1. INTRODUCTION

1.1. Statement of the problem

In many African countries, sorghum is used for the production of malt and sorghum beer, also known as opaque beer (Novellie and De Schaepdrijver, 1986). It is also used as an ingredient in the preparation of weaning foods in other African countries, a practice that has not found its application yet in South Africa, although its benefits have been recognised by South African researchers (Den Besten, Glatthaar and IjsselmuidenGlatthaar and Ijsselmuiden, 1988; Thaoge, Adams, Sibara, Watson, Taylor and Goyvaerts, 2003). In Southern Africa some 200 000 tons of sorghum is malted commercially annually (Taylor and Dewar, 2001). In South Africa most sorghum malting is by traditional outdoor floor malting, whereby sorghum grain is steeped for about 8 hours, left outdoors to germinate in an uncontrolled environment, without being turned and then dried (Novellie and De Schaepdrijver, 1986).

The germination conditions, especially not turning the grain, encourages entangling of the roots and shoots growing from the grains. This then leads to the formation of matted clumps (hot spots), which encourages the growth of bacteria and fungi (Briggs, 1998). Heavy microbial populations may negatively impact on the quality of the malt by causing discoloration of the malt and development of unacceptable off flavours (Noots, Delcour, Michiels, 1999). Of more concern is the colonization of the sorghum malt with coliforms and moulds because coliforms are associated with diarrhoeal diseases (Jay, 2000) and moulds have the potential of producing mycotoxins, which are toxic to animals and humans (Gourama and Bullerman, 1995a).

The moulds found in sorghum malt are brought in on contaminated grain or can originate as contaminants that develop during the malting process (Briggs, 1998). Common moulds that were shown to occur in sorghum malt samples (65 commercial and 22 industrial) obtained from different South African commercial and industrial maltsters are, *Saccharomyces* spp., *Rhizopus/Mucor* spp., *Aspergillus clavatus Aspergillus flavus, Phoma sorghina, Penicillium* spp., and *Fusarium* spp. (Rabie and Lübben, 1984). South African sorghum malt was found to contain on average 2.18

µg/kg of aflatoxin (Trinder, 1988) and zearalenone has also been detected in it (Odhav and Naicker, 2002). In other African countries, mycotoxins such as aflatoxin B_1 and zearalenone have been shown to occur in home-brewed beer (Alozie, Rotimi and Oyibo, 1980; Sibanda, Marovatsanga and Pestka., 1997).

Consumption of mycotoxins poses a very high health risk as some mycotoxins are very carcinogenic and can therefore lead to cancer of liver, kidney, central nervous system, alimentary canal and the reproductive system (D'Mello and McDonald, 1997). For example in South Africa, a high incidence of oesophageal cancer in the rural Transkei (Eastern Cape province) amongst elderly black men has been linked with the fumonisins found in the traditional maize beer they consume (Marasas, Jaskiewicz, Venter and Van Schalkwyk, 1988).

The high microbial load and the possible presence of mycotoxins in sorghum malt implies that sorghum malt is not a very safe product to be used for beer brewing and for the preparation of weaning foods. In order to avoid potential hazards associated with the high microbial load and the possible presence of mycotoxins in sorghum malts, a method that can prevent or inhibit the growth potentially harmful microorganisms during the sorghum malting process should be implemented.

1.2. Literature review

1.2.1. Sorghum malting

Sorghum (*Sorghum bicolor*) (L). Moench) is a major food crop and is ranked fifth in terms of world cereal grain production after wheat, rice, maize and barley (Doggett, 1988). It is drought-tolerant and thus has an advantage over other cereals because it can yield a crop under harsh environmental conditions (Doggett, 1988). Sorghum has similar nutritional value to maize and is consumed as a major source of energy and protein by millions of people in the semi-arid areas of Africa and Asia (Serna-Saldivar and Rooney, 1995). Much sorghum is malted to brew opaque beer in most parts of Africa, including South Africa and European type beer (e.g., lager) and non-alcoholic malt beverages in several African countries (Taylor and Dewar, 2001). Malting is the limited germination of cereals in moist air, under controlled conditions, with the objective of mobilizing the endogenous hydrolytic enzymes, especially α-amylase of the grain which attacks the α-(1-4) glucosidic bonds in starch molecules (Taylor and Belton, 2002). The malting process also modifies the structure of the grain so that it will be readily solubilised during the brewing process to produce fermentable wort**.**

Another application of sorghum malt is as an ingredient during the preparation of weaning foods, a common practice in the rural communities of some African countries (Mosha and Svanberg, 1990). Protein-energy malnutrition (PEM) and other nutritional deficiencies during infancy are still a problem in developing countries. This is mainly due to the thin liquid gruels (porridges) based on the local staple food, usually a cereal such as maize, millet, sorghum, rice and cassava that are used as weaning porridges (Gopaldas, Deshpande and John, 1988; Mensah, Ndiokwelo, Ugwaegbute, Van Boxtel, Brinkman, Nout and Ngoddy, 1995). The thin gruel may be more easily consumed but its energy density is too low to meet the energy requirements of the young. The addition of a small amount of malt flour, sometimes called Amylase-Rich Flour (ARF) or Power Flour (PF), to the already prepared thick starch-based food could be the solution to the dietary bulk problem (Svanberg and Sandberg, 1988). Small quantities of ARF when added to freshly prepared thick gruels, liquefy them due to the action of amylases, reducing their viscosity without

lowering their nutrient and energy density (Svanberg and Sandberg 1988; Thaoge *et al*., 2003).

Other benefits that result from malting are the reduction of anti-nutritional factors (e.g. phytate), enhancement of the vitamins riboflavin, niacin, pyridoxine and ascorbic acid content (Malleshi and Klopfenstein, 1996), improvement of the minerals Ca, Mg, Zn and P availability (Glennie, Harris and Liebenberg., 1983) and imparting flavour and sweetness to the porridge (Taylor and Dewar, 2001). Malting has also been shown to improve the *in vitro* digestibility of sorghum protein (Bhise, Chavan and Kadam, 1988) and starch (Wang and Fields, 1978), improve the composition and content of essential amino acids (lysine, methionine and tryptophan) (Wang and Fields, 1978; Taylor, 1983). The benefits of sorghum malting and the uses of sorghum malt are summarized in Table 1.1.

Table 1.1. Benefits of sorghum malting and the uses of sorghum malt.

Sorghum malting
1. Results in high levels of amylases
2. Reduces anti-nutritional factors
3. Enhances vitamin content
4. Improves mineral content
5. Improves *in vitro* digestibility of sorghum protein
6. Improves the composition and content of essential amino acids
7. Increases the *in vitro* starch digestibility
The use of sorghum malt
1. Make grains to be readily solubilised during the brewing process
2. Reduces the viscosity of porridges whilst maintaining their nutrient and energy density
3. Imparts flavour and sweetness to porridge

1.2.2. Sorghum malting process

Like barley malting, sorghum malting involves three main steps: steeping, germination and drying/kilning (Novellie and De Schaepdrijver, 1986), as shown in Figure 1.1.

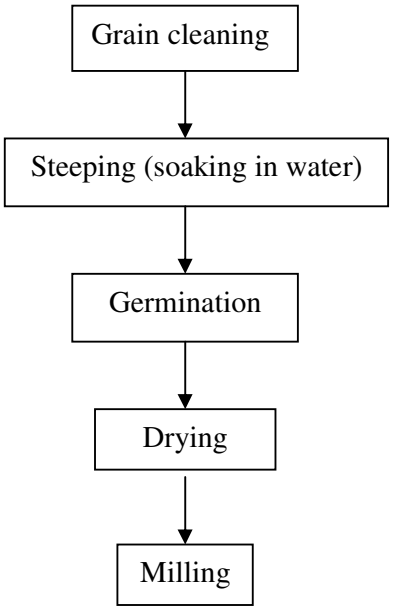


Figure 1.1. Schematic presentation of sorghum malting

1.2.2.1. Steeping

Steeping, the first step of the malting process, is the immersion of the grain in water. It is practised chiefly as a means of achieving imbibition of water by the dormant grain and thereby initiating biochemical processes leading to seed germination (Brookes, Levett and Mcwilliam, 1976; Briggs, 1998). Steeping is also carried out to clean and to remove broken grains. A moisture content of 33 to 35% (wet basis) should be achieved during steeping of sorghum grains (Daiber and Taylor, 1995). The more water that is taken up during steeping (within limits) the higher is the resulting malt quality (Dewar, Orovan and Taylor, 1997b).

Factors associated with the grain that affect the rate at which the grains absorb water include: grain structure- softer grains absorb more water than hard grains, and grain size- smaller grains absorb moisture more rapidly (Pitz, 1989). The temperature, time

and aeration required for steeping can also affect the rate at which the grain absorbs water and are therefore chosen to achieve a good level of hydration in order to produce good malt (Olkku, Reinikkanen and Carregal, 1991). The optimum steeping temperature and time required for sorghum grains to reach appropriate water content is 25–30°C and 16-40 hours, respectively (Briggs, Hough, Stevens and Young, 1981; Morrall, Boyd, Taylor and Van der Walt, 1986; Dewar, Taylor and Berjak, 1997a). Traditional South African sorghum malting employs a continuous steep of 6-8 hours (Taylor and Belton, 2002). Aeration, either by draining the water from the grain periodically (air-resting) or by sparging air through steeping water, is necessary for production of a good quality malt (Novellie and De Schaepdrijver, 1986).

During steeping, the grain swells and softens, while the living tissues resume their metabolism (Briggs, 1998). There is a breakdown of complex carbohydrates and nutrients leach out from the grain into the steep water (Pathinara, Sivayogasundaram and Jayatissa, 1983).

1.2.2.2. Germination

Germination normally takes about 6 days. The germination of sorghum occurs rapidly between 20°C and 30°C with an optimum of 25–28°C (Morrall *et al*., 1986; Palmer, 1989; Dewar *et al*., 1997a). The germination phase of sorghum is physiologically very active. Important physiological processes associated with the germination phase are the synthesis of amylases, proteases and other endogenous hydrolytic enzymes (Palmer, 1989). The hydrolytic enzymes migrate from the germ into the endosperm (Figure 1.2) where starch and protein are hydrolysed to sugars and amino acids, respectively (Glennie *et al.*, 1983). These are then transported into the germ where they are further metabolised by the growing seedling (Taylor and Evans, 1989; Priest and Campbell, 1996). During germination the hard endosperm is converted into a more friable malt. The conditions that can impact greatly on the quality of the finished sorghum malt during the germination phase are grain moisture content, temperature, length of germination time and oxygen availability. Germinating sorghum grains have the tendency to rapidly lose water taken up during steeping and therefore it has been found necessary to spray germinating grains at intervals during the germination phase because the higher the level of moisture content (within limits), the higher the resulting malt quality (Morrall *et al*., 1986; Palmer, 1989; Dewar *et al*., 1997a). Other

ways to maintain good humidification are to germinate the grain in an atmosphere of near-water saturation (Palmer, 1989) or by continuous passage of moist air through the malting environment (Morrall *et al*., 1986). The germination step is complete when the whole of the endosperm (the storage part of the grain) which naturally sustains the development of the growing embryo or germ (the living part) during germination, has modified (partially attacked by enzymes) (Briggs *et al,* 1981; Dewar, Taylor and Joustra, 1995).

1.2.2.3. Drying/kilning and milling

Drying/kilning is the final stage of the malting process. The purpose of drying is to stop the growth of the green malt at the end of the germination process and to produce a shelf-stable product complete with active enzymes by reducing the moisture content and water activity (a_w) (Novellie and De Schaepdrijver, 1986). During this phase, the germinated sorghum grains are dried at a temperature of about 50˚C for 24 hours. In South African sorghum malting, the dried malt is then milled without the removal of external vegetative parts from the grain. The resultant product has a moisture content of around 10% (Daiber and Taylor, 1995).

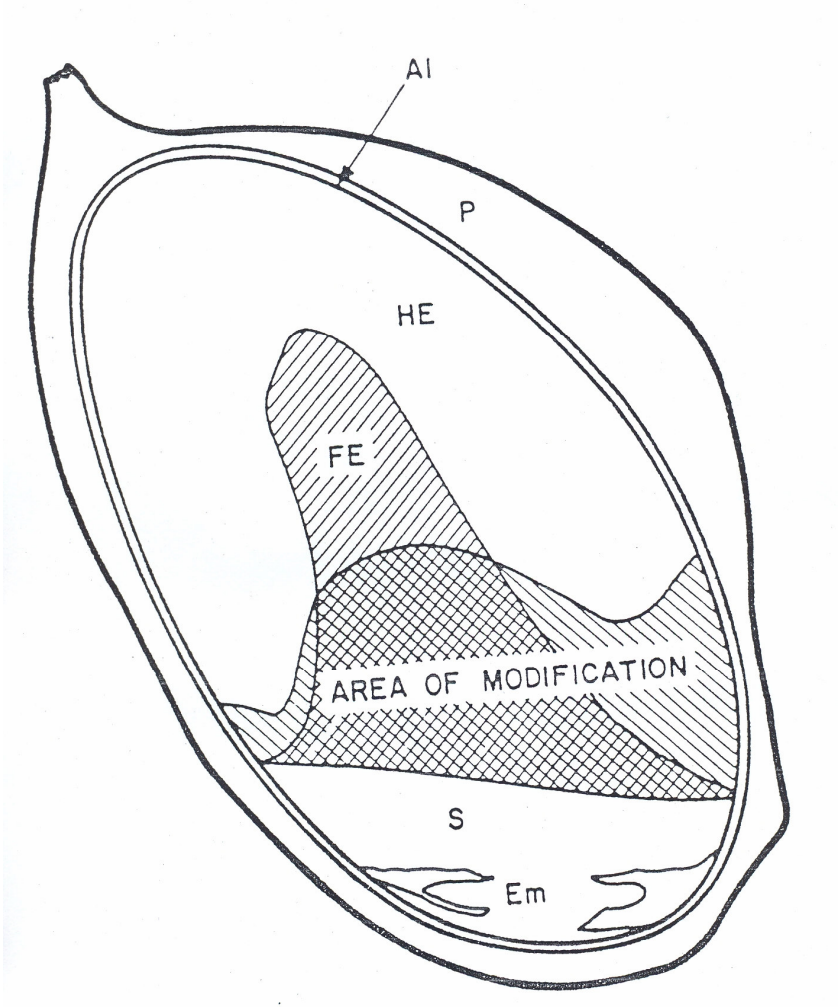


Figure 1.2. Pattern of endosperm modification during sorghum germination. Al – Aleurone layer, P – pericarp, HE - Horny endosperm, S – Scutellum, Em – embryo, FE –Floury endosperm (Glennie *et al.*, 1983).

1.2.3. Sorghum malting technologies in South Africa

Commercial sorghum malting in South Africa is carried out using two different processes; pneumatic malting, referred to as industrial malting, and floor malting, referred to as commercial malting (Novellie and De Schaepdrijver, 1986). Products of the latter technology are widely sold for home brewing but not to the brewing industry, while malt produced by the former are employed in industrial scale brewing of the opaque sorghum beer. The steeping process is common in both processes and the differences occur during the germination and drying processes.

1.2.3.1. Floor malting

Floor sorghum malting takes place outdoors whereby steeped grain is spread in a layer of 10–30 cm thick on a concrete floor that is slightly sloped to allow drainage of water (Taylor and Dewar, 1993). The layer of grain might be covered with shade cloth to reduce evaporation and to help prevent birds and rodents from feeding on the grain. Grains are watered intermittently with a hosepipe and are not normally turned. The temperature of the malt bed can only be controlled to some degree by making the bed thicker or thinner. A thick bed retains metabolic heat and is used during the winter season, while a shallow bed which allows faster dissipation of metabolic heat is employed during summer. Malted grains are dried under the sun or by forced draught as with pneumatic malting (Novellie and De Schaepdrijver, 1986; Priest and Campbell, 1996). The uncontrolled weather conditions and not turning the grains during floor malting encourages the formation of "hot spots" with a high load of bacteria and often result in malts with low and inconsistent quality (Briggs, 1998). Not turning also encourages the stratification of the grain bed into different layers that are mainly differentiated by the water activity, whereby the malt is progressively wetter from the top of the bed to the bottom layer (Taylor and Belton, 2002).

1.2.3.2. Pneumatic malting

Pneumatic sorghum malting is most commonly carried out in Saladin boxes, which comprise of a rectangular chamber with a perforated steel false floor containing the malt (1–1.5 metres deep) and a second chamber or plenum below it (Novellie and De Schaepdrijver, 1986). Air is blown by means of a fan into the lower chamber and through the bed of malt to provide oxygen for germinating grain, to remove carbon

dioxide and to maintain a uniform temperature throughout the malt bed. The germinating grain is watered at intervals by spraying and it is turned by means of helical screws mounted on a carrier which traverses up and down the length of the box. The malt is then force dried with a flow of warm, dry air from a furnace (Novellie and De Schaepdrijver, 1986). The uniform temperature that is maintained and turning the grain in intervals during germination discourages the entangling of roots and shoots, the development of "hot spots" and by so doing, the microbial growth is reduced. Generally, pneumatic malting produces better quality malt than floor malting due to better control of germination temperature and moisture. However, it is more expensive, it requires more energy and maintenance, whereas floor malting requires no sophisticated equipment and very minimal energy costs (Dewar, Taylor and Joustra, 1995). For these reasons most sorghum malting in South Africa is still by the floor malting procedure even if it produces inferior malts as compared to the pneumatic malting method.

1.2.4. Sorghum malt quality

In the Southern African sorghum malt industry, sorghum malt quality for opaque beer brewing is defined mainly in terms of its diastatic power (DP), which is the measure of the joint, α- and β-amylase activity measured in sorghum diastatic units (SDU) per gram of malt (South African Bureau of Standards, 1970). Generally, sorghum malt has a lower DP than barley and is required to have a minimum DP of approximately 28 SDU/gram for brewing the South African opaque beer (Dewar *et al.,* 1995). Free Amino Nitrogen content (FAN), which comprises of small peptides and amino acids (the products of protease activity) is another parameter used to define sorghum malt quality. A minimum of 110 mg FAN/100 g is required for opaque beer brewing (Dewar *et al.,* 1995). The type of the sorghum grain cultivar is also another factor that can contribute to the malt quality. In South Africa, the sorghum cultivars used are divided into condensed tannin-free (GM) or high-tannin (tannin) (GH) types (National Department of Agriculture, 1990). To produce good quality malt, it is essential that the tannins be inactivated, otherwise they can bind to the malt enzymes and affect hydrolysis of starch and proteins during brewing (Beta *et al*., 2000). It is also required that a high proportion of the grain must germinate for the production of a good quality malt. A measure of the percentage of grains which can be expected to germinate if the

grain is malted normally at the time of the test is referred to as the germinative energy (GE) and is recommended to be greater than 90% (Dewar *et al*., 1995). The resistance of the grain to mould infection is also sometimes taken as a quality criterion because grains that are susceptible to infection by moulds do not malt well (Briggs, 1998).

1.2.5. Microbial proliferation during sorghum malting

Fresh sorghum grain carries a variable microbial population that comprises mainly of bacteria and fungi (yeasts and moulds) (Rabbie and Lubben, 1984; Ogundiwin, Ilori, Fessehatzion, Babalola and Olajuyigbe, 1991). Therefore high levels of fungal and bacterial proliferation can result during germination if the grain was not properly cleaned (Agu and Palmer, 1997). Except for the initial sorghum microbial contamination, the activity and evolution of the microbial population during the different stages of the malting process depends on the availability of nutrients (e.g. sugars and amino acids), the moisture content, temperature, aeration and the interaction between members of the different microbial populations as it applies in barley malting (Noots *et al*., 1999).

Steeping is a critical stage at which the grain microorganisms start to proliferate due to the favourable elevated moisture content (33-35%) (Noots *et al*., 1999). During steeping bacteria, yeasts and moulds multiply and dormant spores are activated. The availability of nutrients that the microorganisms feed on leads to further microbial proliferation which result in a large number of microorganisms in steep water (Peters, Flannigan and Austin, 1988). It is therefore advisable to change the steep water more frequently or to rinse the grain at the end of each drain in order to remove the residual surface film of dirty water, the dissolved materials, the microbes growing on them and undesirable microbial metabolites (Kelly and Briggs, 1992).

Bacteria, yeasts and moulds which proliferated during steeping continue to develop during the germination stage (Agu and Palmer, 1999). The warm temperature and the humid/wet moisture content conditions in the grain bed are favourable for microbial growth during the germination stage. The increase is also attributable to the release of readily metabolizable components as a result of enzymatic activity in the germinating sorghum grain which the microorganisms feed on (Peters *et al*., 1988). There is,

therefore, a progressive increase in microbial population from steeping, during germination until the green malt is ready to be dried (Peters *et al*., 1988).

Drying reduces the microbial growth in the germinated green malt because the relative humidity of the grain becomes too low to permit proliferation of microorganisms. However, there are still substantial levels of microorganisms that survive the drying process. As a result the final malt product carries complex microbial population that mainly consists of bacteria, yeasts, and filamentous fungi (moulds) (Priest and Campbell, 1996). Some bacteria and yeasts are essential during brewing but some could also be spoilage organisms. Further, some bacteria (coliforms) and moulds could be harmful to humans if consumed (Priest and Campbell, 1996).

1.2.5.1. Coliforms

Coliforms are a group of bacteria that are members of the Enterobacteriaceae and are normal inhabiotants of the intestinal tract (Jay, 2000). The group includes the genera *Escherichia, Enterobacter, Klebsiella, Serratia* and *Citrobacter.* Coliforms grow optimally at moisture contents of about 18% and temperatures of about 35ºC and therefore the temperature and moisture content during sorghum malting will be ideal for the growth of coliforms (Agu and Palmer, 1997). *Enterobacter cloacae, Klebsiella aerogenes* and *Escherichia coli,* have been detected in sorghum grain and malt (Ogundiwin *et al*., 1991). Thaoge *et al.* (2003) found the presence of coliforms at levels of 10^4 cfu/g in South African sorghum malt.

1.2.5.2. Moulds

Moulds are fungi that grow in multicellular colonies by the production of long filaments called hyphae (Jay, 2000). Hyphal growth allows the moulds to colonize a food source as well as to grow from one food source to the other (Whitlow and Hagler, 2002). A network of hyphae is referred to as mycelium. This hyphal network is responsible for "cementing" grains together in grain piles resulting in column of grains that cannot be separated (Priest and Campbell, 1996). Moulds grow over a temperature range of 10–40ºC, a pH range of 4-8 and above 0.7 activity (a_w) (Bilgrami and Choudhary, 1998). Moulds can grow on a dry surface and on feeds containing 12-13% moisture content (Whitlow and Hagler, 2002). Reproduction of

moulds occurs through the production of spores and these spores are generally formed asexually but under certain circumstances can be formed sexually (Rodericks, Hesseltine and Mehlman, 1977).

Mould infection of grains can begin in the fields (field fungi), in or on the crop itself or during storage (storage fungi) and continues during malting (Agu and Palmer, 1999). The field fungi (e.g. members of the genera *Alternaria, Chlamydosporum* and *Fusarium*) populations decreases during storage, while that of the storage fungi (e.g., *Aspergillus* and *Penicillium)* increases (Papadopoulou, Wheaton and Muller, 2000). Both intrinsic and extrinsic factors encourage fungal growth when grain is malted. Intrinsic factors include high water activity (a_w), a high redox potential and pH around 6, while extrinsic parameters include temperature of about 30ºC, relative humidity of about 70% and available oxygen (Briggs, 1998). A large variety of moulds have been associated with sorghum malt (Rabie and Lübben, 1984). The dominant moulds (52–100% grains infected) isolated from both commercial and industrial South African sorghum malts by these authors are shown in Table 1.2. The first three most dominant fungi in both the industrial and the commercial sorghum malts were the yeasts (*Saccharomyces* spp), *Rhizopus*/*Mucor* and *Fusarium* spp. *Aspergillus flavus* and *Alternaria alternata* were the next most dominant at 26-29% grains infected in the commercial malt, whereas in the industrial malt *Aspergillus clavatus* and *Aspergillus flavus* followed with 23-26% grains infected.

The temperature of about 50ºC used during the drying process lowers the moisture content to a point where growth of moulds is inhibited or at least stops (Agu and Palmer, 1999).

Table 1.2. Incidence of the dominant mould spp. (% grain infected) in commercial and commercial sorghum malt (Rabie and Lübben, 1984)

Isolates	Grain infected (%)	
	Commercial malt	Industrial malt
Yeasts (*Saccharomyces* spp),	86	100
Rhizopus/ Mucor spp.	82	73
Fusarium spp.	52	64
Aspergillus clavatus	9	45
Aspergillus flavus	29	23
Phoma sorghina	8	36
Alternaria alternata	26	14
Oidiodendron spp.	8	18
Aspergillus fumigatus	6	5
Aspergillus niger	6	0
Aspergillus glaucus	5	0
Piptocephalis spp	3	0
Sclerotium folfsii	2	0
Streptomyces spp	2	0
Trichoderma viride	2	0
Gonatobotrys spp	2	0

1.2.6. Effect of a high microbial load on the quality of sorghum malt

Microbial contamination can alter physical characteristics of grains such as its colour (Noots *et al*., 1999). Highly stained grains are more contaminated by yeasts or filamentous fungi. It has long been known that obviously mouldy grains, or heavily stained or weathered grains (grains discoloured because they are, or have been, heavily infected with microbes that were able to multiply as a result of wet weather around harvest time) are water sensitive and have decreased maltability (Briggs, 1998). Microbes play other significant roles in malting and influence malt quality even when their presence is not immediately apparent (Kelly and Briggs, 1992).

During malting, microbes respire utilizing oxygen in competition with the grain (Mayer and Poljakoff-Mayber, 1989). This competition for oxygen is believed to be at least partly responsible for grain dormancy and water sensitivity.

Several types of microbes produce and release enzymes. Some produce substances with plant-hormone-like properties and others produce metabolites that are phytotoxic (damaging to the grain) (Bilgrami and Choudhary, 1998). Some microbes influence malt so that beer made from it is prone to gushing (overfoaming; gas instability) or to become hazy (loss of colloidal stability) (Agu and Palmer, 1997). Of more concern is that some mould species that have been found in sorghum grain are capable of producing mycotoxins that are harmful to animals and humans (D'Mello and McDonald, 1997). As a result, the presence of moulds in the sorghum malt might be an indication of the risk of possible contamination with mycotoxins.

1.2.7. Occurrence of Mycotoxins

Mycotoxins are secondary metabolites produced by moulds (Bilgrami and Choudhary, 1998). Mycotoxins are chemically diverse, representing a variety of chemical families and range in molecular weight from 200–500 Da. Mycotoxins are considered non-essential for the growth of the organism as compared to primary metabolites like amino acids and nucleic acids. Several genera of moulds are capable of producing mycotoxins. The most important are: *Aspergillus, Fusarium* and *Penicillium* (Sweeney and Dobson, 1998).

Production of mycotoxins can either occur in the field, during storage or a combination of the two or during processing of foodstuff (Sweeney and Dobson, 1998). In the field, mould growth and mycotoxin production are related to weather extremes (drought/ floods or excessive heat/ frost) and insect damage. After harvest, temperature, moisture content and insect activity are the major factors influencing mould and mycotoxin contamination of grains (Williams and McDonald, 1983). *Fusarium* species normally produce mycotoxins before, or immediately after harvesting, whereas production of mycotoxins by *Penicillium* and *Aspergillus* species occurs during drying and storage (Sweeney and Dobson, 1998). Visual observation of fungal growth does not necessarily indicate the presence of mycotoxins in samples

just as the inability to visually detect fungi does not indicate the absence of mycotoxins (Gourama and Bullerman, 1995b).

The five most common mycotoxins found in food are aflatoxin, ochratoxin, fumonisins, deoxynivalenol (DON, vomitoxin) and zearalenone (ZEA) (D'Mello and McDonald, 1997). Mycotoxins may be classified according to the primary anatomical part they affect, e.g. hepatotoxins affect the liver, nephrotoxins affect the kidney, while neurotoxins, cytotoxins and oestrotoxins affect the central nervous system, alimentary canal and the reproductive system, respectively (Husein and Brasel, 2001). Mycotoxins may also be carcinogenic (cancer causing) or mutagenic and teratogenic (capable of causing foetal malformation) (Bilgrami and Choudhary, 1998). Table 1.3 shows the fungal species that produce these five mycotoxins and the main effects observed in human and animals. Ingestion of small doses of mycotoxins over a prolonged period of time could results in chronic effects to the consumer, whereas ingestion of large amounts of mycotoxins in a short period of time will cause acute toxicity leading to death (Shima, Takase, Iwai and Fujimoto, 1997; Husein and Brasel, 2001).

