001 |

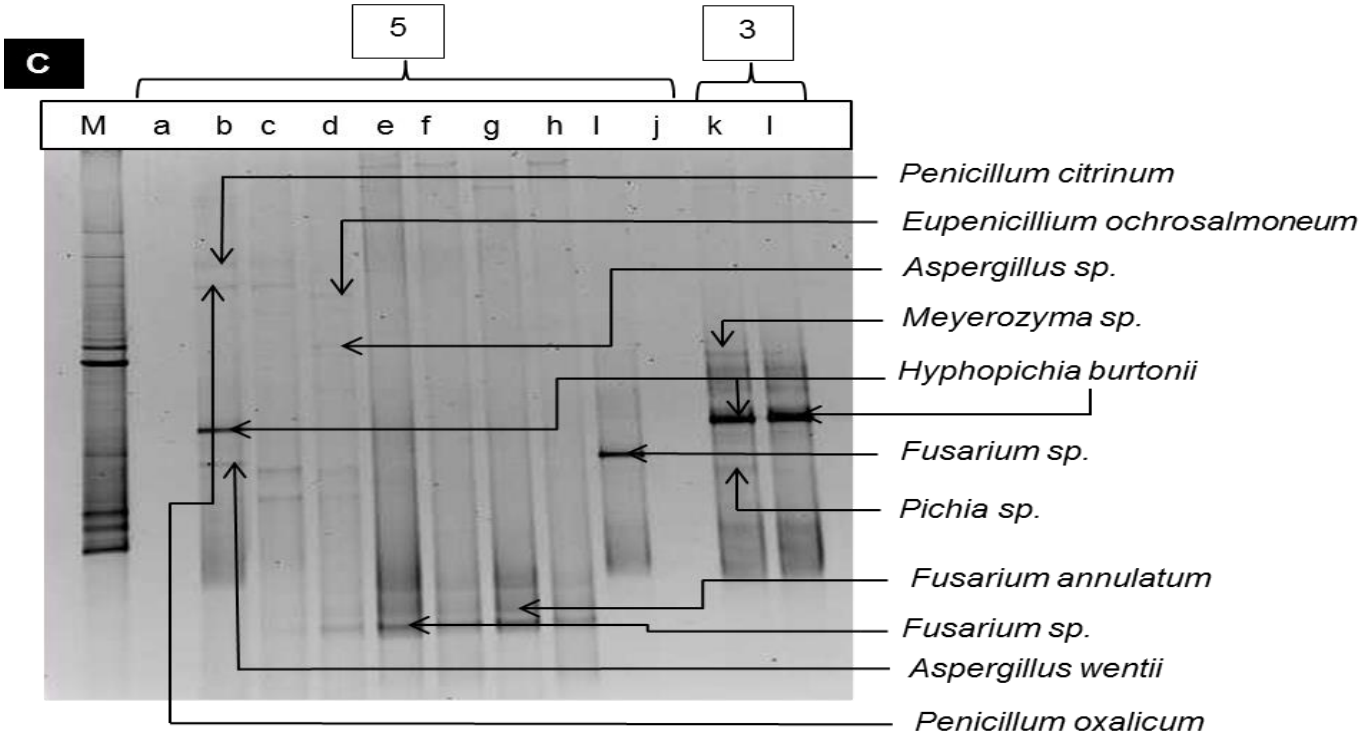
Probability, P (rand >= data), for PhiPT is based on standard permutation across the full data set.