Table 1.3. Mycotoxins, moulds that produce them and the main effects observed in human and animals (adapted from D' Mello and McDonald, 1997)

Mycotoxin	**Fungal source**	**Effects of ingestion**
Aflatoxin B_1	*Aspergillus parasiticus* *Aspergillus flavus*	Carcinogenic immunosuppressive, hepatotoxic, nephrotoxic
Ochratoxin A	*Aspergillus ochraceus* *Penicillium viridicatum* *Penicillium cyclopium*	Carcinogenic, immunosuppressive, nephrotoxic
Fumonisin B_1	*Fusarium verticillioides*	Carcinogenic, dermatotoxic, neurotoxic
Deoxynivalenol	*Fusarium culmorum* *Fusarium graminearum* *Fusarium sporotrichioides*	Carcinogenic, dermatotoxic, Neurotoxic
Zearalenone	*Fusarium culmorum* *Fusarium graminearum* *Fusarium sporotrichioides*	Oestrogenic, dermatotoxic

1.2.7.1. Commonly occurring mycotoxins

1.2.7.1.1. Aflatoxins

Aflatoxins are the best known mycotoxins. They are both acutely and chronically toxic compounds that have been implicated as causative agents in human hepatic and extrahepatic carcinogens (Rustom, 1997). Aflatoxins are produced by *Aspergillus flavus, A. nomius* and *A. parasiticus*. These compounds were named aflatoxins (*A* for *Aspergillus* and fla for flavus) after they were identified from *A. flavus* isolated as a microbial contaminant of a peanut meal after there was a toxic outbreak in England (Turkey-X disease) in 1960 (Ellis, Smith, Simpson and Oldham, 1991). Aflatoxins comprise of four most common subclasses: B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2). The B group fluoresces blue in long wavelength ultraviolet and the G group fluoresces green. The subscripts 1 and 2 designate the chromatographic mobility (Rf values) pattern of the compound on thin layer chromatography (TLC) plates. Biotransformation of aflatoxins in several animal species results in the production of aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) which were first isolated in milk (Van Egmond, 1989). Later, aflatoxin GM_1 and GM_2 were isolated and characterized (Van Egmond, 1999). Of the four major metabolites aflatoxin B_1 (Figure 1.3) is the most predominate and the most toxic (Husein and Brasel, 2001). Aflatoxins bind to DNA disrupting genetic coding, thus promoting tumourgenecity (Revanker, 2003). In Africa, mycotoxins have been implicated in human diseases including liver cancer, Reye's syndrome, childhood cirrhosis, chronic gastritis, kwashiorkor and other occupational respiratory diseases (Sibanda *et al*., 1997). For most species, the LD_{50} value of aflatoxins ranges from 0.5 to 10 mg/kg body weight (Rustom, 1997).

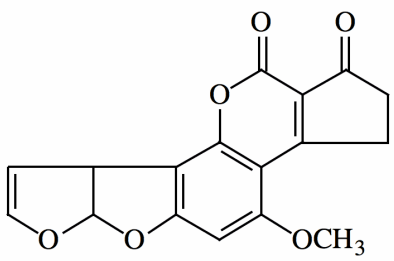


Figure 1.3. Structure of aflatoxin B_1 (European Mycotoxin Awareness Network, 2002).

1.2.7.1.2. Ochratoxin A (OTA or OA)

This toxin is produced by *Aspergillus ochraceus* and some species of *Penicillium* (e.g, *Penicillium verrucosum*). Production of ochratoxin A by *A. ochraceus*, was first described in South Africa isolated from a maize sample (van der Merwe, Steyn, Fourie, De Scott and Theron, 1965). Barley, maize, sorghum, oats and wheat have been found to have Ochratoxin A (Pitt, 1988). Ochratoxin A has been found to cause a human disease called endemic nephropathy and porcine nephropathy in a number of mammalian species (Lowe and Arendt, 2004). Optimal conditions for ochratoxin A production are at a temperature range between 20 and 25°C and moisture content of 16% or above. The LD_{50} of ochratoxin A for rats is 22 mg/kg and values vary greatly in different animals (European Mycotoxin Awareness Network, 2002). The structure of ochratoxin A is shown in Figure 1.4.

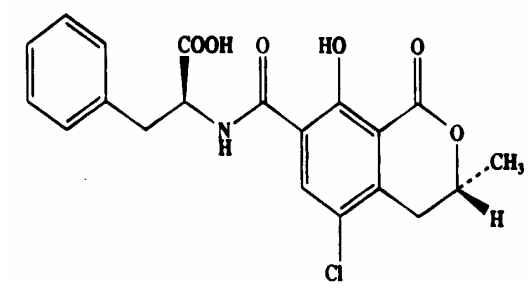


Figure 1.4. Structure of ochratoxin A (European Mycotoxin Awareness Network, 2002)

1.2.7.1.3. Fumonisin

Fumonisins are produced by *Fusarium verticillioides (moniliforme)* and *Fusarium proliferatum* (Bilgrami and Choudhary, 1998). *F. verticillioides* is a soilborne plant pathogen that is found mostly in maize but fumonisins have been found in rice, sorghum, yams, hazelnuts, pecans, and cheeses (Doko, Rapior, Visconti and Schoth, 1995). The most common types of fumonisins are B_1 (FB_1), B_2, (FB_2) and B_3 (FB_3) (Medina-Martinez and Martinez, 2000). The basic chemical structure is a diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amino group (Figure 1.5) (European Mycotoxin Awareness Network, 2002). Fumonisins are an increasingly important group of toxins as they have been postulated as causative agents for several endemic diseases both in humans and domestic animals (Sydenham, Thiel, Marasas, Shepard, Van Schalkwyk and Koch,

1990; Norred and Vos, 1994; Dutton 1996). The presence of high levels of *F. verticillioides* and fumonisins in maize in the Transkei region of South Africa has been correlated with a high incidence of oesophageal cancer (Marasas *et al.*, 1988, Sydenham *et al*., 1990). Fumonisins cause equine leukoencephalomalacia (ELEM), a liquifactive necrosis of the brain of horses, pulmonary oedema and hydrothorax in swine, liver cancer in rats, and abnormal bone development in chicks and pigs (Chu and Li, 1994; D'Mello, Placinta and McDonald*.,* 1999). The general structure of fumonisins is shown in Figure 1.5.

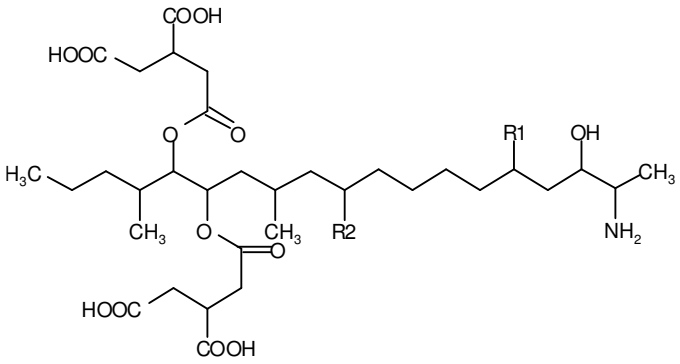


Figure 1.5. Structure of fumonisin. The groups R1 and R2 are different fumonisins (European Mycotoxin Awareness Network, 2002)

1.2.7.1.4. Deoxynivalenol (DON)

Deoxynivalenol (DON or vomitoxin) is produced by *Fusarium graminearum,* which is the most widely distributed toxigenic *Fusarium* species (Jelinek, Pohland and Wood, 1989). The optimal temperature range for *F. graminearum* growth is 21 - 27°C with moisture levels preferred to be greater than 20% (Placinta, D'Mello and McDonald, 1999). Deoxynivalenol has been found in wheat, barley, sorghum and maize (Bilgrami and Choudhary, 1998). Deoxynivalenol is classified under the largest group of *Fusarium* mycotoxins named as the trichothecenes (Miller, Greenhalgh, Wang and Lu*,* 1991). They are tricylic sesquiterpenes characterized by the presence of a double bond at C-9, 10 and an epoxy-ring at C12, 13 and consequently are classed as 12,13-epoxy-trichothecenes and are divided into Type A, B, C and D (European Mycotoxin Awareness Network, 2002). The type A and B are of importance with respect to their presence in grains. Deoxynivalenol is classed under type B. Deoxynivalenol has an immunosuppressant effect in animals and may play a role in

the human alimentary toxic aleukia and has also been identified as a feed inhibitor in pigs (Husein and Brasel, 2001). The structure of deoxynivalenol is shown in Figure 1.6.

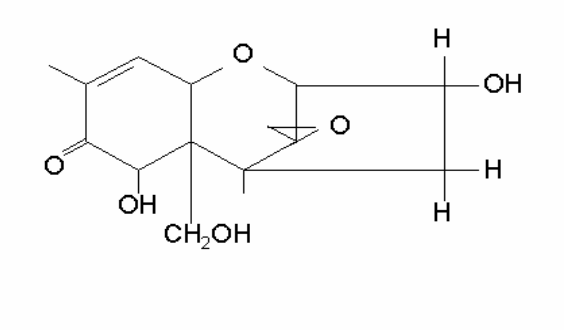


Figure 1.6. Structure of deoxynivalenol (European Mycotoxin Awareness Network, 2002)

1.2.7.1.5. Zearalenone (ZEA)

Zearalenone is produced by *F. graminearum, Fusarium culmorum, Fusarium equiseti, Fusarium poae* and some other *Fusarium* species (Bilgrami and Choudhary, 1998). Zearalenone is commonly found in maize but it has also been detected in sorghum and in beer and sour porridge prepared from contaminated maize and sorghum (Sibanda *et al*., 1997). ZEA is macrocyclic lactone (Figure 1.7) with high binding affinity to oestrogen receptors and only low acute toxicity (Diekman and Green, 1992). In mammals it causes infertility, vulva oedema, vaginal prolapse, mammary hypertrophy in females and feminization in males, complete infertility, constant oestrus and a pseudo-pregnancy syndrome, with pigs being the most sensitive species (Blaney, Bloomfield and Moore, 1984; Diekman and Green, 1992).

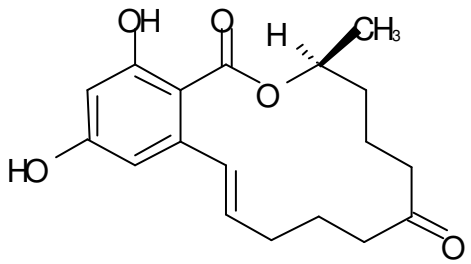


Figure 1.7. Structure of Zearalenone (European Mycotoxin Awareness Network, 2002)

1.2.7.2. Occurrence of mycotoxins in sorghum malt

In South Africa, a 5 years survey of 1190 commercial sorghum malt samples showed that on average the aflatoxin content of sorghum malt was 2.18 µg/kg, with 68% of malts containing ≤ 1 µg/kg, 20% having 1-3 µg/kg and only 4.5% contained more than 10 µg/kg (Trinder, 1988). According to this report, sorghum malt was not a health hazard because the levels of the aflatoxin were within the acceptable levels. On a very small scale, with only 10 sorghum malt samples analysed, Odhav and Naicker (2002) reported the absence of aflatoxin in South African sorghum malt. There are no reports of the presence of the fumonisins or DON in sorghum malt (Rabie and Marais, 2000). However, these authors reported the presence of ZEA in South African sorghum malt at levels of 15-20 µg/kg. ZEA was also found to occur in home brewed South African opaque beers (Odhav and Naicker, 2002).

1.2.8. Legislation

Various governments have set regulatory limits/advisory levels or established tolerance levels for different mycotoxins in food and animal foodstuffs for sale or import. The South African legal limit for aflatoxin B_1 is 5 µg/kg (Department of Health, 1972). There are currently no regulatory limits of DON, ZEA, fumonisins and Ochratoxin A in human foods in South Africa. The United States Food and Drugs Administration (FDA) has the regulatory levels of Aflatoxin B_1 (20 µg/kg), DON (500 µg/kg), fumonisins (2-3µg/g) in foodstuffs and no specifications for ZEA and Ochratoxin A (Food and Drug Administration, 2001). The European Union has of regulatory limits of 10 µg/kg for Aflatoxin B_1, 5 µg/kg for Ochratoxin A, advisory levels of 5 µg/kg for DON 5 µg/kg but has no specifications for ZEA and Fumonisins (Food and Agriculture Organization, 1997).

1.2.9. Techniques for analysis of mycotoxins

Because of the potential hazards that the detection of mycotoxins in foods pose, it is vital that the methods used for the analysis of mycotoxins are precise and reliable (Gilbert, 2002). Every method used should be able to address issues such as detection limits, repeatability, reproducibility and the percentage of recovery.

Many alternatives exist for the analysis of some mycotoxins and only limited choices are available for others (Wilkes and Sutherland, 1998). Often the combination chosen is dependent on operator preference or on the equipment available. A wide range of analytical techniques exist by which the mycotoxin load in food and feed products can be analysed. The most popular techniques are thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and immunoaffinity columns (Holcomb, Wilson, Trucksess and Thompson, 1992). In all these methods, the general steps that should be followed are sampling, extraction, clean up, concentration, ultimate separation and lastly, detection and quantification (Figure 1.8).

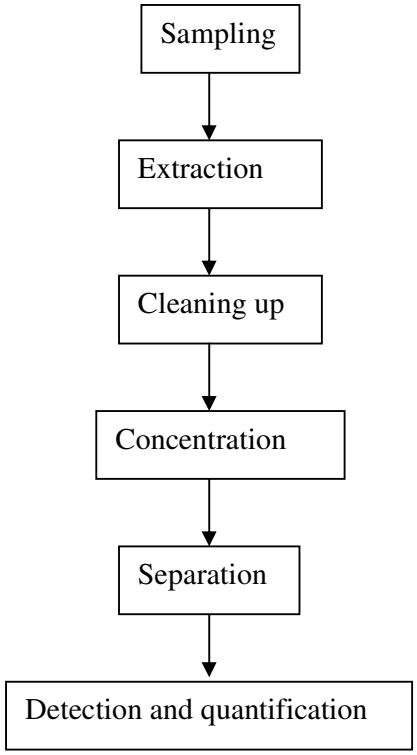


Figure 1.8. A general analytical method procedure for mycotoxin determination (Van Egmond, 1989).

1.2.9.1. Thin Layer Chromatography (TLC)

Thin layer chromatography is one of the oldest of the chromatographic methods and still one of the most popular methods. Thin layer chromatography is based on a heterogeneous equilibrium established during the flow of the solvent called the mobile phase, through a fixed stationary phase to separate components from the mixture carried by the solvent (Touchstone and Dobbins, 1983). During separation, a

polar compound is retained in the stationary phase, whereas a non-polar compound is delayed less by the stationary phase and forms a mark where it stops. For qualitative purposes the Rf value is used, which is the ratio of the distance of the compound from origin over the distance of solvent from origin (Randerath, 1966). Target compounds may be detected after selective coloration by spraying with various reagents (Lin, Zhang, Wang, Wang and Chen, 1998). The presence of spots on TLC with Rf values similar to or identical with those of the particular mycotoxin is a tentative identification. The concentration is then estimated in comparison with the concentration of the standard. In this regard TLC is semi-quantitative. TLC does not require expensive and sophisticated equipment. It is a good screening technique and its processes are easy to follow (Lin *et al*., 1998). There are easy to use, multi-toxin TLC methods, especially for agricultural commodities that enumerates several mycotoxins at once (Dutton and Westlake, 1985) and TLC methods for analysis of specific mycotoxins, e.g. aflatoxins (Trinder, 1988).

1.2.9.2. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a separation technique involving mass-transfer between the stationary and mobile phase (Jasco, 2004). The stationary phase is embedded in a column. The mobile phase is a liquid flowing through the column under high pressure. Separation of sample components depends on the chemical interactions of the sample components with the stationary and the mobile phases. A normal phase column has embedded polar stationary phase, whilst a non-polar mobile phase flows through the column while the reverse phase is vice versa. High performance liquid chromatography is precise, accurate and therefore very reliable. It is automated but it requires well trained personnel and it is an expensive technology (Gilbert, 2002).

1.2.9.3. Immunoassays

Immunoaffinity columns are widely used for cleanup and isolation of mycotoxins extracted from foods (Gilbert, 2002) and biological fluids (Wilkes and Sutherland, 1998). These columns are based on immuno-analytical methods, prepared by binding antibodies specific for a given mycotoxin to a specially activated solid-phase support and packing the support suspended in aqueous buffer solution into a cartridge (Vicam 2001). The mycotoxin in the extract or fluid binds to the antibody, impurities are

removed with water or aqueous solution, and then the mycotoxin is desorbed with a miscible solvent such as methanol. Usually, no clean-up is required and, after extraction of the mycotoxin, the test can be applied directly, giving results very quickly. Quantification is by use of instruments such as a fluorometer. Assays with immunoaffinity columns do not require a lot of work, they reduce time and the amount of solvent used significantly, and they are less expensive than the HPLC (Gilbert, 2002).

Enzyme linked immunosorbent assay (ELISA) involves the binding of the antigen (mycotoxins) to selective antibodies, resulting in a specific antigen-antibody complex (Smith and Solomons, 1994). Antibodies are immobilized on a solid support (microplates). An enzyme labelled antigen conjugate and an enzyme substrate are added to bind to the unbound antibodies. The bound enzyme reacts with the substrate and the resulting colour change is measured spectrophotometrically.

Immunoassays methods are generally rapid, simpler and cheaper than the HPLC methods and are very useful where numerous assays are required. However, there are possibilities of false positives and they are only semi-quantitative (Scott and Trucksess, 1997). Therefore it is required that these methods be confirmed by the use of another method.

The summary of the advantages and disadvantages of the most popular techniques are summarized in Table 1.4.

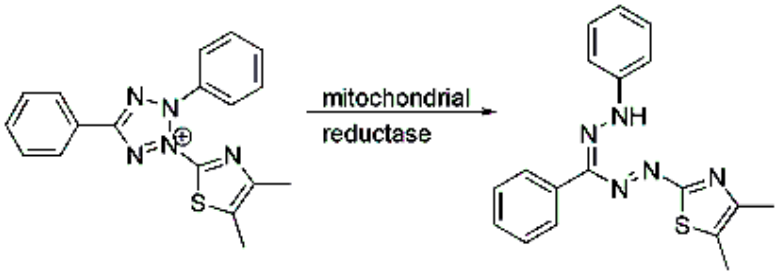
Table 1.4. Summary of the advantages and disadvantages of the analytical techniques for mycotoxins

Technique	**Advantages**	**Disadvantages**
TLC	High throughput; good screening technique	Semi-quantitative
HPLC	Precise and accurate; automated; simultaneous detection of different mycotoxins	Requires well trained personnel; expensive technology
Immunoassays	Minimum sample preparation; quick results;	Semi-quantitative; false positives

1.2.9.4. Cytotoxicity assays

Cytotoxicity studies of samples using cell culture systems provide useful information that will indicate the cytological effect of the mycotoxins in samples. A number of approaches to quickly and properly evaluate cytotoxicity in vitro have been developed. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay is one of the mostly used cytotoxicity method for quantitating viable cells (Hanelt, Gareis and Kollarczik, 1994).

The MTT assay is a colorimetric assay for measuring cellular proliferation (cell growth) (Hanelt *et al*., 1994). The assay is based on the capacity of the mitochondrial reductase enzymes (succinate dehydrogenase) of viable cells to oxidize the yellow MTT tetrazolium salt into a purple coloured product, formazan (Abe and Matsuki, 2000). Figure 1.9 shows the chemical structure of the reactant (MTT) and the product (formazan) following the exposure of the tetrazolium salt to viable cells.



Yellow MTT → Purple formazan

FADH2

Figure 1.9. Chemical structure of the MTT and the formazan (Mossman, 1983)

This oxidation of the MTT takes place only when there are metabolically active (live) cells, and therefore conversion is directly related to the number of live cells (Mossman, 1983).

1.2.10. Prevention and decontamination/detoxification of mycotoxins

The ideal decontamination of mycotoxin procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic or could alter the nutritional and sensory qualities of the food and feed (Piva, Galvano, Pietri and Piva, 1995). There have been attempts of the use of chemical, physical or biological methods to control the occurrence of mycotoxins in feeds and food but no single method appears to be equally effective against the wide variety of mycotoxins (Piva *et al*., 1995). The best way to prevent mycotoxin production is to inhibit mould growth (Samarajeewa, Sen, Cohen and Wey, 1990). If moulds cannot be analytically detected then there is unlikely to be any mycotoxin contamination. The control of the growth of mycotoxigenic moulds in the malting process can be achieved through the use of physical, chemical treatments or biological control methods (Noots *et al*., 1999; Lowe and Arendt, 2004).

1.2.10.1. Physical treatment

A typical example of a physical treatment is turning the grains during germination, as is practised in barley floor malting (Briggs, 1998). Turning limits fungal growth by discouraging the entanglement of the mould mycelia and thus prevents the formation of "hot spots" (Taylor and Dewar, 2001). Rotation of the grains by turning also allows uniformity of the malt bed in terms of temperature and exposure of the grains to oxygen.

1.2.10.2. Chemical treatments

Several chemical treatments like sulphuric acid, salicyclic acid, phosphoric acid, sodium hypochlorite, calcium hypochlorite, formaldehyde, caustic soda and lime have been suggested (reviewed by Briggs, 1998) for controlling microbes in barley steeps but they are disliked for some other characteristic (Table 1.5). As a result there is no worldwide acceptable, agents for controlling microbial growth during malting. Sulphuric acid, salicyclic acid and phosphoric acid were said to be corrosive, sodium and calcium hypochlorite tainted the malts and gave the malt an unpleasant odour and flavour, prolonged exposure to caustic soda (NaOH) and lime ($Ca(OH)_2$) was harmful to the grains. Many commercial maltsters in Southern Africa use dilute formaldehyde to mainly inactivate tannins (Dewar *et al*., 1995). Formaldehyde also inhibits

excessive growth of microorganisms during sorghum malting (Briggs, 1998). However, there are negative sentiments against the use of formaldehyde due to its carcinogenicity and therefore its use in food applications had been discouraged (United States Department of Health and Human Services, 2005).

The use of prolonged (more than 24 hours) steeping of barley grains with up to 1 % NaOH and $Ca(OH)_2$ was discouraged because it was harmful to the grains, although it was able to inhibit the growth of harmful microorganisms (Briggs, 1998). The addition of NaOH and $Ca(OH)_2$ results in a high pH, which distorts the membrane, leading to the death of the microorganisms. On the other hand, steeping sorghum grains with the addition of a very low concentration (0.1–0.5%) of NaOH solution has been shown not to cause adverse effects to the grain (Nelles and Taylor, 2002). Instead, steeping sorghum grains with dilute alkali improves malt's quality with respect to diastatic power (DP) (Okolo and Ezeogu, 1996a), FAN (Okolo and Ezeogu, 1996b), carbohydrates, protein mobilization (Dewar *et al*., 1997b) and inactivation of tannins (Palmer, 1989; Ezeogu and Okolo, 1999; Nelles and Taylor, 2002). Therefore steeping sorghum grain in dilute alkali is a potential method to control microbial growth (especially fungi) during malting.

Table 1.5. Chemicals which could be used as antimicrobial agents and disadvantages in their use (Compiled form information in Briggs, 1998).

Chemical	**Disadvantage**
Sulphuric acid, salicyclic acid, phosphoric acid	Corrosive
Formaldehyde	Potential carcinogen
Caustic soda, lime	Harmful to grain during prolonged exposure
Sodium and calcium hypochlorite	Taint the grain
	Gives malt an unpleasant odour and flavour

1.2.10.3. Addition of microbial cultures

Biological control methods, involving inoculation with lactic acid bacteria (LAB) and yeast (*Geotrichum candidum*) starter cultures (Boivin and Malanda, 1997), have shown promise for the control of unwanted bacteria and fungi during barley malting.

Bacterial cultures have also been added to the grain during malting to inhibit the growth of fungi (Lowe and Arendt, 2004). It has been demonstrated that the microflora during malting can be controlled by promoting the growth of desirable microbial cultures selected as natural control agents and inhibiting mycotoxin producing moulds (Agu and Palmer, 1999; Noots *et al*., 1999). The addition of *G. candidum* to steeping water during malting of barley resulted in the inhibition of undesirable micro-organisms such as yeast and filamentous fungi and in a stimulation of the growth of LAB (Boivin and Malanda, 1997). The antifungal effect was attributed to competition, because during malting *G. candidum* was detected in all analysed barley kernels in higher counts than of other microflora.

Lactic acid bacteria (LAB) have long been known to have the ability to inhibit growth and survival of the normal spoilage microflora and pathogens (Holzapfel, Geisen and Schillinger, 1995). Certain LAB starter cultures (e.g. *Lactococcus lactis*, *Lactobacillus acidophilus, Streptococcus lactis, Lactobacillus plantarum* and *Pediococcus pentosaceus*) have been found to inhibit the growth and survival of some fungi (Batish, Lal and Grover, 1990 and 1997; Suzuki, Nomura and Morichi, 1991; Gourama and Bullerman 1995a and 1995b; Roy, Batish, Grover and Neelakantan, 1996; Gourama, 1997; Noots *et al*., 1999; Laitila, Alakomi, Raaska, Mattila-Sandholm and Haikara 2002; Lowe and Arendt, 2004). The various factors contributing to the antimicrobial activity of LAB are low pH due to the production of organic acids (lactic acid and acetic acid), carbon dioxide, hydrogen peroxide, ethanol, diacetyl, depletion of nutrients and microbial competition (Batish *et al*., 1990 and 1997; Roy *et al*., 1996; Boivin and Malanda, 1997; Laitila *et al.*, 2002). Other beneficial effects with the application of LAB starter cultures during barley malting are reduced proportion of grains contaminated with *Fusarium*, decreased barley water sensitivity, increased FAN and α-amylase activity, better malt modification, improved wort filterability and decreased gushing tendency (Lowe and Arendt, 2004). The use of LAB and yeast during sorghum malting to reduce contamination by mycotoxigenic strains in sorghum malting have not been explored up until now.

1.2.10.4. Thermal treatment

An alternative way of reducing microbial load during malting, described by Delrue, Coppens, Delcour, Noots and Michiels, (2005), is by the use of thermal treatment.

During this process moisture is applied on the surface of the grains and then the grains are heated to a temperature of between 60 and 90ºC for about 1 to 30 seconds before steeping and germination. This process apparently reduces the microbial count of the grains without affecting the germinative energy.

1.3. Conclusions

In South Africa sorghum malt is an important product for brewing. In addition to brewing, sorghum malt is also used as an ingredient during the preparation of weaning foods in other parts of Africa. However, the high contamination with bacteria and fungi, and possibly mycotoxins during the malting process remains a problem. There is therefore a need for improvements in the production of sorghum malt in order to eradicate the problem of excessive microbial contamination and the presence of mycotoxins. According to literature, turning of the grain during germination, addition of dilute alkali to steep water, addition of LAB or yeast starter cultures to the steep water might suppress microbial growth and production of mycotoxin. Additionally, some of these methods might even improve the quality of the malt in terms of the DP of the malt. However, with thermal treatment, the elevated temperatures of between 60 and 90ºC might damage the sorghum grains. Furthermore, maintaining those elevated temperatures uniformly for only about 30 seconds would require sophisticated temperature control equipment. For these reasons, thermal treatment was not investigated in this study.

The three processes chosen for investigation – turning of the grains, alkaline steeping and steeping with cultures appear to have potential for reducing malt microbial proliferation and the hazards associated with it, including mycotoxins, and thereby producing sorghum malt with improved safety.

1.4. Hypotheses

1. Turning the grains during outdoor floor sorghum malting will prevent entangling of the roots and shoots and equalize the temperature of the malt bed, thereby preventing the growth of coliforms, fungi and the prevention of the production of mycotoxins.

2. The addition of dilute alkali during sorghum malting will inhibit the growth of coliforms, fungi and prevent the production of mycotoxins in the malt. The inhibition mechanism of alkaline pH is a function of the ability of a high pH to distort the bacterial membrane and thereby eventually killing the microorganism.

3. The addition of microbial starter cultures during sorghum malting will inhibit the growth of coliforms and moulds and prevent the production of mycotoxins in the malt. Low pH, depletion of nutrients and the production of antimicrobial substances will inhibit the growth of bacteria (coliforms) and moulds.

1.5. Objectives

Thus, the objectives of the study were:

1. To determine the total microbiological quality, different mould species, cytotoxicity and production of mycotoxins and malt quality of outdoor floor malted sorghum.

2. To determine the effect of turning the grain during germination on the microbiological quality, the cytotoxicity and mycotoxins and malt quality of the sorghum malt.

3. To evaluate the growth inhibition of unwanted bacteria and fungi, prevention of production of mycotoxins, effect on the cytotoxicity and the DP by steeping with the addition of dilute alkali solutions.

4. To evaluate the growth inhibition of unwanted bacteria and fungi, prevention of production of mycotoxins, effect on the cytotoxicity and DP by the addition of microbial cultures during the steeping process of sorghum malting.

2. RESEARCH

The research was divided into 3 parts which addressed the objectives stated in section 1.5. The three parts are as follows:

2.1. The microbial contamination, toxicity and quality of turned and unturned outdoor floor malted sorghum

2.2. Effect of dilute alkaline steeping on the microbial contamination and toxicity of sorghum malt

2.3. Antimicrobial activities of bacterial and yeast cultures in sorghum malting

2.1. The microbial contamination, toxicity and quality of turned and unturned outdoor floor malted sorghum

2.1.1. Abstract

Turned and unturned outdoor floor malted sorghum were studied for their total microbial contamination, nature and extent of contamination by moulds, cytotoxicity (IC_{50}) and quality in terms of diastatic power (DP). The presence of aflatoxins, fumonisins, deoxynivalenol and zearalenone were also investigated. Total microbial counts were high (10^7-10^8 cfu/g) in both turned and unturned samples. All samples showed contamination by different moulds, with the dominant being *Mucor species, Rhizopus oryzae, Fusarium verticillioides* and *Phoma sorghina. Aspergillus flavus* and *Alternaria alternata*. The latter four are known for producing mycotoxins. Malt samples had very low cytotoxicity (IC_{50} from 62.5 to > 500 mg/kg), though all contained fumonisins, deoxynivalenol and zearalenone at levels of < 0.25-2 µg/g, 15-20 and 10-15 µg/kg, respectively. Malt DP was generally lower in turned samples compared to unturned samples probably because the heat conserved in the latter ensured better germination conditions. Overall, turning during germination did not affect the microbial load, mould population and levels of deoxynivalenol and zearalenone in sorghum malt but decreased sorghum malt DP. Thus, alternative methods of controlling sorghum malt microbial load will have to be sought.

Keywords: Sorghum malt, mycoflora, mycotoxins, cytotoxicity

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2.1.2. Introduction

In Southern Africa some 200 000 tons of sorghum is malted commercially annually and is mostly used to brew sorghum beer (Taylor and Dewar, 2001). In South Africa, most sorghum malting is by traditional outdoor floor malting. Floor malting involves germination of the steeped grain outdoors for 4–6 days in layers of about 10 to 30 cm, on a very slightly sloped floor (generally rough concrete) to allow drainage of rain water (Taylor and Dewar, 2000). The germinating grain is sprayed once or twice daily using a hose. Unlike in barley floor malting, it is general practice in sorghum floor malting that the grain is not turned. Rather, sorghum grains are allowed to germinate undisturbed, resulting in the stratification of the malt bed (Taylor and Belton, 2002). The germination bed temperature and aeration are difficult to control under such conditions. Hence the practice whereby thinner beds are employed in hot weather (for easier dissipation of heat) as against the use of much thicker beds during winter (better preservation of metabolic heat) (Taylor and Belton, 2002; Thaoge *et al*., 2003). These germination conditions apparently encourage the proliferation of fungi and lead to high levels (10^5–10^6 cfu/g) of fungal counts in South African sorghum malt (Rabie and Lübben, 1984; Taylor and Dewar, 2001). Rabie and Marais (2000), have suggested that sorghum malt has the highest fungal load of all South African foods and feeds. While sorghum malt microflora also include bacteria and yeasts, the mould contaminants have been of the greatest concern because of their potential mycotoxigenicity (Gourama and Bullerman, 1995a; 1995b).

A five-year survey of industrial sorghum beer brewing in South Africa has shown South African sorghum malt to contain on average 2.18 µg/kg of aflatoxin, with 68% of malts containing ≤ 1 µg/kg, 20% having 1-3 µg/kg and only 4.5% yielding more than 10 µg/kg (Trinder, 1988). Recently, zearalenone has also been detected in South African sorghum malt grain samples (Odhav and Naicker, 2002).

Ideally, sorghum malt should be completely free of mycotoxin producing fungi and mycotoxins. The objective of this study was to determine the effect of turning the grain during germination, as is practised in barley floor malting (Briggs, 1998) on the microbiological quality of outdoor floor malted sorghum (Briggs, 1998). In barley floor malting the germinating grain is raked, turned and mixed periodically to

equalize the temperature and to prevent the roots from matting. Matting leads to "hot spots" where mould growth occurs.

2.1.3. Materials and methods

2.1.3.1. Sorghum malting

Sorghum cultivar NK 283, a condensed tannin-free red hybrid, was obtained from Tiger Brands, Potchefstroom, South Africa.

2.1.3.2. Malting

Malting was repeated twice in the winter season with a relatively thin depth of grains (10 cm) to maximally stress the grains. The malting procedure used simulated South African commercial outdoor floor malting conditions. Cleaned sorghum grains (2.5 kg) were weighed into plastic baskets (12 cm depth) to make a 10 cm thick layer. The baskets were steeped continuously in 4.0 L of tap water for 8 hours at room temperature (20˚C). Subsequently grains were germinated outdoors with malt bed temperatures of 18–20˚C and 14–17˚C. Germination was for 7 days with the baskets placed on slightly sloped surfaces (15° angle) to allow drainage of excess water. The surface of the germinating grains was covered with a shade cloth to reduce evaporation and to prevent attack by birds. At intervals (12 h) grains were sprayed with 350 ml of distilled water using an atomizer spray. Temperature could not be controlled because the malting was done outdoors but it was measured three times a day (in the morning, midday and in the evening). To determine the effect of turning, germinating grains in one basket were turned during each watering period, while grains in the other were not turned. At the end of germination, unturned grains were separated into the top, middle and bottom layers (about 3 cm each) according to the different visual estimation of moisture content of the grains (dry top, damp middle and the wet bottom). The grains of the turned sample were randomly separated into the top, middle and bottom layers for comparison. The moisture content of the different layers was determined. The green malt was dried in a forced-draft oven at 50°C for 24 hours and milled with a laboratory hammer mill (Falling Number AB, Huddinge, Sweden) fitted with a 500 µm opening screen.

2.1.3.3. Microbiological analysis

2.1.3.3.1. Microbial population

Malt samples (10 g) were placed in sterile bags and homogenized in 90 ml 0.1 % peptone, 0.85% NaCl, for 30 seconds. Tenfold serial dilutions were prepared and appropriate dilutions spread-plated in triplicate onto Plate Count Agar (PCA) for total aerobic counts, Potato Dextrose Agar (PDA) for yeasts and moulds and MRS (de Man Rogosa and Sharp) agar for lactic acid bacteria (LAB) counts. Pour plate technique with the second overlay with agar was performed with the Violet Red Bile agar (VRB) for coliforms. PCA plates were incubated at 35°C, MRS plates at 30°C, VRB plates at 37°C and PDA plates 25°C for 24 to 48 hrs. All media preparations were according to Pattison, Georgnavas and Von Holy (1998).

2.1.3.3.2. Mould isolation and identification

Mould enumeration, isolation and identification were according to Rabie and Lübben (1984). The direct plating method used to quantify the growth of moulds used is more efficient than dilution plating for detecting moulds from sorghum malt samples (Rabie *et al.*, 1997). Briefly, alcohol-disinfected grains (5) were placed on plates (ten each) of Potato Dextrose Agar (PDA), Malt Salt Agar (MSA), acidified Czapek-Dox Agar and Pentachlorobenzene Agar and incubated at 25°C for 2 to 14 days. The results are reported as a percentage of isolated mould species per sample. Moulds were identified to species level where possible, using morphological identification keys, by light and stereo microscopy, as described in several texts (Ellis, 1971; Nelson, Tousson and Marasas 1983; Pitt, 1988).

2.1.3.4. Diastatic Power (DP)

Diastatic Power (DP) which measures the overall amylase activity, was measured as the parameter to determine the sorghum malt quality because with the Southern African sorghum malt industry, sorghum malt quality is defined in terms of its overall DP (Raschke, Taylor and Taylor, 1995). The South African Bureau of Standards method 235 was used, except that water was used as the extractant and 5 g of malt used. The volume of the extractant was reduced accordingly (South African Bureau of Standards, 1970). Results are expressed as sorghum diastatic units (SDU)/g dry weight.

2.1.3.5. Cytotoxicity assays

2.1.3.5.1. Sample extraction

Sample extraction was by the multi-mycotoxin screen method of Dutton and Westlake (1985), The extracts (neutral fraction) were weighed and dissolved to 1 mg/ml in complete medium containing ethanol and dimethylsulphoxide (DMSO). Samples were sonicated until they dissolved, then filter-sterilised through a 0.22 µm filter.

2.1.3.5.2. Cells and Mycotoxin standards

Sp2/0 cell line from Balb/C mice was used. Cell maintenance was as described in Hanelt *et al,* (1994), except that Dubelcco's Modified Eagle's Medium (DMEM) was used instead of Modified Eagle's Medium (MEM). Aflatoxin B_1, deoxynivalenol (DON) and zearalenone (ZEA) standards were from Sigma–Aldrich (catalogue numbers A6636, D0156 and Z2125, respectively).

2.1.3.5.3. MTT-cell culture test

The cytotoxicity of sorghum grain samples was analysed using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) cell culture assay which monitors a reduction of yellow tetrazolium (MTT) salt by mitochondrial dehydrogenase enzymes of metabolically active/viable cells to purple formazans (Mossman, 1983). The concentration of samples and mycotoxin standards ranged from 16 to 500 mg/kg and 0.25 to 500 µg/g, respectively. Assays were performed according to Hanelt, Gareis and Kollarczik (1994). The absorbance of cultures was measured at 490 nm with an ELISA- reader (Multiscan® ascent, Lab Systems Oy, Helsinki, Finland). Mean extinction values and standard deviations of each sample were compared with those of the corresponding controls and expressed as % cleavage activity in comparison to the cell controls (100%). The cytotoxicity of samples was expressed in terms of their IC_{50} value (Inhibitory $concentration_{50}$ = concentration resulting in 50% inhibition of the MTT cleavage activity).

2.1.3.6. Assay of Aflatoxins, Fumonisins, Deoxynivalenol (DON) and Zearalenone (ZEA)

Aflatoxins and fumonisins were assayed by the Vicam Aflatest™ and Fumonitest™ (Vicam, Watertown, USA) which is based on affinity chromatography. Aflatoxins were also determined by TLC as described by Trinder (1988). Confirmation of the

presence of aflatoxins was carried out by two-dimension TLC. The TLC detection limit is 0.3 µg aflatoxin/kg and the results were expressed as µg/kg of sample. DON and ZEA were determined by a multi-mycotoxin TLC screen method as described by Dutton and Westlake (1985), except that plates were developed and viewed using a method described in Wilbert and Kemmelmeier (2003).

2.1.3.7. Statistical analysis

The experiments were repeated three times to obtain a total of 6 observations for each analysis. Duplicate samples were evaluated during each analysis. Results were analysed using the SAS package, version 8.2. Analysis of variance (ANOVA) was used to evaluate the data based on a 0.05 level of significance. Differences between means were determined using the least significant difference (LSD) test.

2.1.4. Results and discussion

2.1.4.1. Microbial population

For both germination temperatures the sorghum malt displayed microbial counts as follows: 1.3 x 10^7–5.7 x 10^8 cfu/g, total aerobic plate count (APC), 6.3 x 10^6–5.7 x 10^8 cfu/g, LAB count; 2.7 x 10^4–7 1 x 10^6 cfu/g, fungal count and 1.6 x 10^4–8.9 x 10^5cfu/g, coliform count (Table 2.1), all of which were substantially higher ($p< 0.05$) than the microbial load of the unmalted sorghum (APC, 3.2 x 10^5 cfu/g; LAB, 4.7 x 10^4cfu/g; fungi, 2.7 x 10^4cfu/g; and coliforms, 1.8 x 10^3cfu/g). The sorghum malts microbial counts are in the same range with those obtained by Thaoge *et al.* (2003) in which a total aerobic count of 1.3 x 10^8 cfu/g, LAB count of 1.8 x 10^7 cfu/g, yeast count of 8.7 x 10^6 cfu/g, moulds of 6.3 x 10^5 cfu/g and coliforms of 5.4 x 10^5 were obtained from sorghum malts. Ilorri *et al.* (1991) obtained bacterial counts of 2 x 10^6 cfu/ml and – 3.1 x 10^6 cfu/ml, in malt samples made from two sorghum varieties. A typical Southern African sorghum malt specification for total bacterial count is <2 x 10^7cfu/g. The aerobic counts of sorghum malt samples in this study exceed the specification and are therefore regarded as unacceptably high.

Table 2.1. Effect of turning on the bacterial counts (cfu/g) of the unmalted sorghum and the top, middle and bottom layers of sorghum malt when germinated at 14-17°C and 18-20°C.

			APC[1]	LAB[2]	Fungi	Coliforms
Unmalted sorghum			3.2×10^{5a}	4.7×10^{4a}	2.7×10^{4a}	1.8×10^{3a}
Sorghum malted at 18-20°C	Unturned	Top	6.0×10^{7b}	8.9×10^{6b}	5.6×10^{5b}	4.6×10^{4b}
		Middle	3.4×10^{8c}	3.5×10^{7c}	7.1×10^{6c}	5.6×10^{5c}
		Bottom	5.1×10^{8c}	5.7×10^{7c}	5.9×10^{6c}	8.9×10^{5c}
	Turned	Top	6.6×10^{8c}	5.4×10^{7c}	5.3×10^{6c}	7.1×10^{5c}
		Middle	4.9×10^{8c}	5.3×10^{7c}	5.1×10^{6c}	8.9×10^{5c}
		Bottom	5.7×10^{8c}	5.1×10^{7c}	5.6×10^{6c}	7.1×10^{5c}
Sorghum malted at 14-17°C	Unturned	Top	1.3×10^{7b}	6.3×10^{6b}	5.6×10^{5b}	1.6×10^{4b}
		Middle	1.9×10^{8c}	3×10^{7c}	2.9×10^{6c}	3.2×10^{5c}
		Bottom	2.6×10^{8c}	4.6×10^{7c}	5.3×10^{6c}	7.1×10^{5c}
	Turned	Top	2.9×10^{8c}	3.6×10^{7c}	3.3×10^{6c}	5.1×10^{5c}
		Middle	3.0×10^{8c}	3.6×10^{7c}	3.7×10^{6c}	5.1×10^{5c}
		Bottom	3.0×10^{8c}	4.0×10^{7c}	3.8×10^{6c}	5.1×10^{5c}

[1]Aerobic Plate Count.

[2]Lactic Acid Bacteria.

Mean and values with different letters (^{abc}) in the same column are significantly different from each other ($p < 0.05$).

Malt samples germinated at 18-20°C had significantly higher microbial counts ($p< 0.05$) than malt germinated at 14-17°C, probably because the higher malting temperature was more suitable for the growth of the microbial contaminants. Nevertheless, significantly ($p< 0.05$) lower microbial loads occurred within the top layers of unturned samples of both batches in comparison to their middle and bottom layers. This is because much of the water sprayed on the grains during germination passed through the top layer faster than could be absorbed. Some water of the top layer was also lost through evaporation, so that the malt became progressively wetter from the top of the bed to the bottom (Fig. 2.1). The dry environment of the top layer does not encourage microbial growth. The rate of water percolation reduced as water passed down through the sorghum grain layers so that some of it was trapped in the middle layer and made it moist and a lot of it was trapped in the bottom layer and made it wet even though the slope allowed for some drainage (Fig. 2.1). Turning the malt resulted in the moisture being uniformly distributed throughout the malt bed.

2.1.4.2. Mould isolation and identification

A wide variety of moulds were isolated from the sorghum grain and malt (Table 2.2). The mould load of malted sorghum far exceeded that of the unmalted grains. *Fusarium verticillioides, Mucor* spp. *Phoma sorghina* and *Rhizopus oryzae* were the most abundant, being detected in all analysed sorghum malt samples (100% incidence level). *A. alternata* was also detected at high levels (39-74%), while the incidence of *Fusarium chlamydosporum*, *Aspergillus flavus* and *Eurotium* spp. was relatively low (12-35, 5-31 and 3-21%, respectively). *Aspergillus niger*, *Penicillium* spp. *Culvularia* spp. and *Aspergillus fumigatus* occurred at less than 2% (results not shown). Thus the fungal population consisted of field (*Fusarium* spp. *P. sorghina* and *Alternaria alternata*) and storage fungi (*Mucor* spp. *Rhizopus* spp. and *Eurotium* spp), suggesting that an increase in moisture content during malting (Williams and McDonald, 1983; Noots *et al*., 1999) is quite favourable for fungal growth of different genera. The contamination of sorghum malt by a wide range of fungal species agrees with findings by Rabie and Lübben (1984) who earlier demonstrated the contamination of South African sorghum malt samples 65 commercial (floor malting) and 22 industrial (pneumatic malting) by the above fungal species in almost the same order of incidence as was observed in the current study.

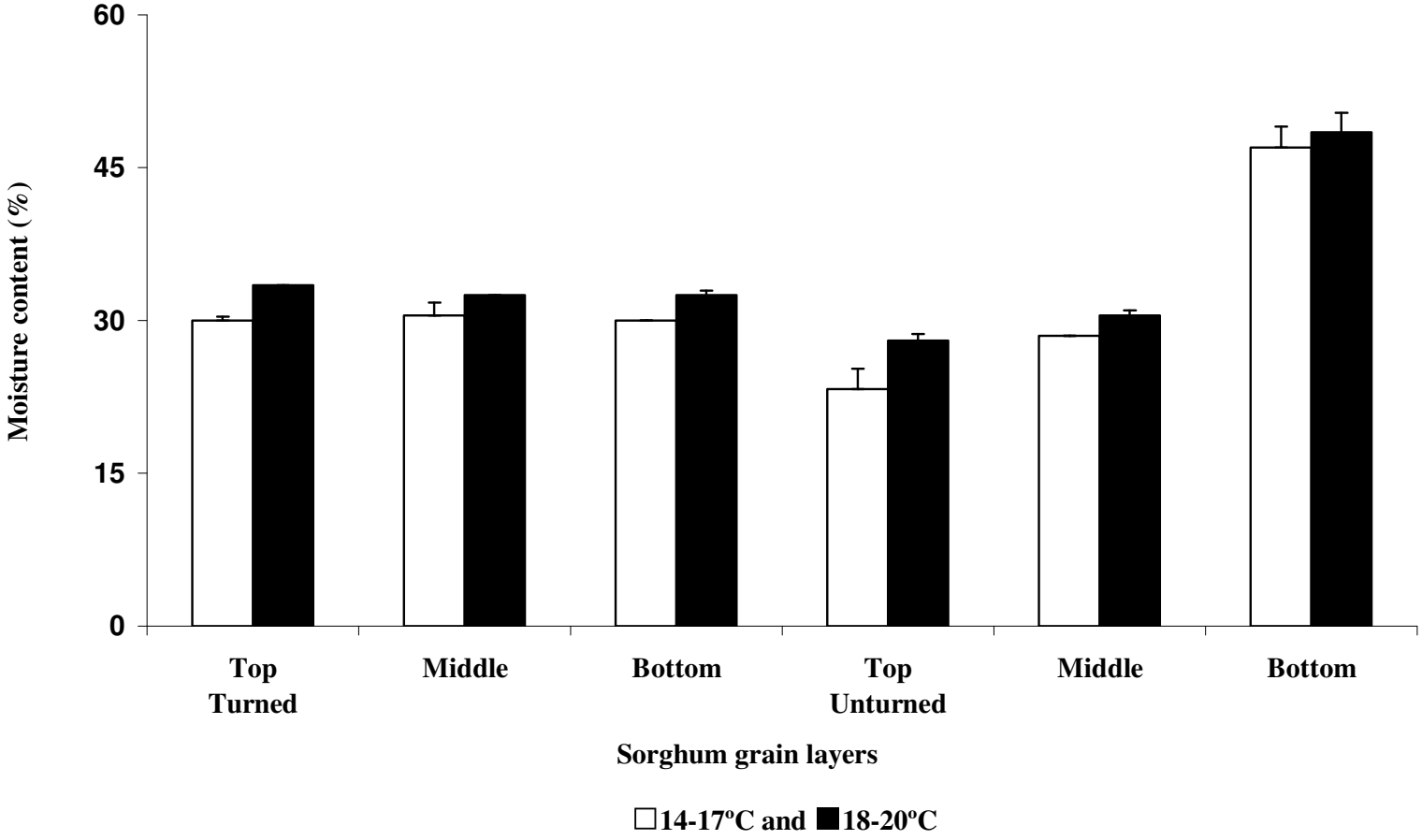

Figure 2.1. Moisture content (%) of the top, middle and bottom layers of the sorghum obtained at temperatures of 18-20 and 14-17ºC. The error bars indicate ± standard deviation.

Table 2.2. Incidence of fungal species (% grains infected) of the unmalted sorghum and the top, middle and bottom layers of sorghum malt when germinated at 14-17°C and 18-20°C

			Aspergillus flavus	*Eurotium species*	*Fusarium Chla -mydosporum*	*Alternaria alternata*	*Fusarium verticillioides*	*Phoma sorghina*	*Rhizopus oryzae*	*Mucor species*
Unmalted sorghum			0	3a[1] ± 1.4[2]	10a ± 3.8	24a ± 5.5	86a ± 4.3	82a ± 9.5	87a± 8.8	87a ± 3.5
Sorghum malted at 18-20°C	Unturned	Top	9a ± 3.0	13b ± 3.4	25c ± 15.6	73d ± 18.3	100b	100b	100b	100b
		Middle	17b ± 1.0	15b ± 6.0	30c ± 16.4	82e ± 19.1	100b	100b	100b	100b
		Bottom	31c ± 4.8	21b ± 2.1	35c ± 10.1	83e ± 21.9	100b	100b	100b	100b
	Turned	Top	6a ± 1.0	5a ± 3.4	22b ± 7.1	53c ± 8.2	100b	100b	100b	100b
		Middle	5a ± 6.6	5a ± 5.7	16b ± 7.3	56c ± 3.8	100b	100b	100b	100b
		Bottom	6a ± 4.6	3a ± 4.3	18b ± 7.2	49c ± 2.1	100b	100b	100b	100b
Sorghum malted at 14-17°C	Unturned	Top	10a ± 1.0	6a± 3.3	18b ± 7.4	34b ± 4.2	100b	100b	100b	100b
		Middle	21b ± 2.0	7a± 2.1	21b ± 2.9	44c ± 4.7	100b	100b	100b	100b
		Bottom	22b ± 4.0	11b ± 3.5	25c ± 15.6	65d ± 8.7	100b	100b	100b	100b
	Turned	Top	7a ± 7.6	6a ± 7.5	15b ± 2.0	27a ± 3.4	100b	100b	100b	100b
		Middle	6a ± 3.8	4a ± 1.9	17b ± 3.3	35b ± 4.5	100b	100b	100b	100b
		Bottom	5a ± 7.8	4a ± 2.7	12b ± 3.0	26a± 3.6	100b	100b	100b	100b

[1]Mean values with different letters in the same column are significantly different from each other ($p < 0.05$).

[2]Mean ± standard deviation

The top layer of the unturned samples for both germination temperatures had, lower ($p< 0.05$) incidence of some mould spp. (*A. flavus, Eurotium* spp. *F. chlamydosporum* and *A. alternata*) compared to both the middle and bottom layers. Unlike unturned samples, the mould distribution of turned sorghum malt samples was not influenced ($p> 0.05$) by either grain bed depth (i.e. whether top, middle or bottom layers), or malting temperature. This is probably due to the fact that the mixing effects of turning reduced differences in temperature, moisture content and gas distribution between the three malt bed layers, ensuring that these conditions were nearly identical throughout the malt bed. Presumably, the blending effects of malt turning ensured even distribution of individual mould propagules across the malt bed. The high levels of mould contamination found in this work raise concerns because of the presence of the potentially mycotoxigenic moulds such as *P. sorghina*, *F. verticillioides* and *A. flavus* and suggests the possibility that the malts could be contaminated with unacceptable levels of mycotoxins. Mycotoxins produced by *P. sorghina* are involved in the aetiology of onyalai disease (Rabie, Van Rensburg, Van der Walt and Lübben, 1975), while toxins of *F. verticillioides* (fumonisins, fusarin C and moniliformin) are involved in several human and animal ailments (D'Mello and McDonald, 1997; Marasas *et al.*, 1979). Beside potential mycotoxin contamination, high levels of moulds in malt may not be desirable for the simple reason that they could make products unpalatable, resulting in reduced consumer acceptance and therefore loss to the producer. From this perspective the high level of contamination of malts by even presumably innocuous moulds such as those of the genera *Rhizopus* and *Mucor* may not be acceptable (Rabbie and Lübben, 1984; Rabie *et al*., 1997).

2.1.4.3. Diastatic Power (DP)

The DPs (11–26 SDU/g dry weight) of malt samples germinated at 18-20˚C were significantly higher ($p<0.001$) than that of the malt samples germinated at 14–17˚C (0.5–6 SDU/g dry weight) (Fig. 2.2), probably due to the higher and lower temperatures, respectively to which the grains were subjected during germination (Dewar, Taylor and Berjak*.,* 1997a). It has been shown that sorghum malts develop DP optimally at temperatures of 24 – 30˚C (Morrall, Boyd, Taylor and Van der Walt, 1986). These results indicate that the low temperature conditions, sometimes used during winter months in South Africa, do not support the production of good quality sorghum malt by outdoor floor malting.

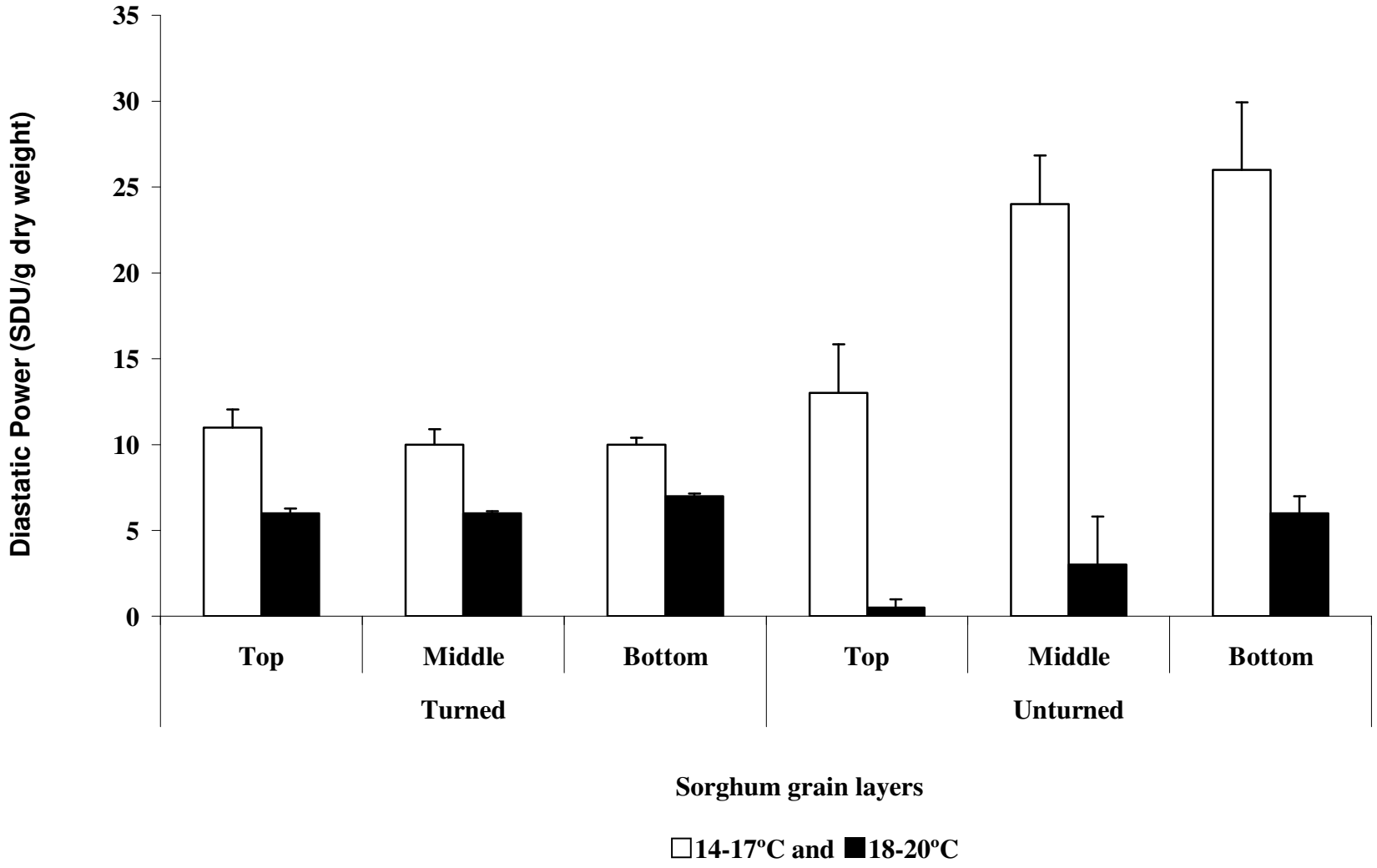

Figure 2. 2. Moisture content (%) of the top, middle and bottom layers of the sorghum obtained at temperatures of 14-17 and 18-20ºC. The bars indicate ± standard deviation.

2.1.4.4. Cytotoxicity and Mycotoxin analyses

The lowest concentration of the DON standard to cause cytotoxic effects in the MTT-bioassay was found to be 1-2 µg/g. ZEA and aflatoxin B_1 did not show any toxicity against the SP 2/0 cells. Unmalted sorghum and the top layer of the unturned, 18-20°C-malted grains showed no toxicity (100% cell growth) even at the highest concentration of 500 mg/kg examined. Generally, high sorghum malt IC_{50} values of 62.5–500 mg/kg were obtained which suggest that the malts are relatively non-toxic as those quantities are very large and not normally consumed by humans. However, it is important to note that this assumption pertains only to acute intoxication and does not preclude the possibility of intoxication due to continuous ingestion of sorghum malt over several years (chronic exposure).