**Appendix 4.5a: DGGE profile of the bacterial community in *ogi* grains by bulk cells and *direct DNA extraction methods.**



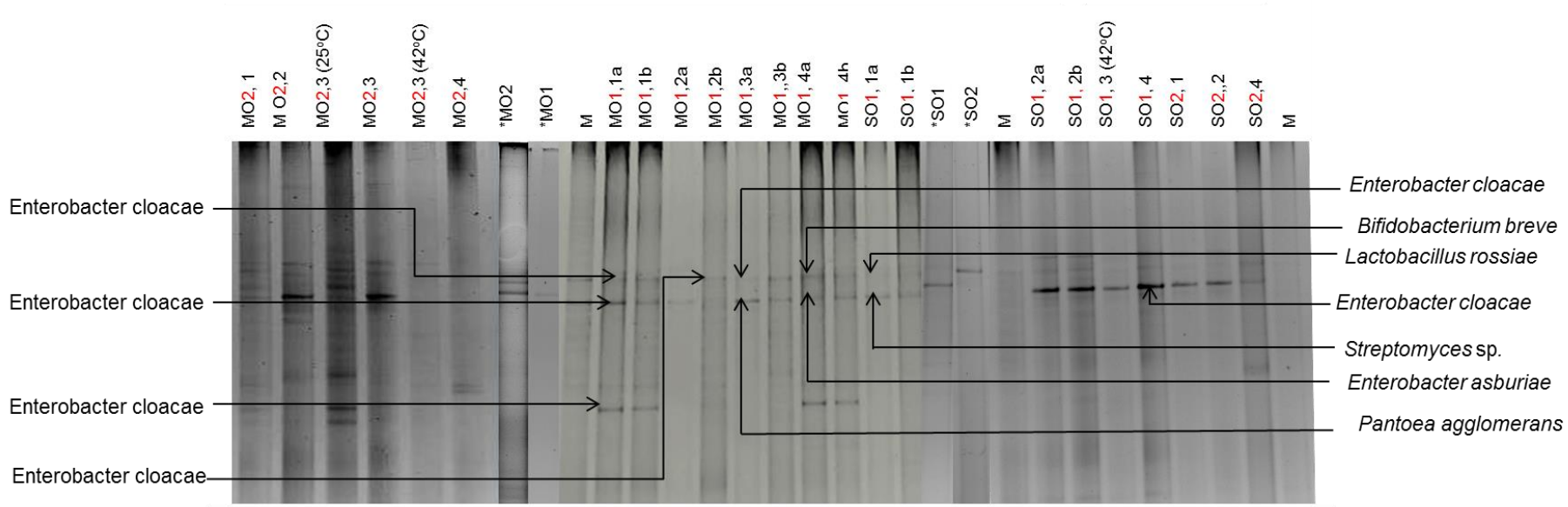

PCR amplicons were analysed on 8% acrylamide gel with denaturing gradient range of 20-60% at 50V for 17h. Numbers on image and in parenthesis represent the culture media**:** 1 = M17 agar; 2= MacConkey agar; 3 = MRS agar; 4 = Nutrient agar; 5 = RBCA. Letters on the lanes represent the DNA samples from two different sources of grains: 1 northern and 2 eastern consisting of maize (M1, M2) and sorghum (S1, S2). **Gel A:** M = strain marker, a = S2 (4), b = M2 (4); c = S1 (4); d=M1 (4), e = S1 (3), 37 $^{o}$C; f = S2 (3),42 $^{o}$C; g= S2 (3), 37 $^{o}$C; h= M1 (3) ,42 $^{o}$C; i= M1 (3),37 $^{o}$C; j = M2 (3),37 $^{o}$C; k= S1 (2) ; l = M1 (2) ; m= M2 (2). **Gel B:** M = strain marker; a = S1 (1) 30 $^{o}$C; b = S1 (1), 37 $^{o}$C; c= S2 (1) ,30 $^{o}$C; d = M1 (1), 30 $^{o}$C; e = M2 (1), 37 $^{o}$C; f = S1*; g = S2*; h = M1*; I = M2*; k = negative control.