Apparently very high levels of aflatoxins were detected in unmalted sorghum (42 µg/kg) and malted samples (52–160 µg/kg) using the Vicam Aflatest™ (Table 2.3). These apparent levels are 8 to 32 times higher than the South African legal limit of 5 µg/kg (Department of Health, 1972) and had never before been obtained in South African sorghum malts. It was therefore necessary to test for aflatoxins using a standard method. TLC was thus performed to confirm the Vicam results. One-dimensional TLC results contrasted with the Vicam Aflatest™ results, indicating that sorghum samples contained about 0.5 µg of aflatoxin B_1/kg. However, in fact that was a false positive because the two dimensional TLC showed that the aflatoxin B_1 suspected spots obtained on the single dimension TLC were not really aflatoxin B_1 spots and that the unmalted sorghum and sorghum malt samples contained less than 0.3 µg of aflatoxin B_1/kg (minimum detection level). This was confirmed by the detection of a spot of 0.5 µg/kg aflatoxin B_1 spiked in an unmalted sorghum sample. These results agree with findings by Odhav and Naicker (2002), who reported the absence of aflatoxin in South African sorghum malt samples. In contrast, Trinder (1988) reported the presence of aflatoxins (2-18 µg/kg) in South African sorghum malt that have been produced by indoor floor malting, so–called industrial malt. We can only hypothesize about the reason why the Vicam test gave false positive aflatoxin results. Perhaps the sorghum phenols bound to the Vicam Aflatest antibodies which resulted in a higher aflatoxin count.

Table 2.3. Concentration of aflatoxins (B_1, B_2, G_1 and G_2) fumonisins (B_1, and B_2), deoxynivalenol (DON), zearalenone (ZEA) and the IC_{50} levels of the unmalted sorghum and the top, middle and bottom layers of sorghum malt when germinated at 14-17°C and 18-20°C.

				Concentration				
			IC^{1}_{50} (mg/kg)	**Afla-toxins[3] (µg/kg)**	**Afla-toxins[4] (µg/kg)**	**Fumo-nisin[3] (µg/g)**	**DON[4] (µg/kg)**	**ZEA[4] (µg/kg)**
Unmalted sorghum			> 500[2]	42	<0.3[5]	< 0.25[5]	<3[1]	<3[1]
Sorghum malted 18-20°C	Unturned	Top	> 500[2]	52	<0.3[5]	< 0.25[5]	15–20	10–15
		Middle	62.5-125	130	<0.3[5]	1	15–20	10–15
		Bottom	62.5-125	160	<0.3[5]	1	15–20	10–15
	Turned	Top	125–250	74	<0.3[5]	< 0.25[5]	15–20	10–15
		Middle	125–250	66	<0.3[5]	< 0.25[5]	15–20	10–15
		Bottom	125–250	69	<0.3[5]	< 0.25[5]	15–20	10–15
Sorghum malted 14-17°C	Unturned	Top	125–250	59	<0.3[5]	< 0.25[5]	15–20	10–15
		Middle	31.2-62.5	140	<0.3[5]	2	15–20	10–15
		Bottom	31.2-62.5	140	<0.3[5]	2	15–20	10–15
	Turned	Top	62.5-125	53	<0.3[5]	1	15–20	10–15
		Middle	62.5-125	68	<0.3[5]	1	15–20	10–15
		Bottom	62.5-125	65	<0.3[5]	1	15–20	10–15

[1] Inhibitory concentration = concentration resulting in 50% inhibition of the MTT cleavage activity.

[2]Maximum concentration

[3]Determined using the Vicam kits

[4]Determined using TLC

[5]Minimum detection limit.

Unmalted and turned samples as well as the top layers of unturned 18-20°C-germinated malt contained less than 0.25 µg fumonisin/g (the minimum detection limit). Conversely, middle and bottom layers of the unturned 14-17°C-germinated malt contained 2 µg fumonisin/g (Table 2.3). Fumonisin levels seemed related to malt sample toxicity to SP2/0 cells. Both batches of malt contained DON values of 15-20 µg/kg and ZEA values of 10-15 µg/kg.

This study is the first report of the presence of fumonisin and DON in sorghum malt. Levels of ZEA obtained in this study (15-20 µg/kg) are substantially lower than that (387 µg/kg) reported by Rabie and Marais (2000), for South African sorghum malt. There are no regulatory levels of DON, ZEA and fumonisins in human foods in South Africa. The United States Food and Drug Aministration (FDA) has the regulatory levels of DON (500 µg/kg) and no specifications for ZEA (Food and Agriculture Organization, 1997). The regulatory levels of fumonisins are 2-3µg/g (FDA, 2001) and therefore fumonisin levels of 1- 2 µg/g were within the limit. The levels of DON, ZEA and fumonisins detected in this work are considered to be too low to be of any concern.

2.1.5. Conclusions

The present study shows that malting sorghum using standard commercial type outdoor floor malting conditions under cold temperatures results in malt with unwanted bacteria (coliforms) and fungi. The malt also contained traces of DON, ZEA and fumonisins but showed very low cytotoxicity. Turning of sorghum grains during germination does not affect the microbial populations, the nature and extent of fungal contamination, the level of aflatoxins, DON and ZEA, or the cytotoxicity but it decreases the DP of the sorghum malt. Notwithstanding the low levels of mycotoxins found, it is imperative that effective methods be found to suppress microbiological contamination of outdoor malted sorghum. Addition of dilute alkaline (NaOH), lactic acid bacteria or yeast culture during steeping could be other alternatives to reduce the microbial load (especially moulds) of sorghum malt (Briggs, 1998; Lowe and Arendt, 2004).

2.1.6. Acknowledgements

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2.2. Effect of dilute alkaline steeping on the microbial contamination and toxicity of sorghum malt

2.2.1. Abstract

Steeping in dilute alkali was investigated with the aim of reducing the moulds, coliforms, and preventing toxicity of sorghum malt. Red tannin-free sorghum varieties NK 283 and PAN 8546 were steeped in 0.1, 0.2 and 0.3% NaOH and 0.1, 0.3 and 0.5% $Ca(OH)_2$ and the effects on aerobic plate count (APC), lactic acid bacteria (LAB), yeasts, moulds, coliforms, the presence of some mycotoxins, the cytotoxicity and diastatic power (DP) of sorghum malt was evaluated. Steeping NK 283 in 0.1% $Ca(OH)_2$ increased the DP from 10.5 to 16 SDU/g. However, it did not reduce the general levels of microflora of the malt. Steeping in dilute NaOH reduced the microflora on the malt. Steeping both grain varieties with 0.2% NaOH resulted in malts with the moulds and coliforms reduced to ± 3.5 and 2.0 log cfu/g, respectively and the percentages of some mould spp. to very low or undetectable levels. The DP of the 0.2% NaOH steeped malts were increased to 16.2 SDU/g with NK 283 and 26.9 SDU/g with PAN 8546. There were no detectable amounts of mycotoxins and no indication of cytotoxicity in the 0.2% NaOH steeped samples. Steeping sorghum grains in 0.2% NaOH is recommended as a method for the control of bacterial and fungal contamination during sorghum malting.

2.2.2. Introduction

The presence of high quantities of undesirable microbes and their toxic products in sorghum malt is a concern (Rabie and Lübben, 1984; Agu and Palmer, 1997). Therefore, there is a need for efficient and safe ways to control microbial growth during sorghum malting. As shown in Chapter 2.1, the physical measure of turning germinating grains did not result in inhibited microbial growth. Steeping is probably the most critical stage at which microbial proliferation begins (Noots, Delcour and Michiels, 1999). Therefore, it is probably the best stage at which the growth of moulds and coliforms could be inhibited or reduced in order to give a microbiologically better quality sorghum malt. In South Africa, dilute formaldehyde is used to inhibit microbial growth during sorghum malting. However, there are some negative sentiments against the use of formaldehyde in food applications due to its carcinogenicity (United States Department of Health and Human Services, 2005).

Alkalis have been used as antimicrobial agents since ancient times and their antimicrobial ability is a function of the high pH (9-13) slowing down the metabolic activities by saponifying lipids within the cell membrane of microorganisms and thereby disrupting it, which then leads to cell death (Maris, 1995). Papadopoulou, Wheaton and Muller (2000) showed that microbial proliferation, especially yeast and fungi during barley malting were reduced by the use of alkaline washes. However, a combination of an acidified wash followed by an alkaline one as part of the steep schedule was somewhat more effective.

The addition of dilute calcium hydroxide [$Ca(OH_2)$/ (lime)] produced when calcium oxide is added to water and sodium hydroxide (NaOH) during barley steeping has been shown to inhibit microbial growth but prolonged exposure was harmful to the grains (Briggs, 1998). $Ca(OH)_2$ forms undesirable chalky and dusty deposits that remained on the malt. However, Ogundiwin *et al.*, (1991) showed that steeping in $Ca(OH)_2$ (2000 ppm) prevented bacteria and mould growth without affecting the malting loss, diastatic power DP, cold and hot water extracts of sorghum malt.

Several researchers have observed that steeping sorghum grain in dilute NaOH can give malts with improved DP and free amino nitrogen (FAN), malting loss, enhanced carbohydrate and protein mobilization without any adverse effects to the grain (Okolo and Ezeogu, 1996a and 1996b; Dewar *et al.*, 1997b; Ezeogu and Okolo, 1999; Nelles and Taylor 2002), but its effects on the microbial population of the malt have not been documented.

The objective of this study was to evaluate the growth inhibition of unwanted bacteria (coliforms) and fungi and the prevention of the production of mycotoxins in sorghum malt by the addition of dilute NaOH and $Ca(OH)_2$ during steeping.

2.2.3. Materials and methods

2..2.3.1. Sorghum grain

Two condensed tannin-free red hybrids - NK 283 and PAN 8546 were used. NK 283 was obtained from Tiger Brands, Potchefstroom, South Africa and PAN 8546 obtained from Free State Malt, Sasolburg, South Africa. NK 283 had a germinative energy of 42% and PAN 8546 had a germinative energy of 88%.

2.2.3.2. Malting

The sorghum grain samples (150 g) were rinsed and then steeped at 25°C for six hours in 300 ml still tap water (control), still solutions of NaOH [0.1, 0.2 and 0.3% (m/v)] and $Ca(OH)_2$ solutions [0.1, 0.3, 0.5 % (m/v)]. The water was then drained and the grain was rinsed and then steeped in fresh water for 2 periods of 1 hour to complete the 8 hours steeping regime. Steeping in $Ca(OH)_2$ was not performed with PAN 5846.

The steeped grains were transferred to 500 ml plastic containers (17 x 12 cm). These were placed in an incubator set at a temperature of 28˚C. Germination was for 6 days with the containers slightly opened to allow aeration. Twice daily, the grains were sprayed with about 5 ml distilled water using an atomizer spray. Excess water was drained out by tilting the containers for about 15 minutes. During spraying, the grain was

turned to avoid stratification of the grain into different layers and meshing of the roots. After germination, the green malt was dried in a forced-draught oven at 50°C for 24 hours. The amount of water taken up during steeping and germination was determined. The whole malt including roots and shoots was then milled with a laboratory hammer mill (Falling Number AB, Huddinge, Sweden) fitted with a 500 µm opening screen.

2.2.3.3. Analyses

2.2.3.3.1. pH

pH was determined on the steep water that was drained after the first six hours of steeping.

2.2.3.3.2. Moisture content

The amount of water absorbed during germination was expressed as a percentage of the the pre-washed, non-steeped grain weight and was calculated using the following equation (Gomez, Obilana, Martin, Madzvamuse and Monyo, 1997).

$$\% \text{ Water absorption (\% WA)} = \frac{B - A}{A} \times 100$$

Where B = the final weight of the grain; and A = the grain's initial weight.

2.2.3.3.3. *Other analyses*

The dried malts were assayed for the following as described in Chapter 2.1.

1. Microbial population: total aerobic plate counts (APC), lactic acid bacteria (LAB), yeasts, moulds and coliforms.
2. Mould isolation and identification.
3. Diastatic Power (DP).
4. Aflatoxins, Fumonisins, Deoxynivalenol (DON) and Zearalenone.
5. Cytotoxicity.

2.2.3.4. Statistical analysis

Duplicate samples were evaluated during each analysis and the experiments were repeated three times to obtain a total of 6 observations for each analysis. The data were analysed using the STATGRAPHICS 5.0 program (Statistical Graphics Corporation,

Rockville, USA). Analysis of variance (ANOVA) was used at 0.05 level of significance. Differences between means were determined by the least significant difference method.

2.2.4. Results and discussion

The two sorghum grain samples used in this study, NK 283 and PAN 8546, were selected on the basis of their germinability, 42 and 88% respectively. NK 283 represented very poor grain quality potentially more susceptible to microbial proliferation, whereas PAN 8546 represented a better quality grain.

2.2.4.1. Microbial population

Steeping NK 283 sorghum grains in $Ca(OH)_2$ caused a significant increase ($p< 0.05$) in all microbial counts (Figure 2.3). The increase in microbial counts was the highest at 0.1% $Ca(OH)_2$, lower with the 0.3% and further decreased or remained the same with the 0.5% $Ca(OH)_2$ concentration. The increase was from 6.8 (APC), 6.3 (LAB), 6.2 (yeasts), 5.2 (moulds), and 4.3 log cfu/g (coliforms) of the control to 7.1 (APC), 6.7 (LAB), 6.8 (yeasts) and 6.7 log cfu/g (moulds and coliforms) of the malt made with the 0.1% $Ca(OH)_2$ steeped grains.

With regard to steeping in dilute NaOH, the microbial population of NK 283 control malt (Figure 2.4a) was significantly higher ($p < 0.05$) than of the PAN 8546 control malt [APC (4.9 log cfu/g), LAB (4.2 log cfu/g), yeasts (4.5 log cfu/g), moulds (3.8 log cfu/g) and coliforms (3.5 log cfu/g)] (Figure 2.4 b). With the malts made from both sorghum varieties, steeping in NaOH caused a reduction in all microbial counts (Figure 2.4a and b). The reduction in microbial counts was directly related to NaOH concentration up to 0.2%. At 0.2% NaOH the microbial population was reduced to 3.0 (APC), 3.2 (LAB), 2.9 (yeasts), 3.3 (moulds) and 3.2 log cfu/g (coliforms) with the NK 283 malts and to 3.3 (APC), 2.3 (LAB), 2.7 (yeasts), 2.6 (moulds) and 2.3 log cfu/g (coliforms) with the PAN 8546 malts.

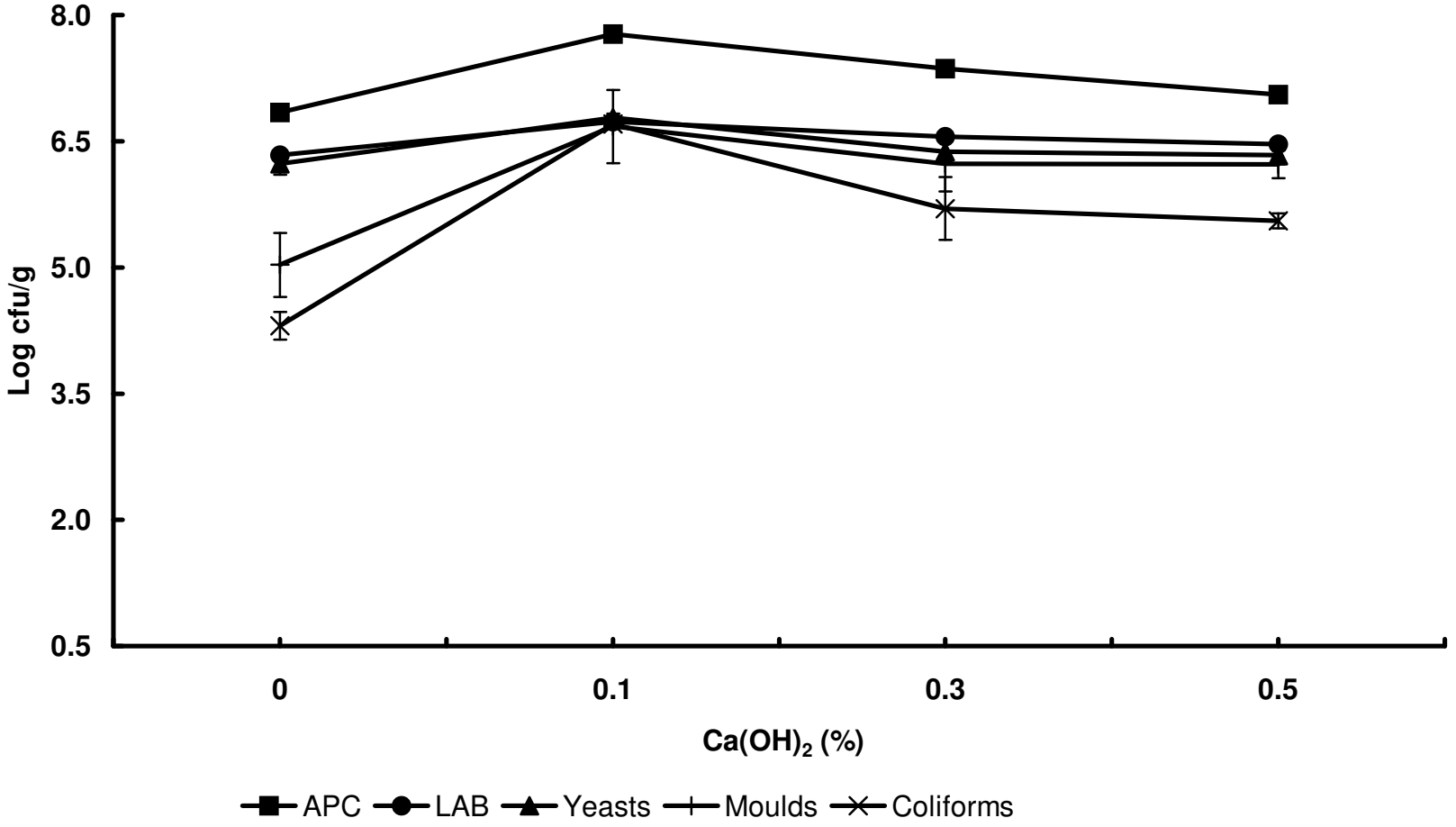
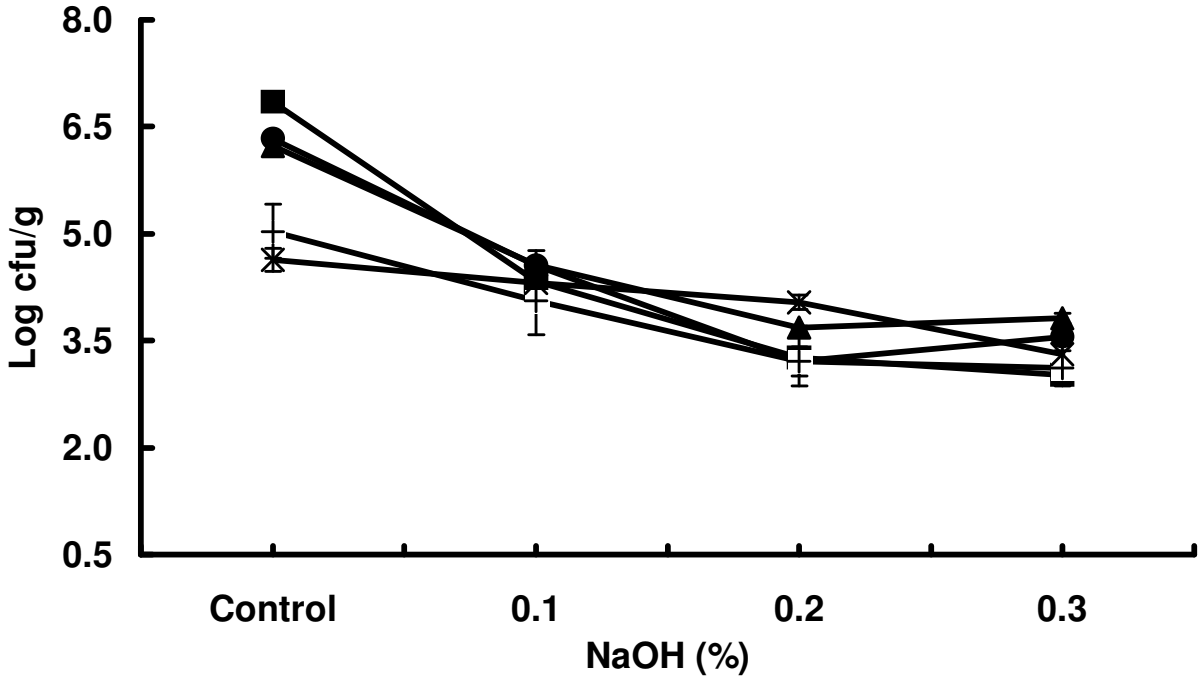
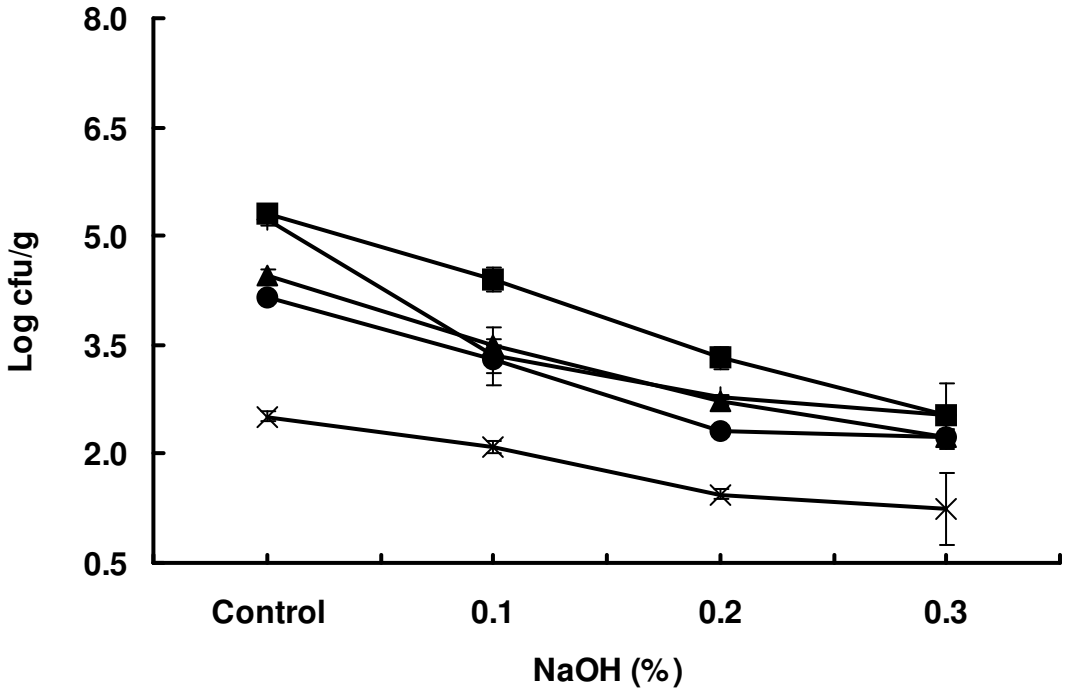


Figure 2.3. Effect of steeping NK 283 sorghum grain in different concentrations of $Ca(OH)_2$ on the microbial loads of the malt. The error bars indicate the ± standard deviation.



(a)



(b)

Figure 2.4. Effect of steeping the NK 283 (a) and PAN 8546 (b) sorghum grains in different concentrations of NaOH on the microbial loads of the malt. ■–APC (Aerobic Plate Count); ●–LAB (Lactic Acid Bacteria); ▲-Yeasts; x- coliforms; +-moulds. The error bars indicate the ± standard deviation.

The reason that $Ca(OH)_2$ caused the microbial load to increase might be the fact that $Ca(OH)_2$ is a weak base, that makes a good buffer against the lactic acid produced by LAB, thus giving the microflora a medium with a pH that is not too alkaline to inhibit their growth. Steeping NK 283 sorghum grains in $Ca(OH)_2$ resulted in a pH of 9.2–10.0 at the end of 6 hours (Figure 2.5). The pH of the NaOH steep water (pH 12.0–12.8) was significantly higher than of the $Ca(OH)_2$ steep water at the end of the 6 hours steeping regime (Figure 2.5). Moulds and coliforms do not survive the higher pH values (ICMSF, International Commission on Microbiological Specifications for Foods, 1980), such as those in the NaOH steeped water, observed in this work.

2.2.4.2. Mould isolation and identification

The NK 283 control malt had associated with it a large variety of moulds: *Fusarium. chlamydosporum* (46% contaminated), *Penicillium* spp. (26% contaminated), *Eurotium* spp. (14% contaminated), *Alternaria alternata* (60% contaminated), *Phoma sorghina* (52% contaminated), *Fusarium verticillioides* (78% contaminated), *Rhizopus oryzae* and *Mucor* spp. (both 100% contaminated) (Figure 2.6). Steeping NK 283 grains in $Ca(OH)_2$ did not have any significant effect ($p > 0.05$) on the numbers of *P. sorghina*, *F. verticillioides*, *Rhizopus* and *Mucor* spp. whereas *F. chlamydosporum*, *Penicillium* spp. *Eurotium* spp. and *A. alternata* were significantly reduced with an increase in the $Ca(OH)_2$ concentration to 34, 14, 12 and 46 % contaminated, respectively at 0.5% $Ca(OH)_2$.

Steeping NK 283 sorghum in NaOH caused a great reduction in mould counts (Figure 2.7a). The reduction of mould counts was directly related to the NaOH concentration. The 0.3% NaOH resulted in complete elimination of *Penicillium* spp. *Eurotium* spp. and *A. alternata*, whereas a decrease to 4% contamination (*P. sorghina*), 6% contamination (*F. chlamydosporum* and *F. verticillioides*), 18% contamination (*Rhizopus oryzae*) and 12% contamination (*Mucor* spp) was observed. The PAN 8546 control mould population consisted of *F. chlamydosporum* (20% contaminated), *Penicillium* spp. (26% contaminated), *Eurotium* spp. (14% contaminated), *A. alternata* (60% contaminated), *P. sorghina* (12% contaminated) *F. moniliforme* (33% contaminated), *R. oryzae* and *Mucor* spp. (both 24% contaminated) (Figure 2.7b).

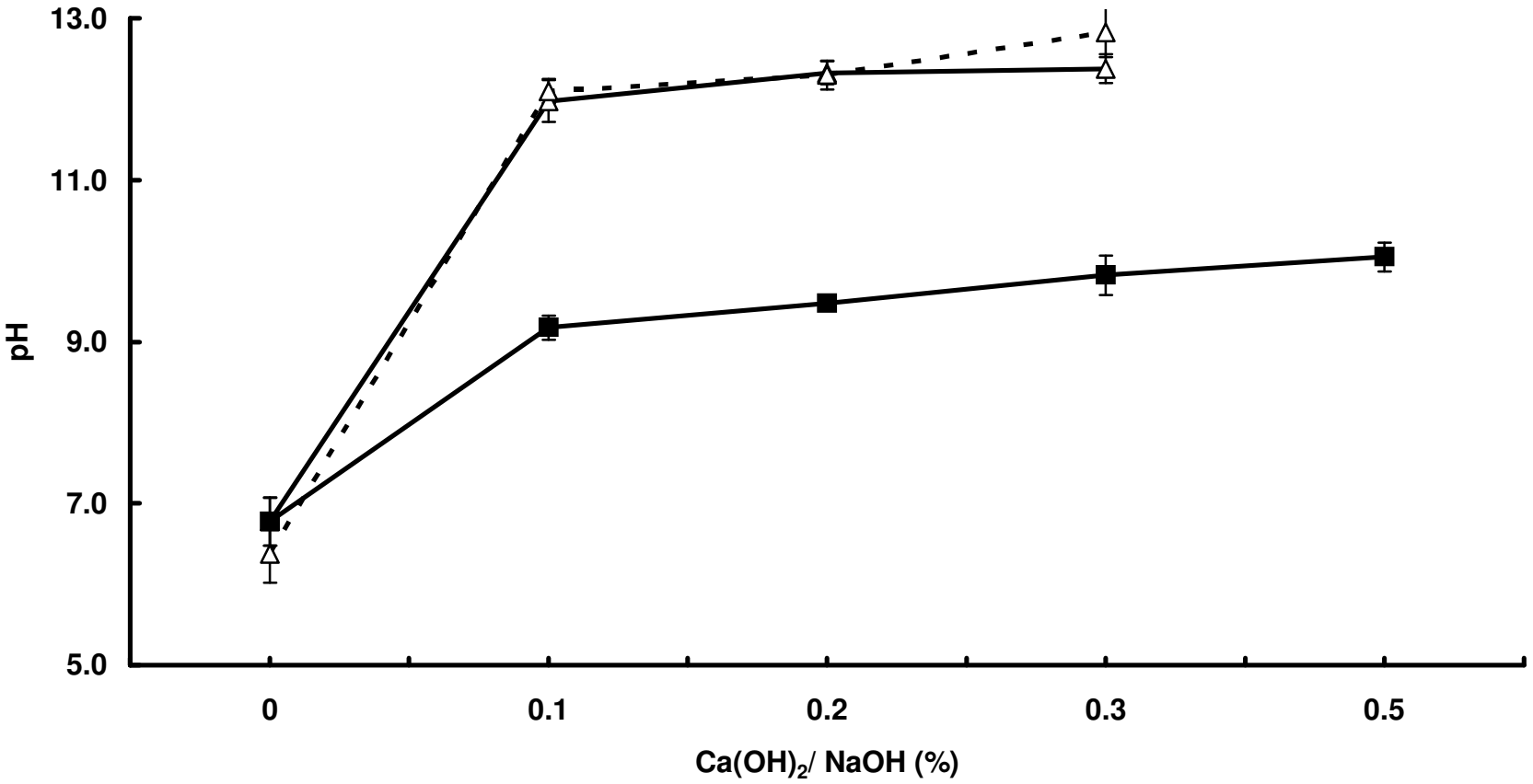


Figure 2.5. Effect of NaOH and $Ca(OH)_2$ concentration on the pH of the NK 283 and PAN 8546 steep waters at the end of the 6 hours steeping regime. The error bars indicate the standard deviation. Δ - Na OH and ■ –$(CaOH)_2$;—NK 283; ----PAN 8546;. The error bars indicate the ± standard deviation.

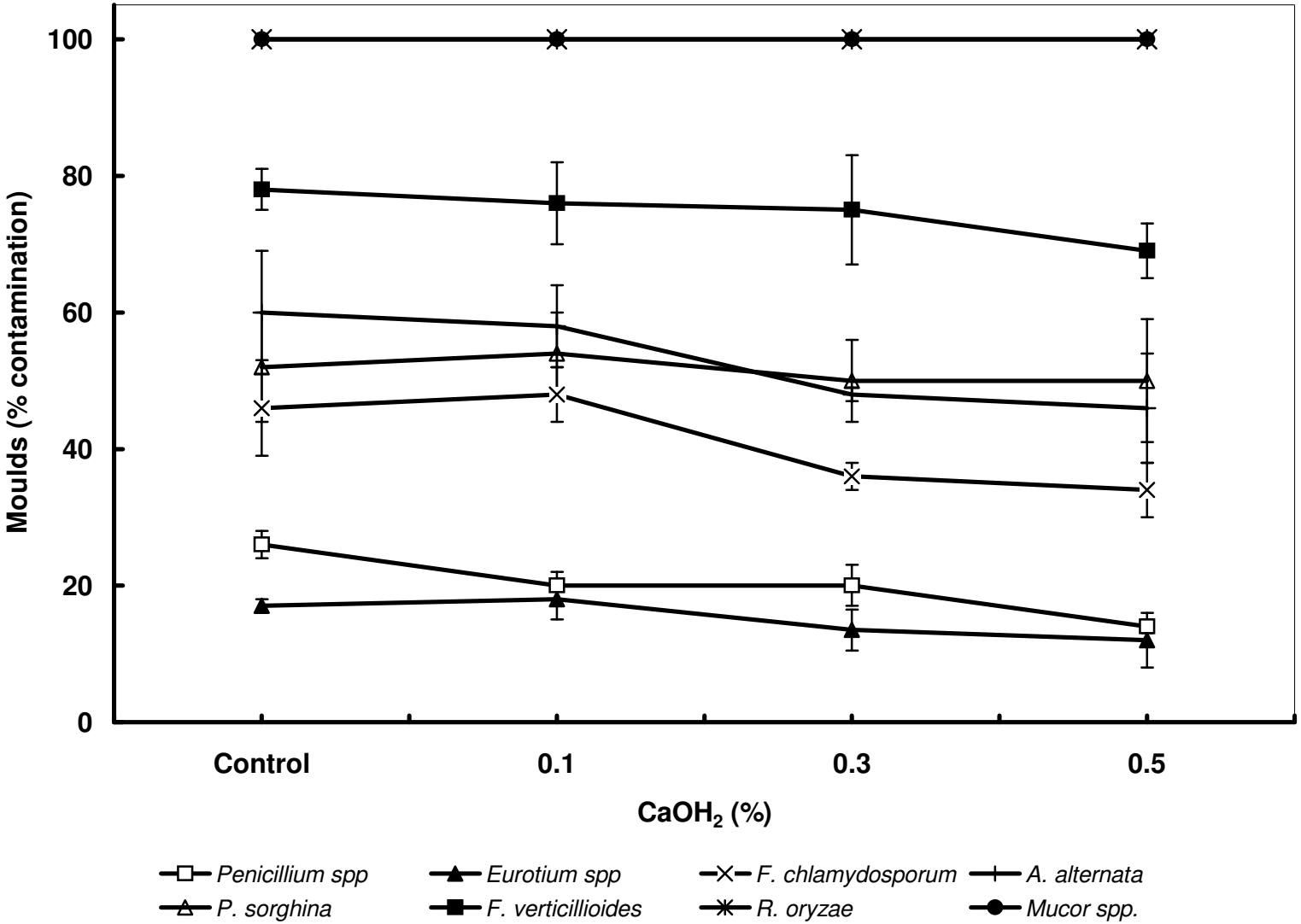
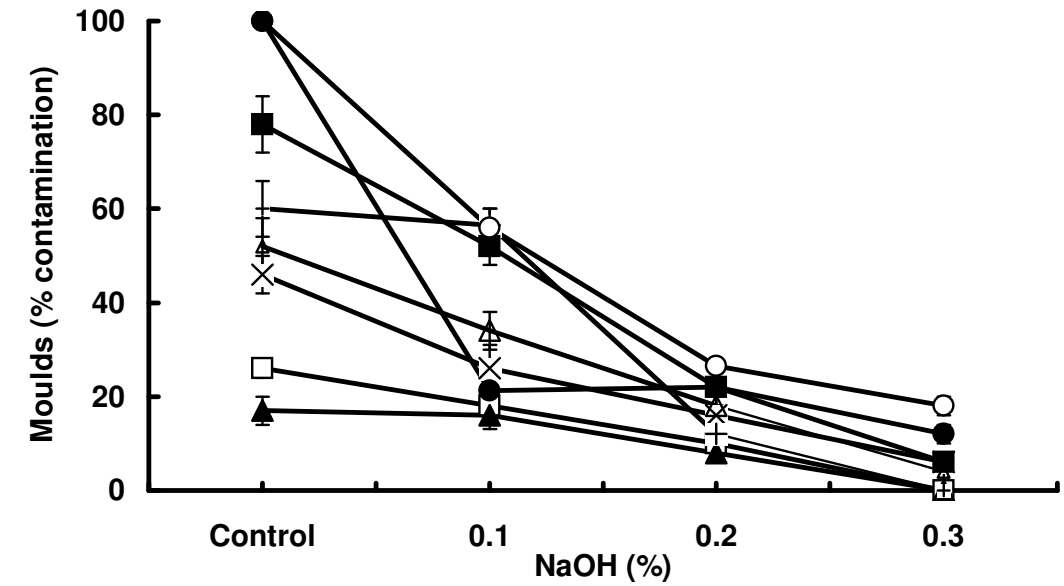
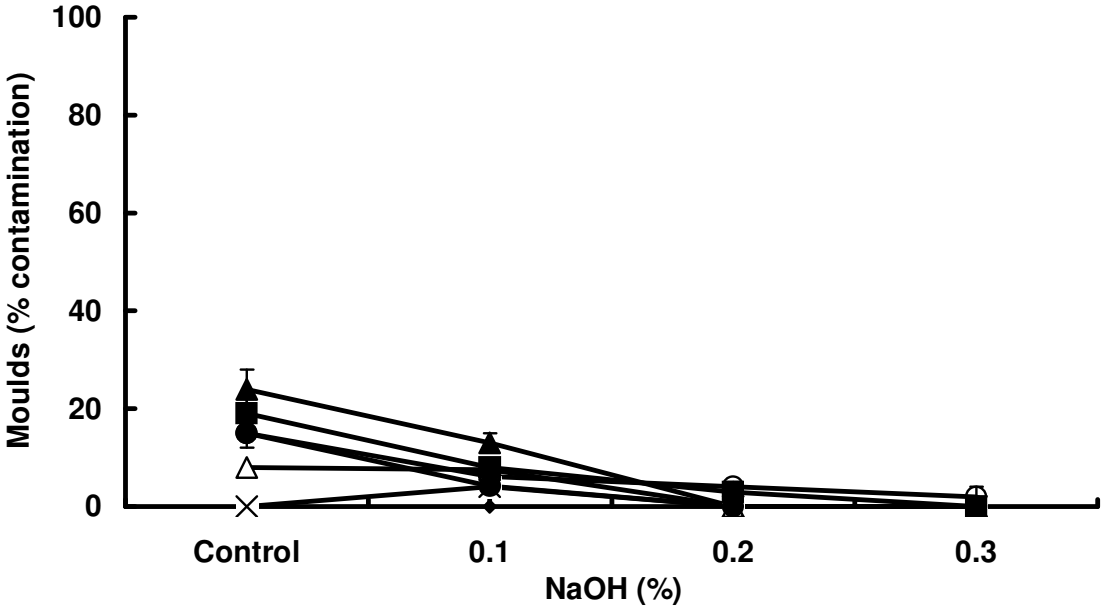


Figure 2.6. Effect of steeping NK 283 sorghum grain in different concentrations of $Ca(OH)_2$ on the different moulds spp. in the malt. The error bars indicate the ± standard deviation.



(a)



(b)

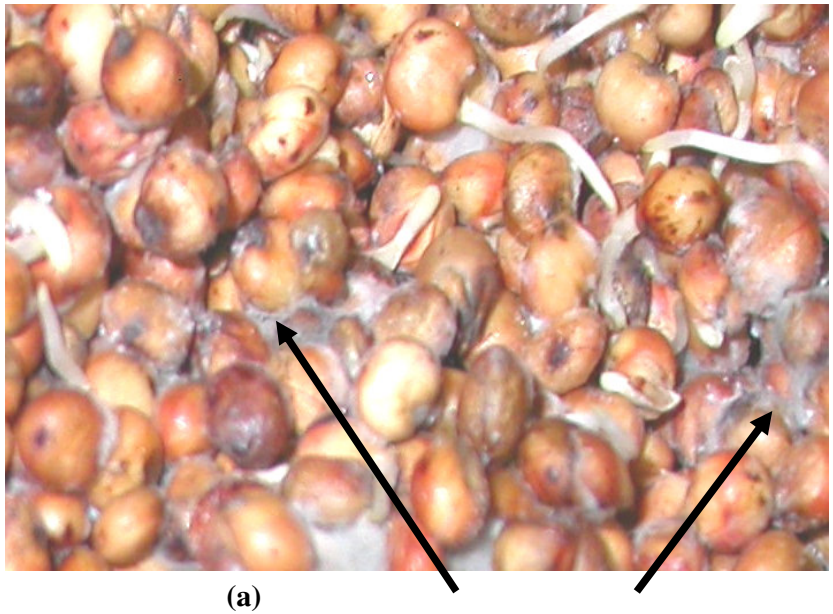
Figure 2.7. Effect of steeping NK 283 (a) and PAN 8546 (b) sorghum grains in different concentrations of NaOH on the different moulds spp. in the malt. □-*Penicillium* spp; ▲-*Eurotium* spp; x-*F. chlamydosporum*; +-*A. alternata*; Δ-*P. sorghina*; ■–*F. verticillioides*; ○–*R. oryzae* and ●–*Mucor* spp. The error bars indicate the ± standard deviation.

Steeping PAN 8546 in NaOH also caused a great reduction in all the mould counts as compared to the control. The reduction caused was not significantly different between the concentrations of NaOH (0.1, 0.2 and 0.3%). At 0.2% most moulds were completely inhibited, except for *F. verticillioides* and *R. oryzae* which were still detectable at 6 and 4%, respectively. At 0.3% NaOH *F. verticillioides* was the only mould still detectable at a very low level of 2%. Figure 2.8 shows green malts made from the 0% (control) and the 0.2% NaOH steeped NK 283 sorghum grains. Although there was still some mould growing on the 0.2% sample, the extent of growth was considerably less than of the control. The control shows mouldy clumps. These were absent on the 0.2% NaOH treated sample. Figure 2.9 shows green malts made from the 0% (control) and the 0.2% NaOH steeped PAN 8546 sorghum grains. The control sample had a dark discoloration, an indication of mould contamination (Briggs, 1998), whereas the 0.2% NaOH treated sample has a lighter, more appealing colour.

2.2.4.3. Diastatic Power (DP)

Steeping NK 283 sorghum grains in the 0.1% $Ca(OH)_2$ caused a significant ($p<0.05$) increase in the DP, from 10.5 (control) to 16 SDU/g (Figure 2.10). However, higher concentrations (0.3 and 0.5%) did not have any effect on the DP (10.4 and 10.1 SDU/g, respectively) as compared to the control. There was an increase in DP when NK 283 grains were steeped in 0.1 and 0.2% NaOH. The increase was higher at 0.1% (16.2 SDU/g) and lower at 0.2% (14 SDU/g). At 0.3% NaOH the DP was significantly reduced to 3.8 SDU/g. With PAN 8546 sorghum grain, steeping in NaOH also resulted in an increase in the DP. The DP values (19.7 SDU/g) obtained with the control increased linearly with the increase in the NaOH concentration. At 0.3% NaOH a maximum DP of 26.9 SDU/g was obtained. With both sorghum varieties, the increase in the DP can be attributed to increased water absorption by the grains. Figures 2.11 and 2.12 show that there was a direct relationship between the $Ca(OH)_2$ and NaOH concentrations, and the water uptake by the grains during germination. Dewar *et al*., (1997b) suggested that higher water absorption in alkali steeping was caused by the alkali disrupting the non-starch polysaccharide cell wall material.

NK 283- Control



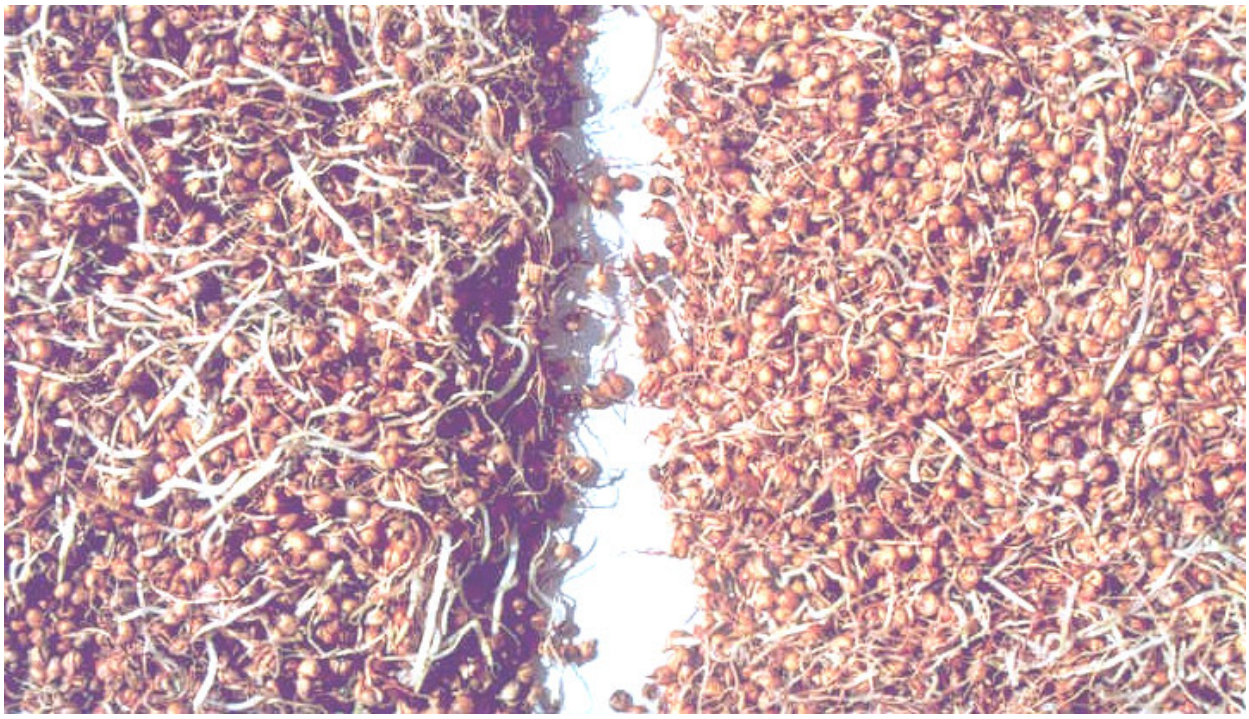
(a)

Arrows show mould growth

NK 283- 0.2% NaOH

(b)

Figure 2.8. Green malts made from the control (a) and the 0.2% NaOH (b) steeped NK 283 sorghum grains



(a) **(b)**

Figure 2.9. Green malts made from the control (a) and the 0.2% NaOH (b) steeped PAN 8546 sorghum grains.

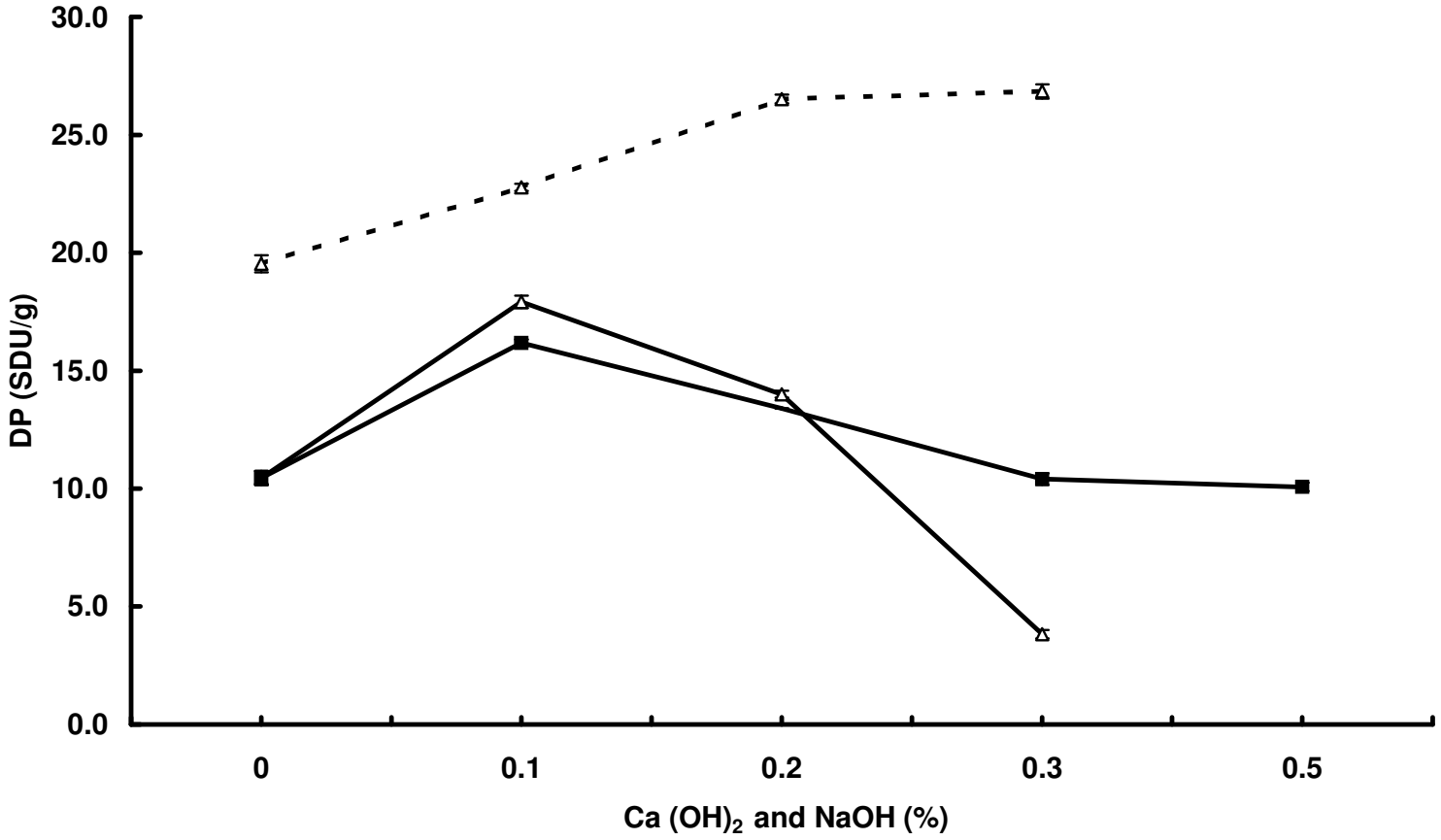


Figure 2.10. Effect of NaOH and $Ca(OH)_2$ concentration on the DP of NK 283 and PAN 8546 sorghum grains. —NK 283; --PAN 8546; Δ - Na OH and ■ –$(CaOH)_2$. The error bars indicate the ± standard deviation.

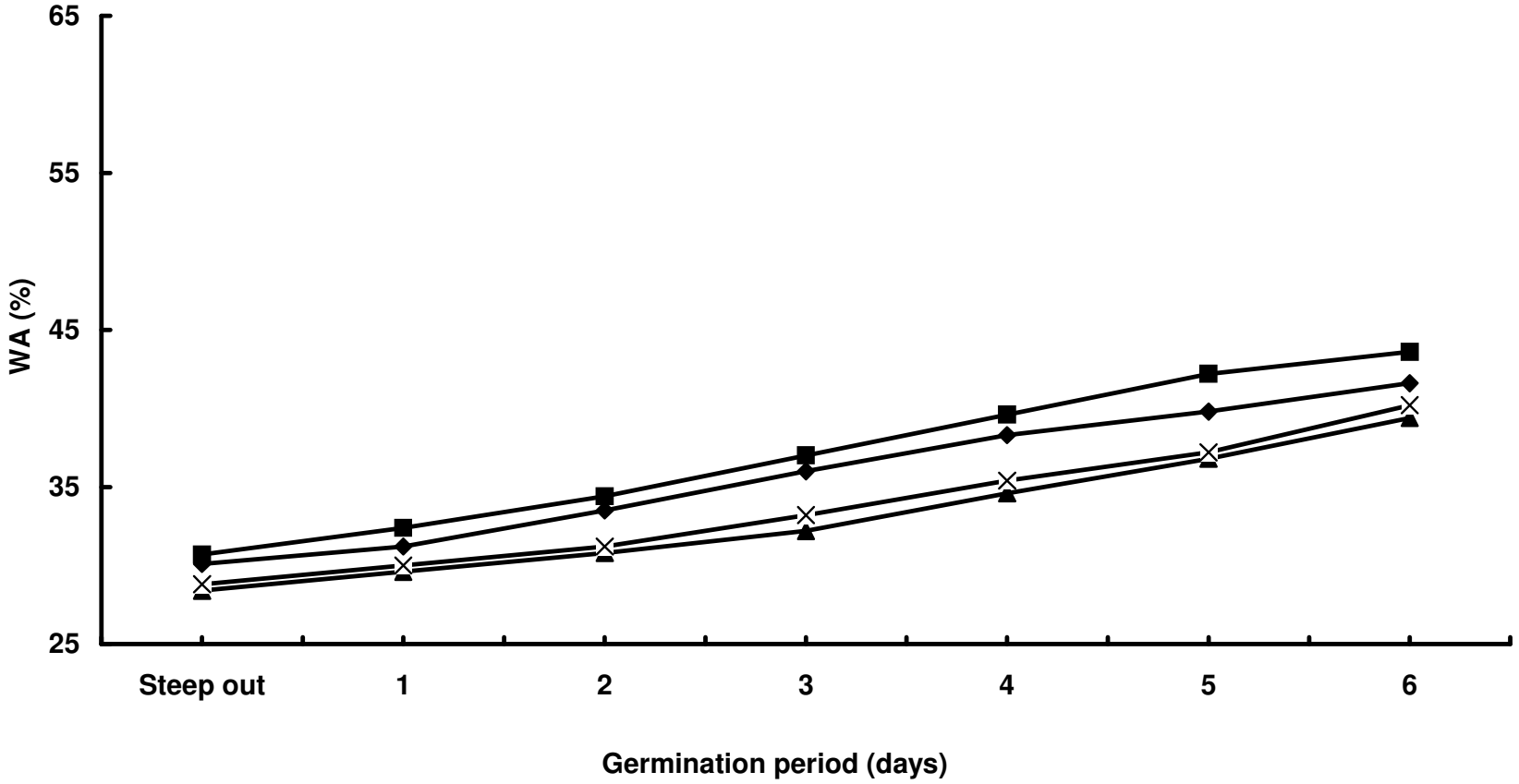
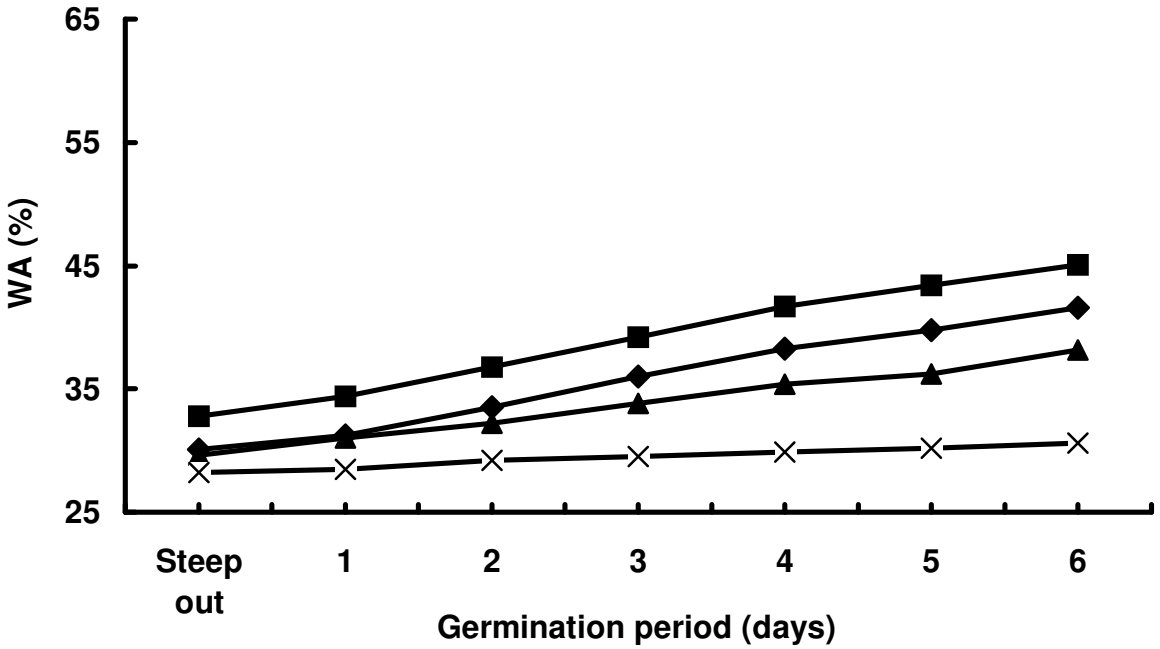
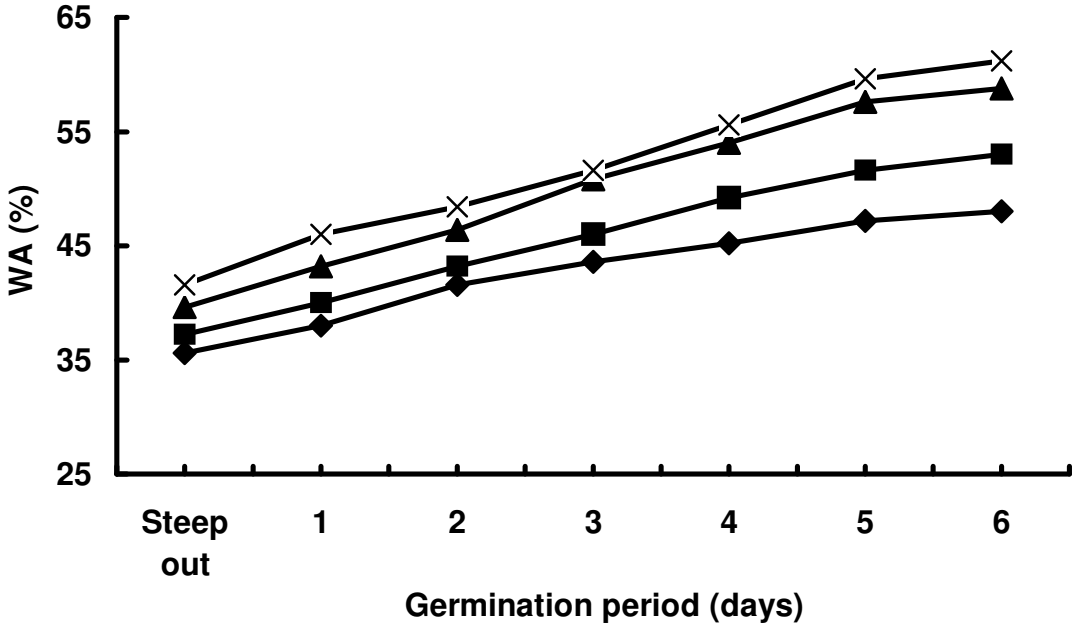


Figure 2.11. Effect of steeping NK 283 sorghum grains in different concentrations (0.1, 0.3 and 0.5 %) of $Ca(OH)_2$ on the water absorption (WA%) by the grains during germination. ◆–control; ■–0.1, ▲-0.3 and x-0.5 %. The error bars indicate the ± standard deviation.