**Appendix 4.5b: DGGE profile of the fungal community in *ogi* grains (bulk cell).**



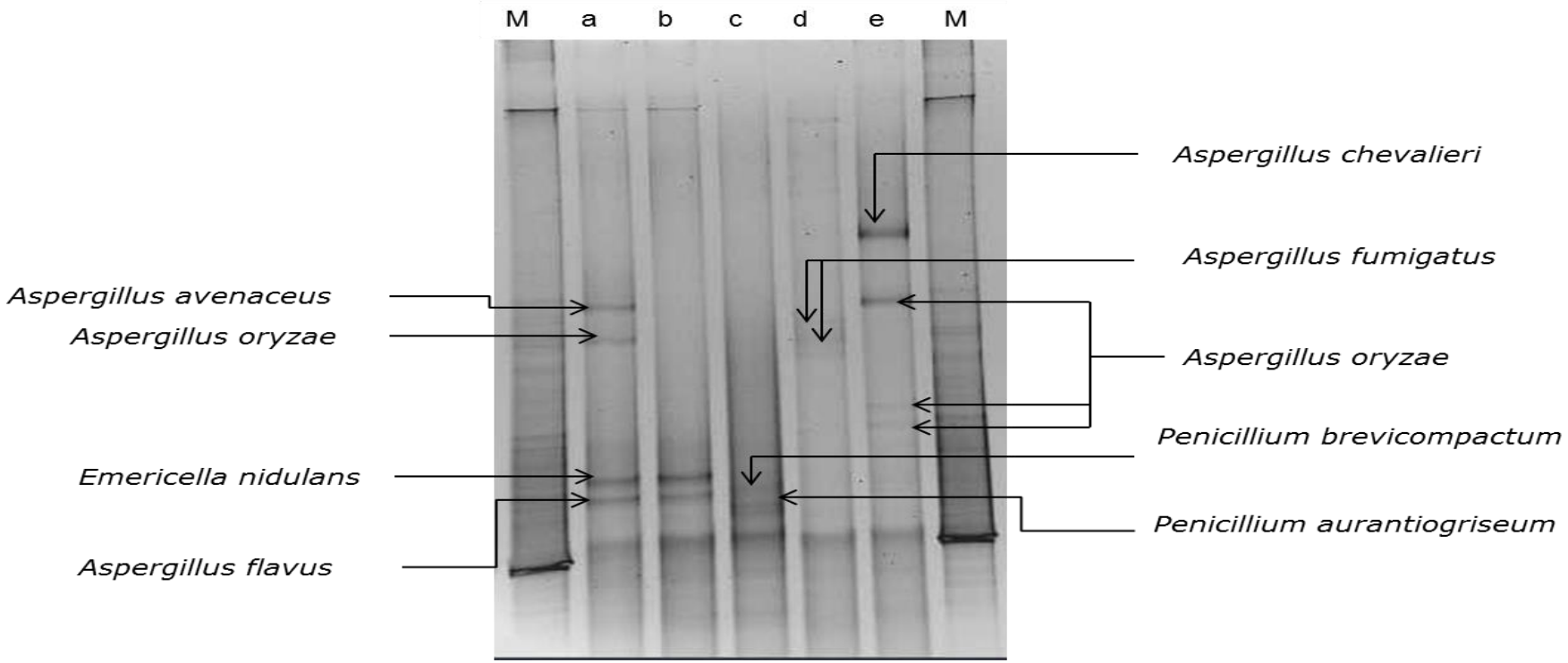

PCR amplicons were analysed on 8% acrylamide gel with denaturing gradient range of 20-60% at 170V for 4h.
**C:** M = strain marker; a - b = M1 (5); c – d = M1 (5); e - h = S2 (5); I - j = S1 (5); k = M1 (3); l = M2 (3).
Letters on the lanes represent the DNA samples from two different sources of grains and *ogi*:
1 northern and 2 eastern consisting of maize (M1, M2) and sorghum (S1).