(a)



(b)

Figure 2.12. Effect of steeping in different concentrations of NaOH (0.1, 0.2, 0.3%) the % water absorption (%WA) of the NK 283 (a) and PAN 8546 (b) sorghum grains during germination. ◆ – control; ■ – 0.1, ▲ - 0.2 and x – 0.3%.

The lower DP values obtained with NK 283 than with PAN 8546 are related to the poorer germinative energy of 42% of the NK 283 compared to 88% for PAN 8546. The poor quality of the NK 283 probably caused it to be sensitive to the 0.3% NaOH, which probably led to the death of most of the grains and thus very low DP values of NK 283. Figure 2.12 shows that there was a very low water uptake during germination of the NK 283 grains steeped in 0.3% NaOH which is indicative of the grains being poorly viable.

2.2.4.4. Mycotoxins and Cytotoxicity

Aflatoxins, Fumonisin, DON and ZEA were not detected in any of the samples analysed. This is in apparent contradiction with the results in Chapter 2.1. However, in that case the steep water was not changed, whereas it was changed twice during malting in this work. Changing the steep water reduced the fungal load and removed dissolved nutrients that would otherwise have supported fungal growth, and thus the production of mycotoxins. The 0.3 % NaOH steeped NK283 sample showed some toxicity with IC_{50} values of 32-62.5 mg/kg as compared to the other samples 250– >500 mg/kg in the absence of any mycotoxins (Table 2.4). This could have been as a result of toxic products from the sorghum caused by the phytotoxicity of the 0.3% NaOH during steeping. Notwithstanding this, the high sorghum malt IC_{50} values of 32–>500 mg/kg indicate that the malts were relatively non-toxic as these quantities are very large.

2.2.5. Conclusions

This report is the first to show that steeping sorghum grains in dilute NaOH reduces the levels of coliforms, reduces mould contamination (to undetectable levels with some mould species) and improves the sorghum malt quality by increasing the DP. Addition of $Ca(OH)_2$ during steeping does not reduce microbial populations, but reduces the level of contamination of some mould species and increases the DP at 0.1% $Ca(OH)_2$. Steeping the grain in up to 0.2% NaOH did not cause the malt exhibit any cytotoxicity. The addition of 0.2% NaOH in steeping water is proposed as a method for the control of bacterial and fungal contamination during sorghum malting.

Table 2.4. Concentration of aflatoxins (B_1, B_2, G_1 and G_2) fumonisins (B_1, and B_2), deoxynivalenol (DON), zearalenone (ZEA) and the IC_{50} levels of malts steeped in different concentrations of NaOH and $Ca(OH)_2$.

		IC^{1}_{50} (mg/kg)		**Aflatoxins[4] (µg/kg)**	**Fumonisin[3] (µg/g)**	**DON[4] (µg/kg)**	**ZEA[4] (µg/kg)**
		NK 283	**PAN 8546**	**NK 283 and PAN 8546**			
$Ca(OH)_2$	Control	250-500	>500[2]	< 0.25[5]	<3[1]	<3	<3
	0.1	250-500	NA[6]	< 0.25	<3	<3	<3
	0.3	250-500	NA	< 0.25	<3	<3	<3
	0.5	250-500	NA	< 0.25	<3	<3	<3
NaOH	Control	250-500	>500	< 0.25	<3	<3	<3
	0.1	>500	>500	< 0.25	<3	<3	<3
	0.2	>500	>500	< 0.25	<3	<3	<3
	0.3	32-62.5	250-500	1	<3	<3	<3

[1] Inhibitory concentration = concentration resulting in 50% inhibition of the MTT cleavage activity.

[2]Maximum concentration

[3]Determined using the Vicam kits

[4]Determined using TLC

[5]Minimum detection limit

[6]NA- Not analysed

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2.3. Antimicrobial activities of bacterial and yeast cultures in sorghum malting

2.3.1. Abstract

The effect of steeping with the addition of lactic acid bacteria (LAB), *Bacillus* spp. and yeast (S*accharomyces* spp) starter cultures on the levels of moulds, coliforms, mycotoxins, toxicity and on the diastatic power (DP) of sorghum malt was investigated. Two red tannin-free sorghum varieties were steeped in the water containing of 10^7–10^8 cfu/ml each of the bacteria and S*accharomyces* spp. starter cultures. A *Bacillus* culture (B1) was the most effective in the inhibition of several mould spp. using the dics assay method but failed to do likewise when inoculated into steep water. *P. pentosaceus* and *Saccharomyces* spp. cultures were more effective in reducing the level of moulds and coliforms contamination than the other LAB and *Bacillus* cultures when added to steep water. Steeping the sorghum with the addition of *Pediococcus pentosaceus* and S*accharomyces* spp. reduced the moulds to low levels of around 10^2 cfu/g. The coliforms were reduced to levels of $<10^1$-10^2 and 10^2 cfu/g by steeping with *P. pentosaceus* and S*accharomyces* spp. respectively. S*accharomyces* spp. reduced more mould spp. than *P. pentosaceus,* although both of them were effective in reducing the different mould spp. Steeping with all the cultures did not have any effect on the malt DP. There were no detectable amounts of mycotoxins and no indication of cytotoxicity in the malts. The use of *P. pentosaceus* and *Saccharomyces* spp. cultures could be an alternative to the alkali treatment.

2.3.2. Introduction

Undesirable sorghum malt microflora (coliforms and potential mycotoxin producing moulds) can be controlled by steeping in dilute NaOH, as shown in the Chapter 2.2. However, a recent development aims to inhibit the growth of unwanted bacteria and moulds by the addition of desirable microbial cultures as natural control agents in steeps (Lowe and Arendt, 2004). Lactic acid bacteria (LAB) and the yeast *Geotrichum candidum* have been shown by Laitila *et al.* (2002) and Boivin and Malanda (1997), respectively, to be effective at inhibiting unwanted microorganisms during steeping of barley grain.

The inhibition of coliforms and moulds by lactic acid bacteria (LAB) is attributed mainly to the low pH, resulting from the production of lactic and/or acetic acid (Nout and Rombouts, 1992). Synthesis of bacteriocins (Gourama and Bullerman, 1995a: Batish *et al*., 1997) and depletion of nutrients (Noots *et al*., 1999) may also play a role. The antimicrobial action of *G. candidum* has been attributed to competition between it and the other microbes because *G. candidum* was present at higher levels during malting than other microflora in all barley kernels analysed (Boivin and Malanda, 1997).

In addition to offering the potential to improve the safety and quality of malt, the use of microbial cultures in steeping is claimed to be easy to use, nature-friendly, inexpensive and not lead to the formation of toxic compounds, nor alter the nutritional and palatability properties of the grain product (Noots *et al*., 1999*;* Lowe and Arendt, 2004).

The objective of this study was to determine the effect of the addition of microbial cultures on the growth of coliforms and moulds, production of mycotoxins, the cytotoxicity and the diastatic power (DP) of sorghum malt.

2.3.3. Materials and methods

2.3.3.1. Sorghum grain

Two red tannin-free sorghum cultivars, NK 283 and PAN 8546 as described in Chapter were used.

2.3.3.2. Microorganisms used

Differerent types of microorganisms (10 LAB, 5 *Bacillus* spp. and 2 *Saccharomyces* spp) were analysed for their antimicrobial properties. The LAB cultures (*Lactobacillus plantarum, Lactococcus lactis, Pediococcus pentosaceus,* and *Leuconostoc* spp) were selected based on the fact that some of them, or their related strains, have been previously shown by other researchers to inhibit the growth and survival of some fungi (Batish *et al*., 1990; Suzuki *et al*., 1991; Gourama and Bullerman 1995a; Roy *et al*., 1996). The *Saccharomyces* spp. and *Bacillus* spp. cultures were selected because of the antifungal activities they showed during the microbial population analyses mentioned in Chapters 2.2 and 2.3. Table 2.5 shows all the test cultures used in the study.

Nine moulds (*Aspergillus flavus, Phoma sorghina, Curvularia* spp. *Fusarium oxysporum*, *Fusarium chlamydosporum*, *Fusarium verticillioides*, *Penicillium* spp. *Aspergillus fumigatus* and *Alternaria alternata*) were used. These were selected as they were the most dominant moulds identified in the sorghum malt samples in Chapter 2.1.

2.3.3.3. Maintenance of microorganisms

The LAB cultures were grown on MRS agar at 30ºC and stored on MRS plates at 5ºC. Liquid suspensions of the cultures were grown in MRS broth at 30ºC. The *Saccharomyces* spp. cultures were grown and stored on malt extract broth and/or agar. The *Bacillus* spp. cultures were grown on nutrient agar at 35ºC and stored on nutrient agar and/or broth. The mould cultures were cultivated and maintained on PDA slants in McCartney bottles at 5ºC until further use. The cultures were subcultured at monthly intervals.

2.3.3.4. Disc diffusion assay

To select cultures that could subsequently be used to inhibit the growth of moulds and coliforms during sorghum malting, the antifungal activity of the LAB, *Bacillus* and *Saccharomyces* spp. was determined against the eight moulds by the disc diffusion method (Piddock, 1990; Niku-Paavola, Laitila, Mattila-Sandholm and Haikara 1999). Moulds were incubated for 5 days at 25ºC. Mould growth on slants was harvested into sterile saline (9%) containing 0.1% Tween-80 by brushing the slant surface with a sterile loop. The mould suspension was subjected to vigorous agitation using a vortex mixer to break the clumps. The spore count was then determined with the aid of a haemocytometer and the suspension standardised to a final concentration of 10^7 spores per ml. A 100 µl aliquot of mould spore suspension was transferred to PDA plates and spread uniformly over the agar surface with a sterile bent glass rod. Sterilized filter paper discs (Whatman no 1, 5 mm diameter) were saturated with 100 µl of each of the test cultures listed in Table 2.5. The discs were allowed to air-dry. The dry discs were placed on inoculated PDA plates. Each culture was tested in duplicate. Each plate also contained one disc with cyclohexamide (1mg/ml in sterile distilled water) as a positive control and another disc with a sterile uninoculated LAB or PDA broth as a negative control. The plates were incubated at 25ºC for 24 hours. Zones of inhibition indicating antifungal activity formed around each disc, if present, were measured in mm. Clear zones of more that 0.5 mm were considered as positive for inhibition (El-Adawy, 2001). The test cultures that showed positive inhibition against more than two mould species were selected and subsequently tested for their ability to inhibit the growth of moulds and coliforms during laboratory scale malting of naturally contaminated sorghum grain.

Table 2.5. LAB, *Bacillus* spp. and *Saccharomyces* spp. cultures tested for antifungal activity with the disc diffusion assay.

Cultures (Experimental number)	Original culture numbers	Source
Lactic acid bacteria		
Lactobacillus spp. (L1)	NA	Sorghum malt, University of Pretoria, South Africa
Lactobacillus spp. (L2)	NA	Sorghum malt, University of Pretoria, South Africa
Leuconostoc spp. (L3)	NA	Sorghum malt, University of Pretoria, South Africa
Lactobacillus plantarum (L4)	NA	Sorghum malt, University of Pretoria, South Africa
Pediococcus pentosaceus (L5)	Culture no 13	Cottage cheese, University of Pretoria, South Africa
Pediococcus pentosaceus (L6)	Culture no 79	Cottage cheese, University of Pretoria, South Africa
Lactococcus lactis (L7)	B 841 (DSM 20384)	CSIR culture collection, Pretoria, South Africa
Lactobacillus plantarum (L8)	B 846	CSIR culture collection, Pretoria, South Africa
Lactobacillus plantarum (L9)	B 845	CSIR culture collection, Pretoria, South Africa
Lactobacillus lactis (L10)	B 842 (DSM 20661)	CSIR culture collection, Pretoria, South Africa
***Bacillus* spp.**		
Bacillus spp. (B1)	NA	Rice, University of Pretoria, Pretoria, South Africa
Bacillus spp. (B2)	NA	Rice, University of Pretoria, Pretoria, South Africa
Bacillus spp. (B3)	NA	Sorghum malt, University of Pretoria, South Africa
Bacillus spp. (B4)	NA	Sorghum malt, University of Pretoria, South Africa
Bacillus spp. (B5)	NA	Sorghum malt, University of Pretoria, South Africa
***Saccharomyces* spp.**		
Saccharomyces spp. (Y1)	NA	Sorghum malt, University of Pretoria, South Africa
Saccharomyces spp. (Y2)	NA	Sorghum malt, University of Pretoria, South Africa

NA- Not available

2.3.3.5. Laboratory scale malting

Samples (150 g) of the two sorghum cultivars were rinsed and then steeped at 25°C for 8 h in 300 ml of still tap water (control). The test cultures (LAB, *Bacillus* spp. and *Saccharomyces* spp), including cells and spent media were added into the 300 ml steeping water to make a final concentration of cultures of about 10^7–10^8 cfu/g. Mixed cultures, were also investigated with different proportions of the *P. pentosaceus* (L5) and *Saccharomyces* spp. (Y1) cultures, prepared as follows: 50% L5 + 50% Y1; 65% L5 and 35% Y1 and 35% L5 + 65% Y1, to make up the 10^7–10^8 cfu/ml of the mixed cultures in steep water. The inoculated grain was similarly steeped at 25ºC for 8 h. After steeping, the grain was rinsed and then germinated as described in Chapter 2.2.

2.3.3.6. Analyses

2.3.3.6.1. pH

The pH was determined on the steep water that was drained after steeping for 8 h.

2.3.3.7. Other analyses

The following assays were performed as described in Chapter 2.1 on the sorghum grain at 0 h, at 8 h before rinsing, at 8 h after rinsing, on the second and fourth day during malting (green malt) and on the dry malt.

1. Microbial population: Aerobic plate counts (APC), Lactic acid bacteria (LAB), yeasts, moulds and coliforms.
2. Mould identification
3. Diastatic Power
4. Aflatoxins, Fumonisins, Deoxynivalenol (DON) and Zearalenone.
5. Cytotoxicity

2.3.3.8. Stastical analysis

Duplicate samples were evaluated during each analysis and the experiments were repeated three times to obtain a total of 6 observations for each analysis. The data were analysed using the STATGRAPHICS 5.0 program (Statistical Graphics Corporation, Rockville, USA). Analysis of variance (ANOVA) was used at 0.05 level of significance. Differences between means were determined by the least significant difference method.

2.3.4. Results and discussion

2.3.4.1. Microbial growth inhibition

2.3.4.1.1. Disc diffusion assay

Table 2.6 shows the antifungal activity of the LAB, *Bacillus* spp. and *Saccharomyces* spp. cultures against the eight moulds, using the disc diffusion assay. *Bacillus* spp. (B1) showed inhibition against all mould species. *P. pentosaceus* (L5) showed inhibition against five moulds. *Bacillus* spp. (B4) and *Saccharomyces* spp. (Y1) were antagonistic against four moulds and *L. plantarum* (L9) against three moulds. The other cultures were antagonistic against 2 or less moulds or showed no antagonistic activity against any of the moulds. The inhibition of moulds observed in this assay could be attributed mainly to the production of extracellular compounds by the LAB, *Bacillus* and *Saccharomyces* cultures. It has been found that some strains of *L. plantarum* and *P. pentosaceus* (Batish *et al*., 1989; Suzuki *et al*., 1991; Gourama and Bullerman 1995a; Roy *et al*., 1996; Gourama, 1997) and *Bacillus* (Wang, Shih, Wang, Tseng, Chang, Twu, Ro and Wang, 2002; Kim and Chung, 2004) produce antifungal proteins. Antifungal activity of *Saccharomyces* is not documented.

The cultures which showed inhibition against more than two moulds (B1, B4, L5, L9 and Y1) were tested for their ability to inhibit the growth of moulds and coliforms during laboratory scale malting.

Table 2.6. Antimicrobial activity (inhibition zones in mm) of the LAB, yeast and *Bacillus* spp. cultures tested against different mould cultures using the disc diffusion assay

	A. flavus	*P. sorghina*	*Curvularia* spp	*F. oxysporum*	*F. chlamydosporum*	*F. verticillioides*	*Penicillium* spp.	*A. alternata*	*A. fumigatus*
Positive Control[1]	4.0	5.5	5.0	5.0	4.5	4.8	4.0	4.2	4.5
Negative Control	-[2]	-	-	-	-	-	-	-	-
Lactobacillus spp. (L1 & L2)	-	-	-	-	-	-	-	-	-
Leuconostoc spp. (L3)	-	-	-	-	-	-	-	-	-
Lactobacillus plantarum (L4 & L8)	-	-	-	-	-	-	-	-	-
Lactococcus lactis (L7)	-	-	-	-	-	-	-	-	-
Pediococcus pentosaceus (L5 & L6)	-	2.4	1.0	1.4	2.0	2.4	-	-	-
Lactobacillus plantarum (L9)	-	1.2	1.5	2.1	-	-	-	-	-
Lactobacillus lactis (L10)	-	2.1	-	-	-	1.4	-	-	-
Bacillus spp. (B1)	3.8	6.0	5.5	5.3	5.2	5.3	4.2	2.6	3.2
Bacillus spp. (B2)	-	-	-	-	-	-	3.0	-	-
Bacillus spp. (B3)	-	-	2.4	2.6	-	-	-	-	-
Bacillus spp. (B4)	-	6.0	3.0	3.1	-	-	3.1	-	-
Bacillus spp. (B5)	-	-	-	-	-	3.0	2.5	-	-
Saccharomyces spp. (Y1)	-	-	1.7	-	2.0	-	2.9	-	1.9
Saccharomyces spp. (Y2)	-	-	-	-	-	-	2.9	-	-

[1]1 mg/ml cyclohexamide

[2]No inhibition

2.3.4.2. Laboratory scale malting

The sorghum cultivar NK 283 represented very poor grain quality (40% germinability) and had more microbial contamination than PAN 8546, which represented a better quality grain (88% germinability). Tables 2.7-2.11 show the effect of the addition of different cultures during steeping on the APC (Table 2.7), LAB (Table 2.8), yeasts (Table 2.9), moulds (Table 2.10) and coliforms (Table 2.11) during germination of the two sorghum cultivars. Steeping conditions were ideal for the growth of microorganisms carried on sorghum grains because the total aerobic counts showed an increase from 10^4-10^7 cfu/g at the beginning of steeping, to 10^7-10^9 cfu/g at the end of the 8 hours of steeping, before rinse (Table 2.7). This might be due to the fact that during steeping nutrients leach from the grains into the steep water and become available for microorganisms and therefore they grow well (Priest and Campbell, 1996). At the end of steeping, discarding the steep water reduced the number of microorganisms, i.e. the microorganisms were washed out. The numbers increased again during the four days of germination and then drying the malt decreased the numbers. The addition of all the cultures with both sorghum cultivars increased APC (Table 2.7), except that the APC in the Y1 inoculated sample of the NK 283 was lower throughout the malting process. The reason for this might be that the APC were outgrown by the inoculated yeast. There was also no inhibition of LAB and yeasts (Tables 2.8 and 2.9, respectively) by any of the cultures on either of the sorghum cultivars. The LAB in the L5 and L9 samples were higher than in the B1, B4 and Y1 samples at 0 h and throughout the malting process (Table 2.8). This was because the inoculation was carried out with the *L. plantarum* (L5) and *P. pentosaceus* (L9), which are LAB. Inoculation with *Saccharomyces* spp. (Y1), presumably similarly resulted in elevated numbers of the yeasts at 0 h and throughout the malting process (Table 2.9).

With the malts made from both sorghum cultivars, steeping with both the *Bacillus* spp. cultures (B1 and B4) did not cause a significant reduction ($p > 0.05$) of moulds (Table 2.10). This is contradictory to the disc assay results which indicated that B1 and B4 inhibited more mould spp. than the other cultures. It is possible that the antifungal substance(s) produced when the *Bacillus* spp. is grown on a bacteriological medium are either not produced or are suppressed when it is inoculated during steeping. The reason for that might be that the microbiological media was more

suitable for growth of the *Bacillus* spp. due to the more available nutrients than the malting environment. With both sorghum cultivars, L5, L9 and Y1 caused a significant decrease ($p< 0.05$) in moulds. With NK 283, steeping with L5 and L9 reduced the mould counts from 5.7×10^4 cfu/g to 7.1×10^2 and 4.0×10^3 cfu/g, respectively. With PAN 8546, steeping with L5 and L9 reduced the mould counts from 2.7×10^3 to 8.6×10^2 and 8.8×10^2 cfu/g, respectively. The moulds in the malts made from the Y1 steeped grain were inhibited to low levels of 1.8×10^2 cfu/g (NK 283) and 1.3×10^1 cfu/g (PAN 8546).

With both cultivars, steeping with the LAB cultures (L5 and L9) inhibited the coliforms to significantly lower ($p< 0.05$) numbers. The coliforms in the malts made with NK 283 and PAN 8546 grain steeped in L5 were reduced from to 1.7×10^4 and to 6.3×10^2 cfu/g, respectively to $<10^1$ cfu/g (undetectable levels), whereas steeping with L9 inhibited the coliforms in the malt to 2.6×10^1 cfu/g (NK 283) and $<10^1$ cfu/g (PAN 8546). Steeping with Y1 also caused a significant decrease ($p< 0.05$) in the coliforms with both sorghum cultivars (Table 2.11). The coliforms were inhibited to 7.2×10^1 (NK 283) and 8.7×10^1 cfu/g (PAN 8546).

Production of antimicrobial compounds by *Saccharomyces* spp. (Y1) is not documented. Therefore the antimicrobial effect of the Y1 culture against moulds (Table 2.10) and coliforms (Table 2.11) might be attributable to microbial competition, as is the case with *G. candidum* during barley malting (Boivin and Malanda, 1997). Yeasts are good biocontrol agents because they are capable of utilizing available nutrients to proliferate rapidly, colonize and survive for long periods of time under different conditions and therefore limit nutrient availability to bacteria and moulds (Jones and Prusky, 2002). The detection of yeasts in higher numbers (Table 2.9) than the moulds (Table 2.10) and coliforms (Table 2.11) in the Y1 sample is an indication that Y1 was able to outcompete the coliforms and moulds and inhibit their growth.

Table 2.7. Effect of steeping NK 283 and PAN 8546 sorghum grains in different microbial cultures on the growth of total aerobic plate count (APC) during the malting process

		0 hours	8 hours before rinse	8 hours after rinse	2 days	4 days	Dry malt
NK 283	Control	3.1×10^{5a1}	4.9×10^{7a}	1.8×10^{4d}	1.1×10^{5cd}	2.7×10^{6f}	1.4×10^{4cd}
	L5	2.6×10^{7bc}	1.1×10^{9f}	4.6×10^{4e}	1.4×10^{5de}	5.4×10^{5d}	2.8×10^{4e}
	L9	3.9×10^{7cd}	5.8×10^{8de}	2.8×10^{4d}	6.8×10^{4bc}	3.5×10^{5bcd}	1.6×10^{4d}
	B1	2.5×10^{7bc}	2.1×10^{9g}	3.0×10^{4d}	1.0×10^{5cd}	4.1×10^{5cd}	9.0×10^{4bcd}
	B4	5.5×10^{7d}	3.7×10^{7a}	4.4×10^{4e}	1.6×10^{5e}	1.6×10^{6e}	3.8×10^{4f}
	Y1	6.1×10^{5a}	7.1×10^{7ab}	1.3×10^{4bc}	7.4×10^{3a}	1.9×10^{4a}	7.3×10^{3abc}
PAN 8546	Control	4.2×10^{4a}	9.8×10^{5a}	9.0×10^{2a}	5.5×10^{3a}	1.3×10^{4a}	4.4×10^{2a}
	L5	2.5×10^{7bc}	5.4×10^{8de}	2.7×10^{3a}	4.9×10^{4ab}	2.2×10^{5abc}	4.3×10^{3ab}
	L9	2.0×10^{7b}	2.6×10^{8abc}	1.6×10^{3a}	2.6×10^{4ab}	1.1×10^{5ab}	1.9×10^{3ab}
	B1	9.5×10^{6ab}	4.2×10^{8cd}	6.6×10^{3ab}	3.8×10^{3a}	2.9×10^{4a}	5.2×10^{3ab}
	B4	2.6×10^{7bc}	8.1×10^{8e}	1.1×10^{4b}	4.4×10^{4ab}	1.4×10^{5ab}	6.4×10^{3ab}
	Y1	1.6×10^{7ab}	3.3×10^{8cbd}	1.4×10^{3a}	4.2×10^{3a}	3.1×10^{4a}	4.6×10^{3ab}

[1] Mean and values with different letters (^{abcd}) in the same column are significantly different from each other ($p < 0.05$).

Table 2.8. Effect of steeping NK 283 and PAN 8546 sorghum grains in different microbial cultures on the growth of LAB during the malting process

		0 hours	8 hours before rinse	8 hours after rinse	2 days	4 days	Dry malt
NK 283	Control	2.2×10^{3a1}	5.2×10^{5a}	5.0×10^{2a}	1.2×10^{3a}	8.3×10^{3a}	3.9×10^{2a}
	L5	4.5×10^{7c}	7.5×10^{8d}	8.3×10^{3a}	1.9×10^{4a}	4.9×10^{5a}	2.6×10^{4a}
	L9	2.7×10^{7b}	3.0×10^{8c}	5.9×10^{3a}	8.3×10^{4a}	1.7×10^{6a}	7.8×10^{4a}
	B1	4.5×10^{3a}	2.4×10^{6a}	4.9×10^{2a}	9.1×10^{3a}	2.4×10^{4a}	2.4×10^{2a}
	B4	1.9×10^{4a}	4.7×10^{6a}	7.0×10^{2a}	9.8×10^{3a}	2.9×10^{4a}	1.5×10^{3a}
	Y1	5.9×10^{3a}	2.8×10^{7a}	1.4×10^{6b}	4.5×10^{7b}	9.0×10^{7b}	3.7×10^{6b}
PAN 8546	Control	1.5×10^{3a}	1.4×10^{5a}	1.4×10^{2a}	4.3×10^{3a}	4.7×10^{4a}	1.4×10^{3a}
	L5	4.1×10^{7b}	9.2×10^{7b}	9.3×10^{3a}	4.4×10^{4a}	7.3×10^{5a}	1.8×10^{4a}
	L9	3.4×10^{7b}	8.6×10^{7b}	7.4×10^{3a}	4.3×10^{4a}	6.9×10^{5a}	1.4×10^{4a}
	B1	1.9×10^{3a}	2.5×10^{4a}	2.5×10^{2a}	5.5×10^{3a}	1.0×10^{4a}	2.2×10^{2a}
	B4	1.0×10^{3a}	4.2×10^{4a}	4.2×10^{2a}	4.2×10^{3a}	1.9×10^{4a}	3.3×10^{2a}
	Y1	1.9×10^{3a}	8.6×10^{4a}	1.9×10^{2a}	6.0×10^{3a}	1.5×10^{4a}	6.9×10^{5a}

[1] Mean and values with different letters (^{ab}) in the same column are significantly different from each other ($p < 0.05$).

Table 2.9. Effect of steeping NK 283 and PAN 8546 sorghum grains in different microbial cultures on the growth of yeasts during malting process

		0 hours	8 hours before rinse	8 hours after rinse	2 days	4 days	Dry malt
NK 283	Control	8.9×10^{3a1}	9.9×10^{4a}	5.5×10^{3d}	1.7×10^{4d}	5.1×10^{4de}	2.1×10^{3a}
	L5	4.3×10^{3a}	3.1×10^{4a}	1.9×10^{2a}	9.0×10^{2a}	1.3×10^{4ab}	5.2×10^{2a}
	L9	1.5×10^{4a}	5.7×10^{4a}	7.0×10^{2ab}	2.1×10^{3b}	3.6×10^{4bcd}	1.7×10^{3a}
	B1	6.5×10^{4a}	3.9×10^{4a}	2.6×10^{3c}	7.0×10^{3c}	4.5×10^{4cd}	5.6×10^{3b}
	B4	2.7×10^{4a}	7.0×10^{4a}	1.6×10^{3b}	7.1×10^{3c}	1.9×10^{4abc}	7.8×10^{3b}
	Y1	1.9×10^{7b}	2.0×10^{8c}	6.5×10^{3e}	3.1×10^{4d}	1.6×10^{5f}	2.9×10^{4d}
PAN 8546	Control	5.4×10^{3a}	2.5×10^{5a}	2.8×10^{2b}	7.4×10^{2a}	2.6×10^{3a}	3.0×10^{2a}
	L5	1.8×10^{3a}	7.1×10^{4a}	4.1×10^{2a}	1.0×10^{3b}	8.0×10^{3ab}	4.2×10^{2a}
	L9	2.7×10^{3a}	4.8×10^{4a}	3.3×10^{2a}	1.6×10^{3b}	2.5×10^{4abcd}	9.1×10^{2a}
	B1	4.2×10^{3a}	6.9×10^{4a}	7.0×10^{2ab}	5.2×10^{3bc}	4.8×10^{3a}	2.6×10^{2a}
	B4	6.0×10^{3a}	4.2×10^{4a}	9.0×10^{2ab}	4.8×10^{3bc}	3.2×10^{4abcd}	7.8×10^{3c}
	Y1	7.4×10^{7a}	3.8×10^{7b}	3.2×10^{3c}	3.3×10^{3bc}	8.0×10^{4e}	7.7×10^{2a}

[1] Mean and values with different letters (^{abcd}) in the same column are significantly different from each other ($p < 0.05$).

Table 2.10. Effect of steeping NK 283 and PAN 8546 sorghum grains in different microbial cultures on the growth of moulds during the malting process

		0 hours	8 hours before rinse	8 hours after rinse	2 days	4 days	Dry malt
NK 283	Control	3.2×10^{4a1}	3.8×10^{5d}	1.4×10^{4bc}	5.2×10^{4b}	2.1×10^{5d}	5.7×10^{4c}
	L5	3.6×10^{4a}	9.3×10^{4cd}	1.8×10^{4bc}	9.3×10^{3ab}	3.2×10^{3b}	9.7×10^{2a}
	L9	3.5×10^{4a}	1.5×10^{5b}	3.0×10^{4c}	3.2×10^{4b}	1.1×10^{4bc}	4.0×10^{3b}
	B1	3.5×10^{4a}	9.0×10^{5de}	2.1×10^{4bc}	9.0×10^{4bc}	5.3×10^{5d}	9.0×10^{4cd}
	B4	3.5×10^{4a}	1.7×10^{6e}	2.4×10^{4bc}	8.8×10^{4bc}	2.0×10^{5b}	4.1×10^{4c}
	Y1	3.1×10^{4a}	7.3×10^{5de}	7.2×10^{3b}	1.7×10^{4b}	5.1×10^{3b}	6.8×10^{2a}
PAN 8546	Control	2.9×10^{3b}	7.2×10^{4bc}	1.1×10^{3ab}	3.2×10^{3a}	1.1×10^{4a}	2.7×10^{3b}
	L5	1.8×10^{3b}	4.5×10^{2a}	5.4×10^{2a}	2.1×10^{3a}	4.0×10^{2ab}	8.6×10^{2a}
	L9	1.9×10^{3b}	1.5×10^{3a}	1.1×10^{3ab}	3.8×10^{3a}	1.9×10^{3ab}	8.8×10^{2ab}
	B1	2.2×10^{3b}	8.5×10^{4c}	7.2×10^{2a}	9.0×10^{4bc}	2.5×10^{4c}	2.0×10^{3b}
	B4	1.6×10^{3b}	1.9×10^{5d}	4.2×10^{2a}	2.9×10^{3a}	4.7×10^{4c}	3.0×10^{3b}
	Y1	2.6×10^{3b}	1.2×10^{2a}	3.4×10^{2a}	1.7×10^{4b}	3.4×10^{2a}	1.3×10^{2a}

[1] Mean and values with different letters (^{abcde}) in the same column are significantly different from each other ($p < 0.05$).

Table 2.11. Effect of steeping NK 283 and PAN 8546 sorghum grains in different microbial cultures on the growth of coliforms during the malting process

		0 hours	8 hours before rinse	8 hours after rinse	2 days	4 days	Dry malt
NK 283	Control	8.7×10^{3ba}	1.3×10^{5b}	1.9×10^{2bc}	6.5×10^{2bc}	8.2×10^{4d}	1.7×10^{4c}
	L5	1.3×10^{2} a	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$
	L9	2.7×10^{3ab}	2.0×10^{2a}	2.4×10^{1ab}	2.0×10^{2ab}	1.2×10^{1a}	2.6×10^{1a}
	B1	2.7×10^{4c}	5.3×10^{4ab}	5.3×10^{2cf}	2.7×10^{3e}	7.0×10^{5e}	2.3×10^{4c}
	B4	3.3×10^{3ab}	3.4×10^{4a}	4.3×10^{2de}	7.3×10^{2c}	4.0×10^{3c}	1.9×10^{2b}
	Y1	2.6×10^{3ab}	3.1×10^{3a}	2.5×10^{2cd}	6.0×10^{2bc}	1.8×10^{2b}	5.2×10^{2b}
PAN 8546	Control	2.6×10^{2a}	8.9×10^{3a}	7.2×10^{1abc}	2.5×10^{2abc}	7.2×10^{2bc}	6.3×10^{1ab}
	L5	2.1×10^{2a}	$<10^{1}$	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$
	L9	1.7×10^{2a}	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$	$<10^{1a}$
	B1	1.5×10^{2a}	3.0×10^{3a}	1.3×10^{2abc}	2.9×10^{2abc}	6.7×10^{4d}	8.9×10^{2bc}
	B4	1.8×10^{2a}	1.7×10^{1a}	6.5×10^{2f}	2.1×10^{3d}	5.3×10^{4d}	4.8×10^{2b}
	Y1	1.7×10^{2a}	1.4×10^{2a}	5.0×10^{1ab}	1.6×10^{2ab}	1.0×10^{2b}	8.7×10^{1ab}

[1] Mean and values with different letters (^{abcdef}) in the same column are significantly different from each other ($p< 0.05$).

Table 2.12. Incidence of mould species (% grains infected) and the diastatic power, (SDU/g) of the NK 283 and PAN 8546 malt samples made with L5 and Y1 steeped grains

		Penicillium spp	*Eurotium* spp	*F. chlamyd-osporum*	*A. alternata*	*P. sorghina*	*F. verticillioides*	*R. oryzae*	*Mucor* spp.	Diastatic Power
NK 283	Control	36c ± 6	37d ± 7	56d ± 6	70d ± 10	62d ± 9	88c ± 6	100d ± 0	100e ± 0	8.5a ± 0
	L5	14b ± 4	22c ± 3	28c ± 4	23c ± 4	30c ± 4	40b ± 2	32c ± 4	36d ± 1	9.0a ± 0
	Y1	9a ± 3	7ab ± 1	23bc ± 2	19bc ± 2	25bc ± 4	35b ± 1	26bc ± 2	25c ± 2	8.6a ± 3
PAN 8546	Control	15b ± 3	18bc ± 2	25bc ± 1	6ab ± 2	18abc ± 2	33b ± 2	22b ± 2	21c ± 2	18.5b ± 3
	L5	6a ± 2	10ab± 2[b]	14ab± 1	0a ± 0	8ab ± 2	14a ± 2	10a ± 3	8b ± 1	20.0b ± 9
	Y1	4a ± 1	7a ± 1	4a ± 2	0a ± 0	0a ± 0	6a ± 1	4a ± 1	0a ± 0	19.3b ± 1

[1] Mean and values with different letters ([abcd]) in the same column are significantly different from each other ($p < 0.05$).

[2]Mean ± standard deviation

The malts where the greatest mould inhibition had taken place, i.e. malts made with L5 and Y1 steeped grain, were further analysed to evaluate the extent to which individual mould species were inhibited (Table 2.12). It is notable that PAN 8546 was less contaminated than NK 283. This is probably due to the fact that PAN 8546 was of a better quality than NK 283 with respect to much higher germinability (88% for PAN 8546 and 40% NK 283). Bad quality grains are more susceptible to fungal infections, due to the available excess oxygen that is not utilised fully by the bad grain and therefore the fungi utilize it for their own growth (Briggs, 1998). All the mould species (*Penicillium* spp. *Eurotium* spp. *F. chlamydosporum*, *A. alternata*, *P. sorghina*, *F. verticillioides*, *R. oryzae* and *Mucor* spp) in both NK 283 and PAN 8546 were significantly reduced by steeping with L5 and Y1. The reduction of most of the moulds (*P. sorghina, R. oryzae*, *Mucor* spp. *Eurotium* spp. *A. alternata* and *Penicillium* spp) was significantly greater ($p < 0.05$) in the Y1 steeped malt than in the L5 malt. With the Y1 treatment of PAN 8546, mould contamination of the malt was negligible.

With both the NK 283 and PAN 8546 cultivars, addition of the LAB cultures, L5 and L9, reduced the pH of the steep water from 6.0-6.4 at the beginning of steeping to 3.9-4.0 at the end of the eight hour steeping regime (Figure 2.13). The pH of the controls was also reduced to a small extent, from around 6.0 at the beginning of steeping to 4.5-4.8 at the end of steeping, whereas the pH of the *Bacillus* (B1 and B4) and the yeast cultures (Y1) were not reduced (pH 6.0 or higher at the end of steeping). The very low pH observed with the LAB cultures was due to the production of lactic acid by the cultures and it could have been responsible for the inhibition of coliforms by *P. pentosaceus*. Low pH inhibits coliforms and other food-borne contaminants by dissociating their cell membranes (Stiles, 1996). Previous studies on the antimicrobial substances of the *P. pentosaceus* spp. used in this study (Gurira and Buys, 2005 demonstrated that pediocin was produced by this strain and that the pediocin was responsible for its antimicrobial activities against other bacteria e.g. *Lactococcus lactis*, *Bacillus cereus* and *Listeria monocytogenes*. However, pediocins are not active against Gram-negative pathogens (Holzapfel, Geisen and Schillinger, 1995) and might therefore not have been responsible for the inhibition of the coliforms. The production of low molecular weight compounds (benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2H-

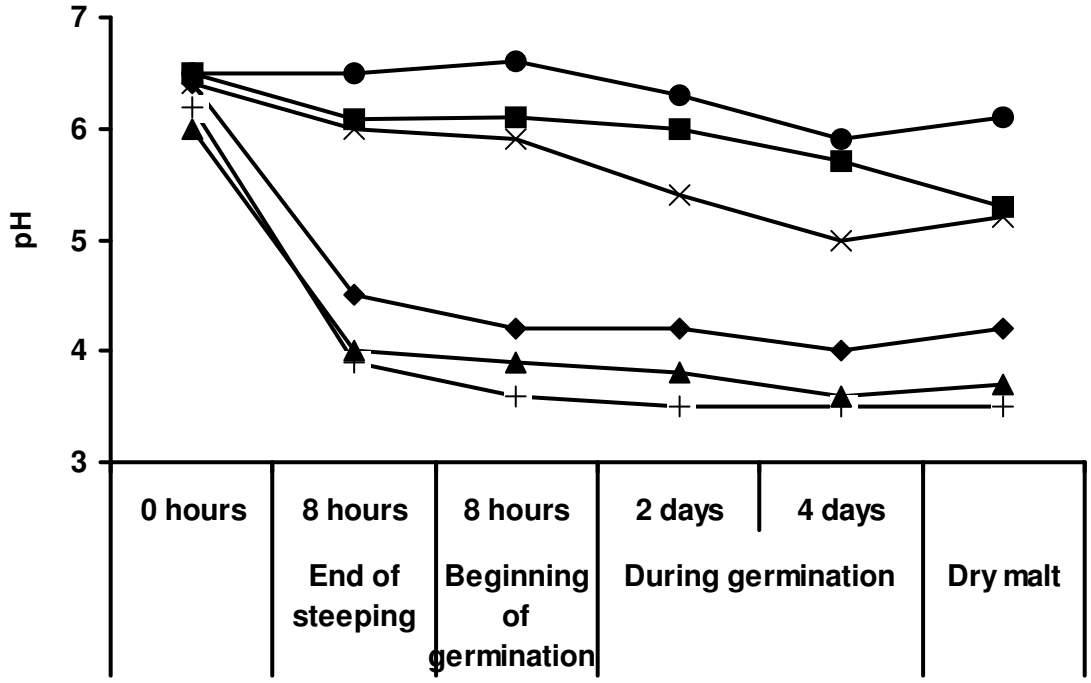
pyran-2-one and 3-(2-methylpropyl) -2,5-piperazinedione) by LAB that are active against Gram-positive, Gram-negative bacteria and moulds have been reported (Niku-Pavoola *et al*., 1999). It is possible that the *P. pentosaceus* spp. used in this study produced one or more of these low molecular weight antimicrobial compounds. It is also possible that the *P. pentosaceus* spp. produced antifungal compounds as it has been shown that numerous LAB exhibit antifungal activities and the active factors have been identified (Lowe and Arendt, 2004). The inhibition of coliforms to undetectable levels by the *P. pentosaceus* (L5) could be attributable to competition for nutrients (Holzapfel *et al*., 1995).

2.3.4.3. Diastatic Power (DP)

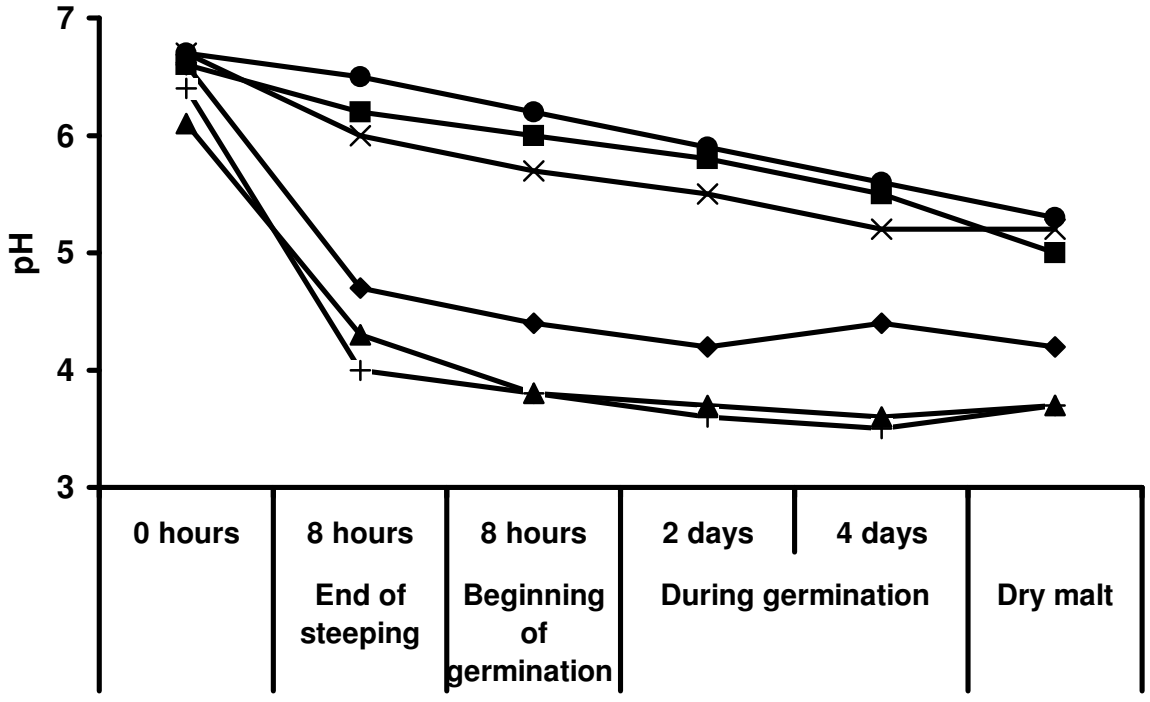
The amylase activities (DP) of the malts which had the greatest mould inhibition, i.e. malts made by steeping with L5 and Y1 were determined. There was no significant effect ($p > 0.05$) of the addition of L5 and Y1 cultures on the DPs of the malts (Table 2.12). The cultures therefore do not contribute to the increase in the amylase activities unlike with steeping with dilute alkali (Chapter 2.2). However, neither did they inhibit the amylase activity.

2.3.4.4. Combined cultures

With both sorghum cultivars the Y1 culture was able to eliminate more mould species than the L5 culture (Table 2.10). However, the L5 culture was able to inhibit coliforms to a greater extent than the Y1 culture did (Table 2.11). Therefore, the two cultures were mixed and added during steeping with the aim that the antimicrobial effects observed when they were used separately would be increased. There was no significant difference ($p > 0.05$) in the inhibition of moulds (Table 2.13) and coliforms (Table 2.14) between the combined cultures and the controls. However, all the combined cultures possessed less antimicrobial activity against moulds and coliforms (Tables 2.13 and 2.14, respectively) as compared to the L5 and Y1 when used individually (Tables 2.10 and 2.11). This was probably the result of competition between the combined cultures for nutrients, leading to them not growing optimally, contrary to the situation when they were inoculated individually.



(a)



(b)

Figure 2.13. Effect of steeping the NK 283 (a) and PAN 8546 (b) sorghum grains in different microbial cultures on the pH during the malting process. ◆-Control; +–L5; ▲ –L9; x–Y1; ■–B1; ●–B4.

Table 2.13. Effect of steeping the NK 283 (a) and PAN 8546 (b) sorghum grains in the combined microbial cultures on the growth of moulds during the malting process

		0 hours	8 hours before rinse	8 hours after rinse	2 days	4 days	Dry malt
NK 283	Control	2.0×10^{3b1}	1.0×10^{5bc}	1.3×10^{3b}	5.0×10^{3b}	2.0×10^{3c}	6.3×10^{2a}
	50% L5 + 50% Y1	3.2×10^{3b}	7.9×10^{4b}	4.3×10^{3b}	4.0×10^{3b}	1.3×10^{3b}	4.0×10^{2a}
	65% L5 + 35% Y1	3.2×10^{3b}	7.9×10^{4b}	2.0×10^{3b}	3.2×10^{3b}	2.0×10^{3b}	3.2×10^{2a}
	35% L5 + 65% Y1	2.5×10^{3b}	1×10^{5bc}	6.3×10^{3b}	3.2×10^{3b}	1.6×10^{3b}	3.2×10^{2a}
PAN 8546	Control	3.4×10^{2a}	1.0×10^{4b}	6.0×10^{2a}	1.8×10^{2a}	7.9×10^{2ab}	1.2×10^{2a}
	50% L5 + 50% Y1	3.6×10^{2a}	5.7×10^{3a}	3.4×10^{2a}	1.6×10^{2a}	1.0×10^{3b}	1.0×10^{2a}
	65% L5 + 35% Y1	4.0×10^{2a}	3.2×10^{3a}	4.3×10^{2a}	4.5×10^{2a}	3.0×10^{2a}	1.6×10^{2a}
	35% L5 + 65% Y1	3.8×10^{2a}	1.5×10^{3a}	5.0×10^{2a}	3.8×10^{2a}	2.5×10^{2a}	1.5×10^{2a}

[1] Mean and values with different letters (^{abc}) in the same column are significantly different from each other ($p < 0.05$).

Table 2.14. Effect of steeping the NK 283 (a) and PAN 8546 (b) sorghum grains in the combined microbial cultures on the growth of coliforms during the malting process

		0 hours	8 hours before rinse	8 hours before rinse	2 days	4 days	Dry malt
NK 283	Control	1.0×10^{4b1}	9.5×10^{4b}	6.3×10^{2ab}	1.6×10^{3b}	1.9×10^{2b}	2.2×10^{2b}
	50% L5 + 50% Y1	3.2×10^{2a}	6.3×10^{3a}	2.3×10^{2a}	1.2×10^{3b}	1×10^{3c}	1.6×10^{2b}
	65% L5 + 35% Y1	4.0×10^{2a}	3.2×10^{3a}	1.5×10^{3}	6.0×10^{2a}	3.2×10^{2a}	1.8×10^{2b}
	35% L5 + 65% Y1	2.9×10^{2a}	1.3×10^{3a}	5.2×10^{2a}	4.0×10^{2a}	2.5×10^{2b}	1.5×10^{2b}
PAN 8546	Control	6.3×10^{2a}	1.0×10^{4b}	1×10^{3b}	6.3×10^{3b}	2.0×10^{3c}	2.4×10^{2b}
	50% L5 + 50% Y1	3.8×10^{2a}	5.9×10^{3a}	5.0×10^{2a}	2.5×10^{2a}	1×10^{2a}	6.0×10^{1ab}
	65% L5 + 35% Y1	3.7×10^{2a}	1.7×10^{3a}	1.0×10^{2a}	1.0×10^{2a}	2.5×10^{1a}	7.0×10^{1ab}
	35% L5 + 65% Y1	4.0×10^{2a}	3.2×10^{3a}	2.0×1^{3b}	1.0×10^{3b}	1.0×10^{3c}	8.7×10^{1ab}

[1] Mean and values with different letters (^{abc}) in the same column are significantly different from each other ($p < 0.05$).

2.3.4.5. Cytotoxicity and Mycotoxins

There were no mycotoxins (Aflatoxins, Fumonisin, DON and ZEA) observed on the controls and malts. This is in apparent contradiction to the results in Chapter 2.1, which showed the presence of Fumonisins (2 µg/g), DON (15-20 µg/kg) and ZEA (10-15 µg/kg) in the NK 283 sorghum malt (control). This difference is probably due to the fact that in the results reported in Chapter 2.1 the germination temperature was not controlled and the excess water after spraying was not removed from the grain. In this work the temperature was maintained at 28ºC and excess water was removed. These factors might have resulted in the moulds not producing mycotoxins, since moulds produce mycotoxins at elevated moisture content and under stressful environmental conditions, like fluctuations in temperatures or extreme (very cold or very hot) temperatures (D'Mello and McDonald, 1997). As in Chapter 2.2, there were also no mycotoxins observed on the treated malts (Table 2.15).

Sorghum malt made from NK 283 had higher cytotoxicity than the PAN 8546 (Table 2.15). This could have been the result of the toxic products that came from the dead grains since it was shown that only 40% of the NK 283 could germinate (meaning that much of the grain was dead), as compared to the 88% of the PAN 8546 grain that could germinate. However, the very high sorghum malt IC_{50} values indicate that the malts were relatively non-toxic.

2.3.5. Conclusions

The use of the *P. pentosaceus* and *Saccharomyces* spp. cultures during steeping has potential as a natural, biocontrol method for microbial growth in sorghum malting. These cultures inhibit moulds and coliforms to very low levels that should not pose health hazards in sorghum malt. The use of such cultures could therefore be an alternative to the alkali treatment discussed in Chapter 2.2.

Table 2.15. Concentration of aflatoxins (B_1, B_2, G_1 and G_2) fumonisins (B_1, and B_2), deoxynivalenol (DON), zearalenone (ZEA) and the IC_{50} levels of the NK 283 and PAN 8546 malt samples made with L5 and Y1 steeped grains.

	NK 283	**PAN 8546**	**NK 283 and PAN 8546**			
	IC^{1}_{50} (mg/kg)		**Aflatoxins[4]**	**Fumonisin[3]**	**DON[4]**	**ZEA[4]**
			(µg/kg)	**(µg/g)**	**(µg/kg)**	**(µg/kg)**
Control	125-250	500[2]	< 0.25[5]	<3[1]	<3	<3
L5	250-500	>500	< 0.25	<3	<3	<3
Y1	250-500	>500	< 0.25	<3	<3	<3

[1] Inhibitory concentration = concentration resulting in 50% inhibition of cells.

[2]Maximum concentration

[3]Determined using the Vicam kits

[4]Determined using TLC

[5]Minimum detection limit

2.3.3. References

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3. GENERAL DISCUSSION

This chapter is divided into three sections. The first section will critically discuss the major methodologies used in this investigation. The second section will explore the possible underlying mechanisms responsible for the main findings of this research, i.e. turning during germination did not have any effect on the microbial populations and levels of certain mycotoxins in sorghum malt but it decreased the DP of the sorghum malt, inhibition of microbial populations and improved amylase activities by steeping sorghum grain in dilute alkali and inhibition of moulds and coliforms by steeping with the addition of microbial cultures. The third section will discuss the relative merits of the two processes and their practical implementation.

3.1. Methodologies

The direct plating method of Rabie and Lübben (1984) was used to enumerate, isolate, identify and count the moulds in the untreated (control) and the treated sorghum malt samples. This method has been shown to be one of the best for the determination of the nature of and extent of the fungal colonization in unmalted and malted grains (Rabie and Lübben, 1984; Rabie, Lübben, Marais and van Vuuren 1997; Ackerman, 1998). The method involved disinfecting the grains using 76% ethanol and placing the grains aseptically on ten plates each of Potato Dextrose Agar (PDA), Malt Salt Agar (MSA), acidified Czapek-Dox Agar (ACA) and Pentachloronitrobenzene Agar (PCNBA).

The reason for disinfecting the grain surface is to eliminate the moulds from the environment that contaminated the grain. It is assumed that all the moulds that have been associated with the grains during the different malting stages would have penetrated the grain and would therefore not be removed by surface disinfection (Kirby, 1987). On this assumption, all the mould species that were enumerated and counted were from within the grain. After incubating the different plates, some of the unmalted grains (control) had no mould growth around them. This was an indication that surface sterilization was effective since it was reported in Chapter 2.1 that the unmalted sorghum was in fact

contaminated with fungi (10^4 cfu/g), which were probably located only on the surface and were, therefore, removed by surface sterilization.

The purpose of using four different types of media is that different moulds have different nutrient requirements for their growth. Therefore, the media, except for PDA (for all fungi) were selective for certain type of moulds (MSA- *Aspergillus, Eurotium* and *Penicillium* spp. ACA and PCNBA- different *Fusarium* spp). This allows a wide variety of moulds to be enumerated and distinguished (Rabie and Lübben, 1984; Rabie *et al*., 1997).

A problem with the direct plating method is that it is only semi-quantitative. The method only gives an indication of the presence of a mould spp. Even just one spore in a grain can germinate and then the grain will be counted as contaminated. It does not give the level of mould contamination per gram of sample, unlike the other common method-dilution plating, which gives results as colony forming units per gram (Rabie *et al*., 1997). However, the direct plating method has been shown to enumerate more types of mould spp. than the dilution plating method (Rabie *et al*., 1997). This is probably because with the dilution plating method, a sample containing different mould species is homogenised in a solution. Therefore, it is possible that one spore of a certain type of moulds spp. might get damaged and be unable to grow or it might be diluted out prior to the final countable dilution plate. With the direct plating, every spore present in the grain is allowed to grow without being disturbed.

A technical drawback of the direct plating method is that a lot of media is used and a very large amount of time is spent on counting the different moulds in each of the grains on all the petri dishes of different media. Some other methods like fluorescent antibody techniques, ELISA and molecular biology techniques do not require that much media and are more qualitative techniques (Flannigan, 1991; Kistner and Johannsen, 1991). They are however, costly and complicated (Flannigan 1991), whereas some simple methods like ergosterol and chitin determination (Roberts, Moore, Graffis, Walgenbach and Kirby, 1987) are very general in that they just give an indication of the presence of moulds. For the purpose of this study, i.e., the detection of all the different types of moulds present in

a sample like sorghum malt, direct plating was a good method to use because a wide variety of moulds were successfully enumerated.

Mycotoxins display a wide variety of chemical structures and therefore it was difficult to use one single method for the detection of the aflatoxins, fumonisins, DON and ZEA. The methods that were used were chosen on the basis of the reliability of the results and the availability of relevant equipment. A multi-toxin analysis using TLC (Dutton and Westlake, 1985) was used for the analysis of DON and ZEA. Fumonisins were assayed with the use of Vicam kits, which are based on immuno-analytical methods. Aflatoxins were assayed with the use TLC by the method of Trinder (1988) and Vicam Aflatest™, which is an immunoassay (Giese, 2000). The fact that the Dutton and Westlake (1985) method is a multi-toxin analysis is good because all the mycotoxins are analysed at the same time. This method does not require complicated equipment and it was easy to follow and to apply. A negative aspect about this method is that although all the four desired mycotoxins (aflatoxins, DON and ZEA) could be enumerated at the same time, there is a clean up step that requires overnight dialysis of the extracts and several TLC plate have to be run for different mycotoxins. Therefore one analysis lasts a minimum of two days, which makes the method not a quick one. However, the multi-toxin analysis is more rapid than the assays for the numerous mycotoxins separately and for that reason it was a good method to use. However, it has to be noted that TLC is semi-quantitative, not always definitive and should preferably be used for screening. Another drawback of TLC is that is has lower sensitivity, particularly for trichothecenes.

The TLC analysis using the method by Trinder (1988) could be completed within a day. This method was specifically designed to identify low concentrations of aflatoxins (0.3 µg/kg) in sorghum beer ingredients and the beer itself and it was a good method to use. Both one and two dimensional TLC techniques were applied in both methods (Dutton and Westlake, 1985; Trinder, 1988). One dimensional TLC was applied to separate the molecules on the basis of partition between the stationary phase and the mobile phase. The same principle also applies with the two dimensional TLC technique, with the second run separating the molecules better by subjecting them to chromatography at 90º to the first chromatographic separation.