**Appendix 4.5c: PCR-DGGE bulk cell analysis of the bacterial community in maize and sorghum *ogi***




MO=maize *ogi* , SO= sorghum *ogi,* M=strain marker; * Direct DNA extraction. Red numbers represent two different sources of grains 1: northern, 2: eastern; Black numbers represent 1: M17 agar, 2: MacConkey agar, 3: MRS agar, and 4: Nutrient agar; a,b: duplicate samples of genomic DNA directly extracted from the samples were amplified with U968 F-GC and L140R primers spanning the V6-V8 regions of 16S rRNA bacterial genes PCR products were analysed on 8% acrylamide concentration ranging from 20-60% at 50V for 17h.

**Appendix 4.5d: PCR-DGGE bulk cell analysis of the fungal community in maize and sorghum *ogi***




Duplicate sample (a, b) PCR amplicons of genomic DNA extracted from cultivated cells were amplified with NL1F-GC and LS2R primers spanning D1 regions of 26S rRNA genes were analysed on 8% acrylamide gel with denaturing gradient range of 20-60% at 170V for 4h. **C:** M = strain marker; a= MO1, 5, b= MO2, 5, c= SO1, 5, d=M1, 5, e= M2, 5. Letters on the lanes represent the DNA samples from two different sources of grains: 1 northern and 2 eastern consisting of maize (M1, M2) and sorghum (S1): S2 (not analysed due to low DNA concentration)**.**

**Appendix 4.6a: Acid tolerance of LAB isolates at pH 3**

| Isolates | Absorbance (600 nm) | | | | | |
|---|---|---|---|---|---|---|
| | 2h | | 3h | | 24h | |
| | (Control) | (Test) | (Control) | (Test) | (Control) | (Test) |
| MO131 | 0.125 | 0.014 | 0.228 | 0.155 | 1.643 | 0.031 |
| MO132 | 0.130 | 0.010 | 0.242 | 0.175 | 1.629 | 0.009 |
| MO134 | 0.132 | 0.012 | 0.241 | 0.182 | 1.647 | 0.021 |
| MO233 | 0.130 | 0.009 | 0.307 | 0.175 | 1.606 | 0.018 |
| SO131 | 0.133 | 0.008 | 0.323 | 0.178 | 1.554 | 0.012 |
| SO132 | 0.073 | 0.004 | 0.130 | 0.165 | 1.876 | 0.010 |
| SO134 | 0.128 | 0.008 | 0.311 | 0.175 | 1.666 | 0.001 |
| SO137 | 0.141 | 0.010 | 0.338 | 0.159 | 1.721 | 0.002 |

Isolates were cultivated in MRS broth adjusted to pH 3.0 with concentrated HCl and incubated at 37 $^{o}$C.

**Appendix 4.6b: Acid tolerance of LAB isolates at pH 2**

| | Absorbance (600 nm) | | | | | |
|---|---|---|---|---|---|---|
| | 2h | | 3h | | 24h | |
| Isolates | (Control) | (Test) | (Control) | (Test) | (Control) | (Test) |
| MO131 | 0.125 | 0.014 | 0.228 | 0.014 | 1.643 | 0.030 |
| MO132 | 0.130 | 0.021 | 0.242 | 0.023 | 1.629 | 0.027 |
| MO134 | 0.132 | 0.003 | 0.241 | 0.027 | 1.647 | 0.007 |
| MO233 | 0.130 | 0.011 | 0.307 | 0.005 | 1.606 | 0.009 |
| SO131 | 0.133 | 0.032 | 0.323 | 0.042 | 1.554 | 0.011 |
| SO132 | 0.073 | 0.022 | 0.130 | 0.028 | 1.876 | 0.013 |
| SO134 | 0.128 | 0.021 | 0.311 | 0.022 | 1.666 | 0.012 |
| SO137 | 0.141 | 0.016 | 0.338 | 0.021 | 1.721 | 0.014 |

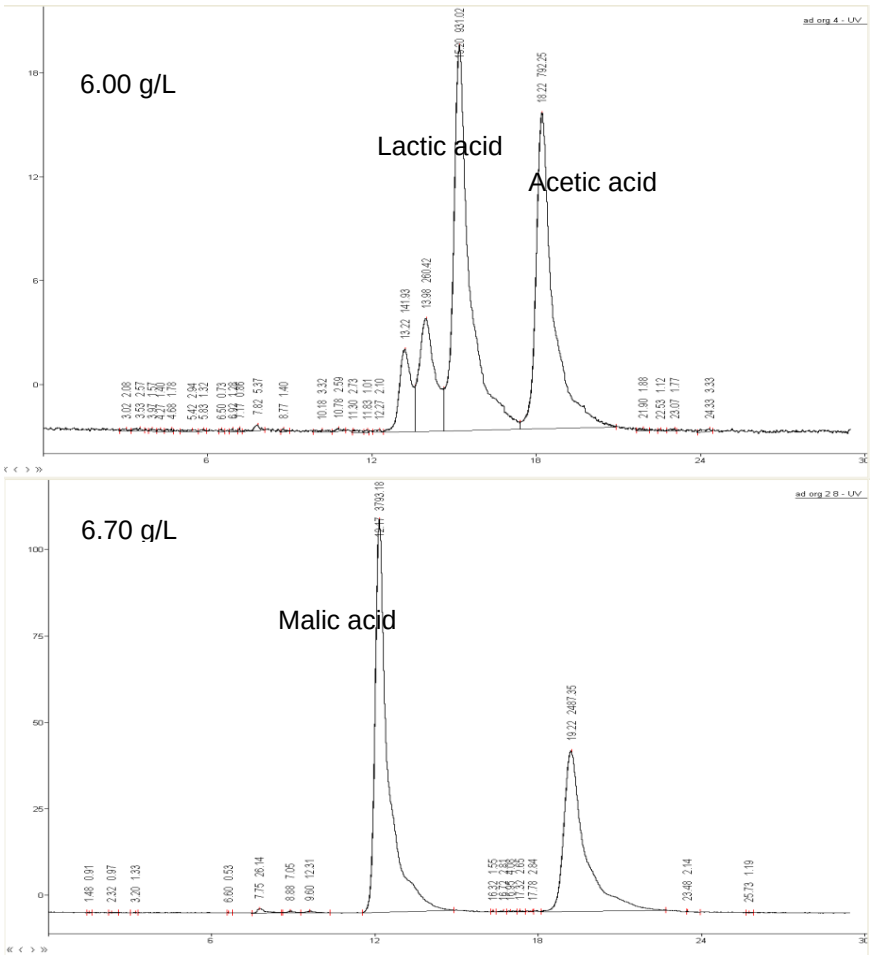
Isolates were cultivated in MRS broth adjusted to pH 2.0 with concentrated HCl and incubated at 37 $^{o}$C.

**Appendix 5.1: Starch and non-starch polysaccharide contents**

| *Samples | Starch (g) | NSP (g) |
|---|---|---|
| M1 | 158.91 (9.53)$^{d}$ | 16.24 (5.72)$^{bc}$ |
| M2 | 162.49 (4.17)$^{d}$ | 21.34 (2.80)$^{b}$ |
| S1 | 183.82 (12.50)$^{e}$ | 14.98 (2.94)$^{c}$ |
| S2 | 171.75 (16.07)$^{de}$ | 17.56 (3.42)$^{bc}$ |
| MO1 | 97.24 (12.00)$^{b}$ | 6.72 (0.52)$^{a}$ |
| MO2 | 103.55 (4.35)$^{bc}$ | 9.10 (1.84)$^{a}$ |
| SO1 | 116.15 (1.60)$^{c}$ | 4.60 (0.51)$^{a}$ |
| SO2 | 87.43 (3.45)$^{b}$ | 4.13 (0.31)$^{a}$ |
| MO1p | 97.24 (2.79)$^{a}$ | 6.72 (0.77)$^{a}$ |
| MO2p | 8.18 (7.39)$^{a}$ | 4.96 (0.51)$^{a}$ |
| SO1p | 17.64 (2.49)$^{a}$ | 4.46 (0.42)$^{a}$ |
| SO2p | 14.21 (7.42)$^{a}$ | 4.18 (0.24)$^{a}$ |

*Two different sources of grains: 1 northern and 2 eastern, M: maize, S: sorghum, O: *ogi*, p: pomace (residue). NSP: Non-starch polysaccharide. Data are means of duplicate determinations and standard deviation in parenthesis. Means with same superscript in the same column are not significantly different (p>0.05).