The Vicam method does not require a lot of work, saves time and is easy to follow. However, as reported in Chapter 2.1 of this work, it was found that results obtained using the Vicam Aflatest™ were highly questionable after it apparently measured very high levels of aflatoxins in unmalted sorghum (42 µg/kg) and sorghum malt (52–160 µg/kg). These values are 8 to 32 times higher than the South African legal limit of 10 µg/kg of which not more than 5 µg/kg should be aflatoxin B_1 (Department of Health, 1972) and had never before been reported in South African sorghum malts. The one and two dimensional TLC method was then performed to confirm the Vicam results. The use of the two-dimensional TLC was very effective in showing that the high levels aflatoxins which the Vicam Aflatest™ detected (Chapter 2.1.) were in fact false positives. One-dimensional TLC results also showed a positive aflatoxin spot, but at a lower level. However, the two dimensional TLC showed that the aflatoxin B_1 suspected spots obtained by one dimensional TLC were not really aflatoxin B_1 spots and that there were no aflatoxins in the samples. To confirm the two-dimensional TLC results, the sorghum malt flour was spiked with 0.5 µg/kg aflatoxin B_1, which was easily detected, meaning that if such high levels of aflatoxin were detected as the Vicam Aflatest™ had shown, then they would be detected on the two-dimensional TLC. A possible explanation to these false positive is that the antibodies used in the Vicam Aflatest™ were probably not reactive towards the aflatoxins only, but might have possibly reacted with the phenols (phenolic acids and flavonoids) in the sorghum. Phenolic acids are the simplest phenolic compounds and are derivatives of benzoic or cinnamic acids (Figure 3.1) (Hahn, Rooney and Earp, 1984). Flavonoids are metabolites with a benzopyran nucleus and have an aromatic substituent (Figure 3.2). Although chemically, the structure of an example of an aflatoxin (aflatoxin B_1) on Figure 3.3 and the phenolic acids in the sorghum malt are different, there might be possibilities of some similarities in the reactive sites towards the antibodies during the assay. Studies on the differences or similarities in the mechanisms by which aflatoxins and sorghum phenols react to the Vicam Aflatest™ antibodies might clarify this issue. Notwithstanding this, the determination of sorghum malt aflatoxins with the Vicam test might require an extra purification step which will remove the sorghum phenols (benzoic acids and cinnamic acids) before it can be used as a reliable assay. Phenols could be removed from the extract by the use of Polyclar clean up. The

extract is passed over an insoluble adsorbent, Polyclar AT, which selectively absorbs the polyphenols (Daiber, 1975).

(a). **(b)**

Figure 3.1. Basic structures of (a) benzoic acids and (b) cinnamic acids. Group R varies for different derivatives (Hahn *et al*., 1984).

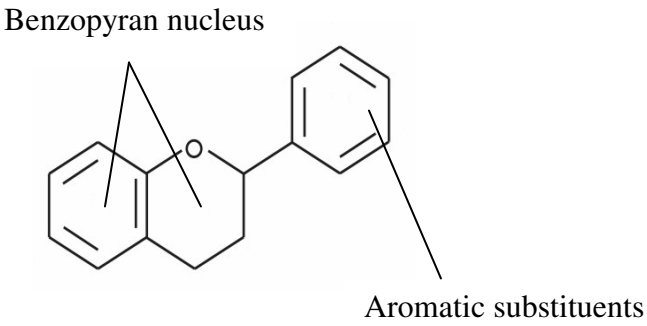

Figure 3.2. Basic structures of flavonoids (Hahn *et al*., 1984)

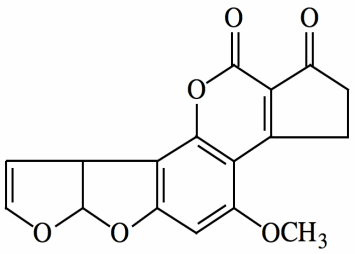

Figure 3.3. Structure of aflatoxin B_1 (European Mycotoxin Awareness Network, 2002)

Alternatives to the methods that were used for the analysis of mycotoxins in this study (TLC and Vicam kits) could be HPLC. The HPLC method has been shown to be more quantitative and faster (Giese, 2000) than the methods used in this study. However, it requires sophisticated and expensive equipment and it is also costly to run the analyses.

To study the toxicity of mycotoxins the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was used. With this assay it was important to make sure that the % of live cells in each test well at the beginning of the assay was not too low or too high as this was going to lead to errors and inaccuracies in the assay itself and the results. To avoid this, the concentration of cells were standardized before the actual analyses

In this study the concentration of the mycotoxin standard was serially diluted from 500 µg/ml (mg/kg) to 0.25 µg/ml (mg/kg). The extracts of sorghum malt were serially diluted from 500 mg/ml (g/kg) to 0.5 mg/ml (g/kg). Dose-response curves for the % live cells against the concentration of the mycotoxin standards and the sorghum extracts were generated. From the curves, the Inhibitory Concentration (IC_{50}), which is the concentration resulting in 50% inhibition of the MTT cleavage activity by the mycotoxin standards and of each sorghum extracts was established. Figure 3.5 shows an example of the calculations of the IC_{50} of an extract. A 100% live cells mean that there is no observed effect level (NOEL) at that concentration of the extract (Crane, Michael and Newman, 2000). The NOEL could be used to give an indication of the safety limits of the sorghum malt extracts. The acceptable daily intake (ADI), which is more relevant in animal toxicity studies than with *in vitro* assays like the MTT-assay, could then be

derived from the NOEL by including a safety factor. A value of 100 is usually chosen as the safety factor (Pariza, 1996). This is to cover by a factor of 10 the possible differences in sensitivity between laboratory animals and humans and by another factor of 10 the variability in sensitivity within the heterogenous human population (due to genotype, sex, age, state of nutrition and health) (Joint FAO/WHO Expert Committee on Food Additives, 2005). Thus the ADI is lower than the NOEL determined.

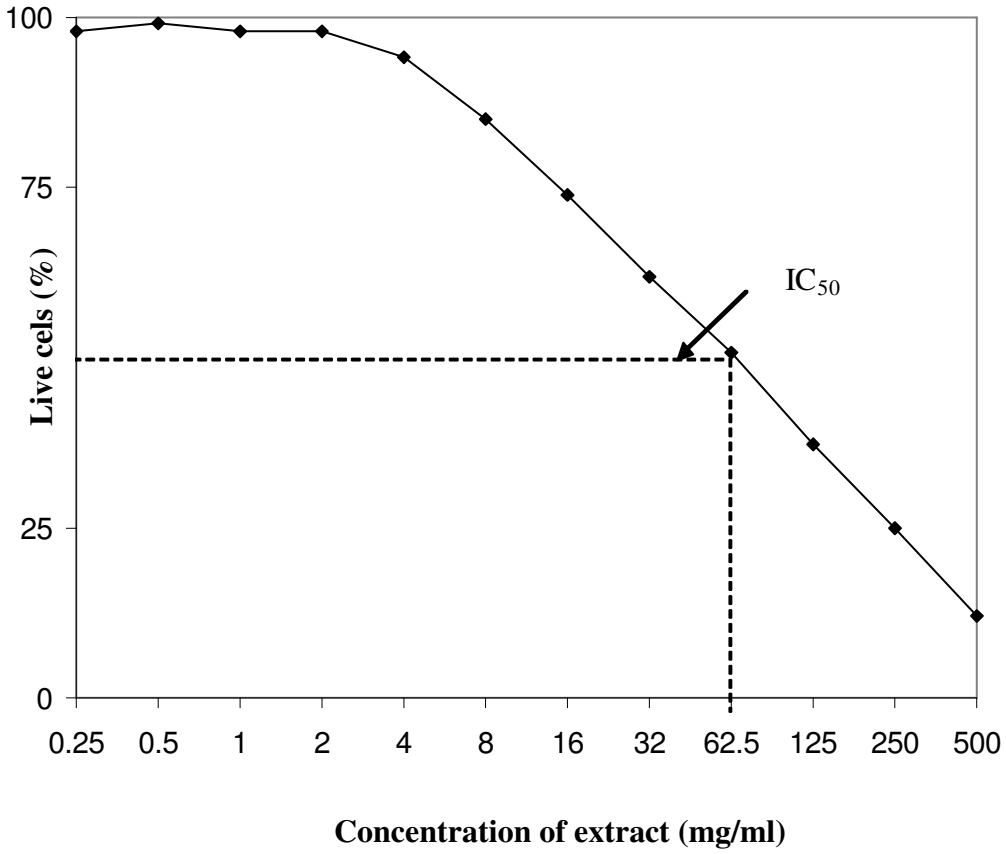

Figure 3.4. Calculation of inhibitory concentration (IC_{50}) of the sorghum malt extracts on the SP2/0 cells.

One of the drawbacks with the use of MTT assay is that every cell line possesses different susceptibility to different mycotoxins (Hanelt *et al.* 1994). The cell line that was used in this study was the lymphoblast-like, myeloma cell line SP2/0 (BALB/c mouse spleen origin) obtained from the Department of Biochemistry of the University of Pretoria. The SP2/0 cells have been shown to work well in other cytotoxicity assays performed in the Department of Biochemistry of the University of Pretoria (personal

communication, Mrs S. Van Wyngaardt, Department of Biochemistry University of Pretoria, South Africa).

In initial work during this investigation assays were performed to establish that the mycotoxin standards used were toxic to this cell line and that the % live cells increased with a decrease in the concentration of the standards. However, there was no measurable cytotoxity caused by either ZEA, which is not cytotoxic but has strong oestrogenic effects, nor Aflatoxin B_1 on the SP 2/0 cells despite the fact that Aflatoxin B_1 (LD_{50} = 0.5 mg/kg in rats) is known to be more toxic than DON (LD_{50} = 46 mg/kg in rats) and ZEA (LD_{50} = 20 000 mg/kg in rats) (Husein and Brasel, 2001). DON showed toxicity and the inhibitory concentration decreased with the increase in the DON standard concentration. The IC_{50} of the DON standard was found to be 1-2 µg/g. DON was therefore the only standard used thoroughout the assay.

The reason that ZEA and aflatoxin B_1 did not show any toxicity might be that the two mycotoxins did not dissolve in the cell culture medium. Mycotoxins are mostly apolar and, therefore, difficult to dissolve in cell culture media (Hanelt *et al*. 1994). Organic solvents are added in order to obtain get a homogenous solution. However, aflatoxin B_1 and ZEA did not dissolve even in different organic solvents (ethanol, methanol, acetonitrile, acetone) investigated. A foam was formed when the solution was sonicated. As part of the method the solution has to be filtered to eliminate contaminants and this step might have also eliminated the undissolved mycotoxins, hence the absence of any toxicity. The other reason might be that the SPO/2 cells probably lack the michrosomal cytochrome P450 monooxygenases, which is found in the liver and is required to transform aflatoxin B_1 to its mutagenic form (Kuilman, Maas and Fink-Gremmels, 2000). The other reason might be the insensitive nature of the mouse to aflatoxins due to the fact that the mouse has a high capacity of detoxification mechanisms (Yanagimoto, Itoh, Sawada and Kamataki, 1997).

A further possible reason might be that the cell line SP2/0 was not susceptible to ZEA and aflatoxin B_1. Hanelt *et al*., (1994) showed that different cell lines possess different susceptibility to different mycotoxins. The main factor responsible for the differences in

effects on different cell lines is the ability of the particular mycotoxin to bind to cellular receptors and/or penetrate cell membranes. The results obtained in this work are in accordance with the findings of Hanelt *et al*. (1994) even though different cell lines were used. Table 3.1 shows a comparison between the results obtained in this study with the SP2/O cell line and three of the cell lines (SK, MDCK and Hela) used by Hanelt *et al.* (1994) on their susceptibility towards aflatoxin B_1, DON and ZEA. The SK cells were not susceptible to aflatoxin B_1 and ZEA even at the highest concentration tested but were highly susceptible to DON. MDCK cells were not susceptible to any of the mycotoxins whereas the Hela cells were mostly susceptible to Aflatoxin B_1.

Table 3.1. Inhibitory concentrations of Aflatoxin B_1, DON and ZEA against the SP2/O, SK, MDCK and Hela cell lines

Cell line	**SP2/O** **[IC_{50} (µg/ml)][1]**	**SK** **[IC_{20} (µg/ml)][2]**	**MDCK** **[IC_{20} (µg/ml)][2]**	**Hela** **[IC_{20} (µg/ml)][2]**
Aflatoxin B_1	>[3]500	>[3]50	>[3]200	25
DON	1-2	0.8	>[3]200	100
ZEA	>[3]500	>[1] 50	>[3]100	100

[1]Findings from this study

[2]Findings from Hanelt *et al*., (1994)

[3]Exceeding the highest concentration tested

The MTT assay has been employed by Reubel, Gareis and Amselgruber (1987); Shier, Abbas and Mirocha (1991); Visconti, Minervini, Lucivero and Gambatesa (1992) and Hanelt *et al*. (1994), to mention a few, to successfully assess the cytotoxicity of specific mycotoxins, unlike in this study where extracts of samples, which were obtained with the Dutton and Westlake (1985) method, were assayed for cytotoxity. Therefore this MTT assay as it was used in this study could not show exactly what mycotoxin was responsible for the cytotoxity of the extracts. Another potential problem with measuring general cytotoxicity is that the chemicals (chloroform, iso-octane, acetronitrile, acetone, potassium chloride, sodium bicarbonate and sulphuric acid) that were used during the extraction of mycotoxins or during steeping when chemicals [NaOH and $Ca(OH)_2$] could

potentially have caused the cytotoxity that was observed. Therefore to rule out that possibility, an extraction without a malt sample (but with just the chemicals) was done and no toxicity was found.

The value of the MTT assay data can be assessed by comparison with the quantities of specific mycotoxins in the samples. The mycotoxin which was found in different quantities amongst the samples, as presented was fumonisin (Chapter 2.1). The unmalted sorghum and the top layer of the unturned, 18-20°C-malted grains which contained less than 0.25 µg/g fumonisin (the minimum detection limit) showed no toxicity (100% cell middle growth) even at the highest concentration of 500 mg/kg examined. Conversely, the middle and bottom layers of the unturned 14-17°C germinated malt which contained 2 µg/g fumonisin were the most cytotoxic at levels of 31.2-62.5 mg/kg. However, due to fact the actual toxins that caused the cytotoxicity of the malt extracts is not known, it cannot be stated for certain that it was the fumonisins that caused the cytotoxity. For the purpose of indicating whether the malt extracts were toxic or not, this method was effective.

3.2. Mechanisims of microbe inhibition

In an attempt to explain why steeping sorghum grain in dilute alkali and the addition of microbial cultures inhibited the growth of moulds and coliforms, this discussion will focus on the possible antimicrobial effects that might have occurred.

A high pH (12-12.8) was observed in the steep water when NaOH was used (Chapter 2.2) and a low pH (3.9-4) was observed when *Pediococcus* spp. was added (Chapter 2.3). Although pH was the only parameter measured, it is a likely cause of observed inhibition of moulds and coliforms observed in Chapters 2.2 and 2.3. The antimicrobial effects of these two pH extremes is due to the fact that bacterial growth generally occurs optimally at pH values in the range of 6-7 (Jay, 2000). As the pH decreases/increases away from this range, so the growth rate falls. Critical limits for the growth of different food-borne microorganisms, have been described (International Commission on Microbiological Specifications for Foods, 1980). A high pH (9-13), will slow down the metabolic

activities by saponifying lipids within the cell membrane of microorganisms and thereby disrupting it, which then leads to cell death (Maris 1995).

Pediococcus pentosaceus is an obligate heterofermenter and produces a mixture of about 50% of lactic acid plus 25% acetic acid and/or ethanol and 25% carbon dioxide (CO_2) (Hammes, Weiss and Holzapfel, 1992). The antimicrobial action of the acid pH, due to the production of lactic acid and possibly acetic acid by *Pediococcus* appears to be the result of the ability of these lipophilic, undissociated acid molecules to penetrate the bacterial plasma membrane (Adams and Hall, 1988). In the higher pH environment of the cytoplasm, the acid (an uncoupler) dissociates to release protons and conjugate bases, thereby reducing the intracellular pH. This disrupts the membrane proton-motive force, thus disabling the energy-yielding and transport process dependent upon it, which will ultimately destroy the microbial cell. In addition to low pH, production of CO_2, competition for nutrients and the production of antimicrobial compounds might also have contributed to the overall level of inhibition of moulds and coliforms (Laitila *et al*., 2002) as a result of the addition of the *Pediococcus* spp. The inhibitory mechanisms of some of these factors are discussed below.

Carbon dioxide inhibits the growth of obligate aerobes, such as moulds (Semple, 2004). The mechanism of inhibition by CO_2 is by inhibition of enzymatic reactions, or by disrupting the solute transport across the cell membrane or by interfering with the cell metabolism (Daniels, Krishnamurthi and Rizvi, 1984). Inhibition of microorganisms by CO_2 has been widely exploited on a commercial scale with the modified and controlled atmospheric packaging (MAP and CAP) of foods (Farber, 1991). For example, the use of MAP with increased CO_2 in dairy products has been shown to inhibit several mould species (Floros, Nielsen and Farkas, 1999).

The growth rate and competitiveness of a microorganism is determined by its adaptation to a substrate (Laitila *et al.,* 2002). The *Pediococcus* spp. was inoculated at high levels of (10^7 cfu/g) at the beginning of steeping and it grew rapidly during steeping and germination. It is likely that rapid growth of the *Pediococcus* could have restricted the

growth of other organisms simply by the mechanism of occupation of available space and uptake of the most readily assimilable nutrients (Erdorul, Çetin and Ergün, 2002).

Bacteriocins are polypeptide anti-microbials produced by bacteria and are primarily active against closely related organisms (Abee, Krockel and Hill, 1995). Currently only two bacteriocins, nisin and natamycin (pimaricin), produced by *Lactoccus lactis* and *Streptomyces* spp. respectively, are accepted by WHO as preservatives for foods (Joint FAO/WHO Expert Committee on Food Additives, 2005). Pediococci produce bacteriocins called pediocins. Pediocins A, AcH, PA-1, PC and SJ1 have been isolated from different *Pediococcus* spp. (Bruno and Montville, 1991). So far, the inhibitory spectrum of pediocins has been found to be limited to Gram-positive toxigenic and pathogenic bacteria (Holzapfel *et al*., 1995). Gram-negative bacteria such as the Salmonellae are not sensitive to bacteriocins because of their outer membrane, which excludes the bacteriocins (Delves-Broughton, 1990). The *Pediococcus* strain used in this study has been found to inhibit the growth of *Lactococcus lactis*, *Bacillus cereus* and *Listeria monocytogenes* (Gurira and Buys, 2004). However, its inhibition against Gram-negative bacteria and moulds was not tested for.

Niku-Pavoola *et al*. (1999) reported the existence of biologically active, non-proteinaceous low molecular mass compounds produced by LAB. They have aromatic/heterocyclic structures. These substances (benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one and 3-(2-methylpropyl) -2,5-piperazinedione) differ from bacteriocins in that they have a wide spectrum of activity against both Gram-negative and Gram-positive bacteria and fungi and work in synergy with the lactic acid.

The antimicrobial activity of the *Saccharomyces* spp. could be attributed mainly to competition with the other microorganims. Boivin and Malanda, (1997) reported that the antimicrobial effect of the yeast *Geotrichum candidum* when added during steeping of barley grain was due to competition with other microorganisms. This method of inhibition has also been observed when yeasts were used as biocontrol agents of postharvest diseases (Jones and Prusky, 2002). Janisiewicz, (1991) showed that yeasts

were able to colonize and survive on fruit surfaces for long periods of time under different conditions and they also used available nutrients to proliferate rapidly, limiting nutrient availability to the pathogens. The results obtained in this work with the disc assay (Chapter 2.2) also suggest that the *Saccharomyces* spp. produced some antifungal products because a clear zone formed around the disc inoculated with the *Saccharomyces*. There is no published information on the production of antifungal compounds by *Saccharomyces* spp. Therefore it cannot be stated what types of antifungal compound may have been produced.

3.3. Relative merits of the technologies

A summary of the above mentioned effects of the two technologies on the microbiological safety and quality of sorghum malt is presented in Table 3.2. The total bacterial counts of the untreated sorghum malt samples in this study (Chapter 2.1) exceed the specifications. A typical Southern African sorghum malt specification for total bacterial count is $<2 \times 10^7$cfu/g and the South African specifications on total bacterial counts in weaning and general foods is $<10^2$ and $<10^3$cfu/g, respectively (Department of Health, 2001). Steeping in dilute alkali or with the addition of *P. pentosaceus* and *Saccharomyces* cultures inhibited moulds and coliforms in the sorghum malt to very low levels of 10^2 cfu/g and $< 10^1$–10^2 cfu/g, respectively. The two processes also reduced the number of types of moulds spp. found in the sorghum malt. The reduction of mould spp. was greater with the alkaline steeping than with steeping with either of the cultures. From these results it was concluded that both processes (dilute NaOH steeping and steeping with the addition cultures) have the potential to be used to inhibit the growth of unwanted bacteria and fungi during sorghum malting (Chapter 2.2 and 2.3), respectively.

Other researchers have reported additional advantages associated with the application of the two processes. Advantages of steeping sorghum with dilute NaOH include improved diastatic power (DP), free amino nitrogen (FAN), enhanced carbohydrate and protein mobilization (Okolo and Ezeogu, 1996a; 1996b; Ezeogu and Okolo, 1999; Dewar *et al.*, 1997b; Nelles and Taylor, 2002). As mentioned, advantages of the application of LAB

Table 3.2. Summary of the effects of alkaline steeping and steeping with microbial cultures on the microbiological quality and safety of sorghum malt.

sorghum cultivar			Alkaline steeping		Steeping with microbial cultures		
			Control	**0.2% NaOH**	**Control**	*Pediococcus pentosaceus*	*Saccharomyces* spp
NK 283	Moulds (cfu/g)		1.0×10^5	7.3×10^2	5.7×10^4	9.7×10^2	6.8×10^2
	Coliforms (cfu/g)		4.0×10^4	8.0×10^2	1.7×10^4	$< 10^1$	5.2×10^2
	Mould (% grain contamination)	0 - 10	0	2	0	0	1
		11 - 20	1	4	0	2	2
		21 - 40	1	2	2	6	5
		41 - 60	3	0	1	0	0
		61 - 80	1	0	3	0	0
		80 - 100	2	0	3	0	0
	Mycotoxins		Not detected	Not detected	Not detected	Not detected	Not detected
	Malt cytotoxicity/ IC_{50} (mg/kg)		125-250	> 500	125-250	250-500	250-500
PAN 8546	Moulds (cfu/g)		1.6×10^5	3.7×10^2	2.7×10^3	8.6×10^2	1.3×10^2
	Coliforms (cfu/g)		3.2×10^2	2.5×10^1	6.3×10^2	$< 10^1$	8.7×10^1
	Moulds (% grain contamination)	0 -10	2	8	1	1	8
		11- 20	3	0	4	3	0
		21- 40	1	0	0	4	0
		41- 60	0	0	0	0	0
		61- 80	0	0	0	0	0
		80 - 100	0	0	0	0	0
	Mycotoxins		Not detected	Not detected	Not detected	Not detected	Not detected
	Malt cytotoxicity/ IC_{50} (mg/kg)		> 500	>500	> 500	> 500	> 500

starter cultures during barley malting include reduced proportion of grains contaminated with *Fusarium*, decreased grain water sensitivity, increased FAN and α-amylase activity, better malt modification, improved wort filterability and decreased gushing tendency (Lowe and Arendt, 2004).

Although the main objective of this work was to research into processes that could improve the safety and quality of sorghum malt, the practical issue of the application of the processes are also important. There are several aspects associated with the application of these proccesses which makes steeping sorghum grains in dilute NaOH a more favourable process than steeping with the addition of microbial cultures.

The cost of applying these processes is a major issue. Table 3.3 shows an estimate of the additional running costs incurred when the two proposed treatments are applied. Sodium hydroxide (NaOH) is not very expensive. However, steeping with NaOH requires the use of additional water because of the two washes during the process. Water is always available in malting plants and washing the grains that are in a steeping tank is easy to do. There is no need to implement better quality control systems with NaOH steeping, the pH will already be high at the beginning of steeping and all the other parameters remain the same. Therefore there will not be extra costs in terms of implementation and maintenance of the process. The use of dilute alkaline steeping is currently being successfully used in Nigeria (Professor J.R.N. Taylor, Department of Food Science, University of Pretoria, South Africa).

Table 3.3. Estimation of the running costs in South African Rands (R 6.6 = 1 US$) incurred when alkaline steeping and steeping with microbial cultures are applied, when 10 tons of sorghum grain is malted per day.

	Steeping with 0.2% alkaline steeping	**Steeping with microbial cultures**
Water use (1 ton of sorghum per 1 ton of water) per day	1. Steeping (10 tons of water) 2. First rinse (10 ton of water) 3. Second rinse (10 ton of water)	10 tons of water
Water cost (R 5.63/ton)	R 5.63 x 30 tons = R 168.9	R 5.63 x 10 tons = R 56.30
NaOH pearls cost (R 6.10/kg) and 2 kg NaOH/ ton of water is required.	20 kg for 10 tons of water R 6.10/kg x 20 = R 122.0	Not applicable
Total costs for the malting process	**R 290. 90/day**	**R 56.30/ day**
Employment of a microbiologist	Not applicable	R 150 000 per annum = R 417/day
Microbiological media for maintenance and preparation of cultures	Fixed apparatus	R 5 000/ month = R 167/day
Preparation of cultures (at least 200 litre/ton required = 2 tons of cultures/day)	Not applicable	R 11.26 of water
Employment of a technician	Not applicable	R 80 000 per annum = R 222/day
TOTAL COSTS	**R 290 day**	**R 873.60/day**

Steeping with microbial cultures does not require the two washings. Thus the extra water costs are not incurred. However, working with the cultures could be cumbersome in a malting plant. The cultures have to be continually maintained pure. The services of a microbiologist, which is an additional cost, will be required to do that. A microbiological laboratory where a microbiologist can work with cultures under sterile conditions will also have to be established and that will add further to the costs. There are also added costs of the microbiological media and chemicals that will be required. Many litres of cultures (at least 2 tons) will be required daily to make the 10^7 cfu/g inoculation and these will also require the services of a skilled technician. The steeping conditions like temperature will have to be monitored so that they favour the optimal growth of the cultures. The growth of the microorganisms and the pH should also be monitored during steeping to ensure that it does change accordingly. The general quality control of the malting plant will have to be upgraded in order to make steeping with cultures work. Therefore the implementation and maintenance of steeping with microbial cultures will require additional costs.

The estimation total costs incurred when alkaline steeping and steeping with microbial cultures are applied, when 10 tons of sorghum grain is malted is R 290 and R 873 per day, respectively. Therefore, alkaline steeping is 1/3 the cost of steeping with microbial cultures.

Percentage (%) of income can be calculated as follows:

Sorghum malt sells at approximately R 4000/ton
Income at 10 tons malt/day = R 40 000/day

Cost as a percentage of income:

Alkaline steeping = 0.73%
Steeping with microbial cultures = 2.18%

The overall relative merits of the two processes are summarized in Table 3.4. Both processes results in the decrease of moulds and coliforms to very low levels. However steeping with NaOH is easy to apply and maintain, requires no implementation of

improved quality system, no appointment of additional staff and it increases the DP of the sorghum malt. In contrast, steeping with microbial cultures could be complicated to apply and maintain, requires implementation of improved quality system, appointment of additional staff and does not affect the DP of the sorghum malt.

Table 3.4. Relative merits of steeping with dilute (NaOH) and treating with microbial cultures.

Steeping with dilute NaOH steeping	**Steeping with microbial cultures**
Decreases moulds to 10^2 cfu/g	Decreases moulds to 10^2 cfu/g
Decreases coliforms to 10^2 cfu/g	Decreases coliforms to $< 10^2$ cfu/g
Easy to implement	Difficult to implement
Increases DP by 100%	No effect on DP
No improved quality control systems required	Improved quality control system required
Simple maintenance	Sophisticated maintenance
No appointment of additional staff required	Requires appointment additional of more staff

4. CONCLUSIONS AND RECOMMENDATIONS

Floor sorghum malting, as is carried out in Southern Africa, produces sorghum malt with a high number of unwanted bacteria (coliforms) and fungi. The malts contain traces of some mycotoxins and exhibit some degree (very minimal) of cytotoxicity. Turning of sorghum grains during germination does not affect the high microbial populations, the nature and extent of fungal contamination, the level of aflatoxins, DON and ZEA, or the cytotoxicity. Therefore it appears that not turning the grains during sorghum malting is not the reason for the high number of unwanted bacteria (coliforms) and fungi found in the outdoor floor malted sorghum as it was hypothesized. The uncontrolled environmental conditions (e.g. variable temperatures and very high water activity) and external sources of contamination (e.g. excrement from birds and vermis) during outdoor floor malting are probably the main causes of the high microbial counts.

Steeping in dilute NaOH produces sorghum malt that is microbiologically safe with very low levels of coliforms and fungi. The inhibition of the fungi results in malt which does not contain mycotoxins and has no or very minimal cytotoxicity. Steeping in dilute NaOH also produces malts with increased DP.

The addition of *Saccharomyces* spp. and *Pediococcus* spp. cultures to steep water results in sorghum malt that is microbiologically safe with the absence of or very low levels of coliforms and fungi. However, there is no improvement of the DP.

Alkaline treatment and the use of starter culture can be applied to produce malt safe for brewing and that could even be used to prepare weaning foods. However, based on the above mentioned findings, dilute alkaline steeping is a better method than the use of starter cultures.