**Appendix 5.2: HPLC chromatogram of standard mixtures of organic acids**




Retention times (Rt); Lactic acid, 15.20, Acetic acid, 18.20 and Malic acid, 12.20. Separation was on Rezex™ ROA-Organic Acid $H^+$ column of 5 µm particle size and 300 x 7.8 mm dimension (Phenomenex, UK) with 0.005 N $H_2SO_4$ (Fisher, Scientific) as mobile phase at flow rate, 0.5 ml/min and signal detection by a UV detector (VG Lab., UK) at 220 nm wavelength. Injection volume was 20 µl.

**Appendix 6.1 pH measurement of starter culture fermentations**

| Time(h) | 0 | 6 | 12 | 24 | 30 | 36 | 48 | 54 | 60 | 72 |
|---|---|---|---|---|---|---|---|---|---|---|
| C1 M | 7.26 | 6.73 | 6.57 | 5.31 | 5.11 | 5.03 | 5.24 | 5.32 | 5.25 | 5.52 |
| | 7.43 | 6.78 | 6.63 | 5.26 | 5.09 | 5.03 | 5.24 | 5.24 | 5.22 | 4.97 |
| C1 Mc | 7.20 | 6.68 | 6.60 | 5.42 | 5.47 | 5.50 | 5.26 | 5.23 | 5.24 | 5.03 |
| | 7.21 | 7.17 | 7.14 | 7.00 | 6.97 | 6.95 | 6.90 | 6.87 | 6.76 | 6.73 |
| C1 S | 7.61 | 7.02 | 6.89 | 5.33 | 5.14 | 4.98 | 5.20 | 5.09 | 5.13 | 5.44 |
| | 7.62 | 6.96 | 6.86 | 5.34 | 5.26 | 5.16 | 5.09 | 5.03 | 5.03 | 5.46 |
| C1 Sc | 7.23 | 6.91 | 6.82 | 6.56 | 6.59 | 6.52 | 6.40 | 6.39 | 6.27 | 6.20 |
| | 7.21 | 7.21 | 7.20 | 7.15 | 7.14 | 7.12 | 7.10 | 7.09 | 7.01 | 6.89 |
| C2 M | 7.36 | 6.51 | 5.98 | 5.44 | 5.28 | 5.44 | 5.30 | 5.28 | 5.34 | 5.31 |
| | 7.19 | 6.54 | 5.92 | 5.49 | 5.31 | 5.39 | 5.30 | 5.25 | 5.27 | 5.25 |
| C2 Mc | 7.18 | 7.15 | 7.11 | 7.08 | 7.05 | 7.01 | 7.00 | 6.98 | 6.94 | 6.60 |
| | 7.15 | 7.13 | 7.13 | 7.12 | 7.11 | 7.09 | 7.07 | 7.05 | 7.01 | 7.00 |
| C2 S | 7.50 | 6.92 | 6.28 | 5.44 | 5.36 | 5.34 | 5.26 | 5.20 | 5.27 | 5.18 |
| | 7.51 | 7.04 | 6.33 | 5.47 | 5.40 | 5.38 | 5.30 | 5.24 | 5.20 | 4.78 |
| C2 Sc | 7.19 | 7.17 | 7.13 | 7.08 | 7.04 | 7.00 | 6.97 | 6.96 | 6.90 | 6.50 |
| | 7.20 | 7.19 | 7.17 | 7.14 | 7.12 | 7.10 | 7.03 | 7.00 | 6.97 | 6.88 |
| C3 M | 7.49 | 6.83 | 6.46 | 4.98 | 4.87 | 4.92 | 5.25 | 5.21 | 5.17 | 5.18 |
| | 7.47 | 6.90 | 6.53 | 5.05 | 4.94 | 4.97 | 5.17 | 5.15 | 5.12 | 5.08 |
| C3 Mc | 7.27 | 7.25 | 7.20 | 7.17 | 7.15 | 7.10 | 7.08 | 7.08 | 6.98 | 6.40 |
| | 7.26 | 7.24 | 7.24 | 7.20 | 7.16 | 7.13 | 7.09 | 7.07 | 7.00 | 6.95 |
| C3 S | 7.58 | 6.99 | 6.43 | 5.66 | 5.07 | 5.09 | 5.25 | 5.16 | 5.08 | 4.89 |
| | 7.54 | 7.00 | 6.44 | 5.67 | 5.10 | 5.08 | 5.12 | 5.09 | 5.05 | 4.90 |
| C3 Sc | 7.36 | 7.32 | 7.30 | 7.25 | 7.20 | 7.16 | 7.08 | 7.02 | 6.97 | 6.58 |
| | 7.33 | 7.30 | 7.29 | 7.26 | 7.24 | 7.20 | 7.17 | 7.16 | 7.13 | 7.05 |
| C4 M | 7.60 | 7.02 | 6.00 | 5.50 | 5.47 | 5.42 | 5.41 | 5.47 | 5.46 | 5.46 |
| | 7.57 | 7.09 | 6.58 | 5.78 | 5.72 | 5.55 | 5.50 | 5.48 | 5.48 | 5.42 |
| C4 Mc | 7.61 | 6.97 | 6.98 | 6.95 | 6.94 | 6.94 | 6.93 | 6.92 | 6.90 | 6.90 |
| | 7.60 | 7.05 | 7.02 | 6.98 | 6.96 | 6.95 | 6.91 | 6.90 | 6.90 | 6.89 |
| C4 S | 7.46 | 7.12 | 4.73 | 4.75 | 4.99 | 5.24 | 5.01 | 4.95 | 4.90 | 4.77 |
| | 7.47 | 7.25 | 5.53 | 4.98 | 4.96 | 5.12 | 5.08 | 5.00 | 4.98 | 4.96 |
| C4 Sc | 7.49 | 7.45 | 7.44 | 7.42 | 7.42 | 7.41 | 7.38 | 7.36 | 7.30 | 7.28 |
| | 7.45 | 7.42 | 7.40 | 7.38 | 7.37 | 7.35 | 7.35 | 7.27 | 7.24 | 7.17 |
| C5 M | 7.78 | 7.09 | 6.85 | 6.50 | 6.50 | 6.37 | 5.86 | 5.96 | 5.70 | 5.54 |
| | 7.65 | 7.15 | 6.98 | 6.94 | 6.67 | 6.60 | 5.97 | 5.94 | 5.79 | 5.48 |
| C5 Mc | 7.54 | 7.52 | 7.50 | 7.45 | 7.45 | 7.43 | 7.41 | 7.41 | 7.35 | 7.17 |
| | 7.52 | 7.50 | 7.50 | 7.48 | 7.43 | 7.40 | 7.37 | 7.35 | 7.30 | 7.25 |
| C5 S | 7.60 | 7.56 | 6.95 | 5.75 | 5.29 | 5.23 | 5.22 | 5.13 | 5.08 | 4.97 |
| | 7.55 | 7.52 | 7.12 | 6.41 | 5.43 | 5.32 | 5.29 | 5.20 | 5.05 | 4.94 |
| C5 Sc | 7.51 | 7.47 | 7.45 | 7.45 | 7.40 | 7.38 | 7.35 | 7.30 | 7.14 | 6.98 |
| | 7.48 | 7.45 | 7.43 | 7.41 | 7.36 | 7.35 | 7.28 | 7.26 | 7.18 | 7.00 |